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PROBLEMS OF SPACE BIOLOGY. VOLUME 22. METABOLISM UNDER THE EXTREME CONDITIONS OF SPACEFLIGHT AND DURING ITS SIMULATION

I. S. Balakhovskiy and Yu. V. Natochin

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16. Abstract

The book summarizes the experience of ten years of study of the metabolic processes in cosmonauts who have flown aboard Soviet spacecraft. The changes observed in them are compared with the results obtained in model experiments and data published by the American researchers. The excretion of water and salts directly during the flight and immediately after landing is analyzed in detail. The methodological section of the book deals with a detailed description of the methods of clinical biochemistry, specially devised and applied to problems of space medicine: the complex microchemical analysis of blood dried on pieces of paper, determination of the mass of hemoglobin, etc. General problems of water transport in osmoregulatory organs and the regulation of water-salt exchange are discussed.

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INTRODUCTION

The flight of man into space has subjected him to conditions which are unusual from the standpoint of the entire evolution of living nature — he has become the first form of matter to spend a long period of time in weightlessness. From the biological standpoint, this is a basic factor. In addition, the physiological processes in the organism of a cosmonaut are subjected to enforced limitation of mobility, the absence of the usual alternation of day and night, unusual food, and considerable neuro-emotional stress, which can be manifested in various ways but whose influence cannot be disregarded. /5*

Weightlessness, i.e., in the direct sense of the word, the absence of the force of gravity, affects primarily hemodynamics, since the orthostatic pressure gradient of the blood disappears. This acts as a triggering mechanism for a number of other processes, including changes in metabolism. The situation is complicated by the direct influence of other flight factors on the organism of the cosmonaut.

During his stay aboard a spacecraft, a cosmonaut is not subject to ordinary laboratory methods of investigation, and special methods of biochemical investigation are required in order to obtain the necessary information.

The present book deals with all of these problems; whenever possible, it generalizes the available information on metabolism under spaceflight conditions and in simulation experiments which imitate the conditions prevailing during such flights, as well as corresponding special methodological procedures.

*Numbers in the margin indicate pagination in the foreign text.

PART I /6

METHODS OF SPACE BIOCHEMISTRY

HIGH-SPEED METHODS OF BIOCHEMICAL ANALYSIS

There are many procedures in biochemical analysis which are relatively simpler than others but still retain the basic outlines of classical chemical methods — pouring of solutions, separation of the precipitate, titration or photometry. Such methods, regardless of how much they have been simplified with respect to others, still cannot be called high-speed methods and there is no prospect of their being used aboard spacecraft, where any operations involving solutions are extremely difficult. Under these conditions it is feasible to use only those measures in which the biological fluid being studied (blood, urine) is delivered to the analyzer without any preliminary procedures and the result is read directly on the scale of the instrument.

At the present time, there are two methods of approaching the design of such direct analyzers. The first is the use of indicator paper and the second is the use of biochemical electrodes. Test paper is being used increasingly widely in clinical biochemistry, inasmuch as it is very simple to use and can be employed to monitor the health of patients both by the youngest medical personnel and by the patients themselves. The principle of their use consists in the fact that a drop of the fluid to be studied (urine, blood or blood serum) is placed on special reagent paper whose color change indicates the presence of a certain amount and in some cases permits a semiquantitative evaluation of its concentration. Such methods are most widely used for the qualitative determination of sugar in the urine (glucopan, glucotest), as well as for determining the amounts of protein and acetone in it. The method for determining sugar in the urine is based on glucosidase reaction, when glucose is oxidized in the presence of the enzyme glucosidase to form hydrogen peroxide, and this substance in turn oxidizes a dye by means of the enzyme peroxidase (benzene, dianizidine, etc.). The qualitative determination of protein in the urine by means of reagent paper is based on the ability of certain dyes, /7 particularly thymol blue, to bind selectively with protein, so that its color changes. The semiquantitative determination of chlorides in the urine is conducted by measuring the size of the spot which is formed when a drop of urine is placed on paper that has been dyed with silver nitrate. There are also semiquantitative paper methods for determining urea, sugar and acetylcholine esterase in the plasma or blood serum [53, 63, 94, 208, 284]. The possibility of using these methods in space medicine is limited because they can be used only to detect (or, on the other hand, exclude) coarse pathology, and not functional changes which may be anticipated during flight.

Biochemical electrodes are more suitable for study during space flight; they are the most promising method of analysis in clinical biochemistry. These electrodes have not yet been widely used, but it may be anticipated that during the next ten years they will become as ordinary as the measurement of pH.

Initially, electrodes were developed for determining the activity of cations. They are similar to the glass electrodes used for determination of pH but they are made of special kinds of glass whose potential with respect to the external solution is a function of the concentration of the ions being studied [73, 200, 202, 218, 255]. Electrodes of this kind have been developed for determining sodium, potassium, calcium and ammonium.

Electrodes used for determination of the activity of chlorine and certain other halides, or ions similar to them in terms of their chemical nature, for example, rhodanide [255] are based on a slightly different principle. They use a crystal of silver chloride whose potential with respect to the external solution is a function of the activity of the chlorine ions in it, as well as other compounds which are capable of forming a common crystalline lattice with the silver chloride. Using crystals of silver chloride in which ions of bromine, fluorine or thiocyanogen have been incorporated, it is possible to produce electrodes that possess relatively high selectivity with respect to the ions of the corresponding substances, but it is impossible to achieve total selectivity with them. However, such selectivity is not necessary for the analysis of natural biochemical fluids; of all the substances that have been determined by means of this type of electrode, only chlorides are present whose concentration may be determined and corrected.

When using glass or silver chloride electrodes, it is not the concentration but the activity of the corresponding substances which is determined, so that the results of a determination in the blood may differ markedly from those obtained by ordinary chemical methods. Thus, for example, the activity of calcium in blood serum, determined by means of a calcium electrode, constitutes approximately half its total concentration as determined by the titrimetric method. The residual calcium which forms complexes with protein is not determined by this method. Differences in terms of potassium and sodium between the concentrations and activities in biological fluids are very small, so that results obtained by means of electrodes and in chemical analysis practically coincide. Hence, there are no significant differences between activity and total chlorine content in biological fluids. /8

A device has been developed based on the glass electrode for determining the partial pressure of carbon dioxide and its total content in the blood and certain other indicators of acid-base balance. Of these systems, the one that is most widely used in the Soviet Union is the method of the Danish scientist Astrup. The pH of a patient's blood plasma is measured several times: in the state in which it was obtained from the organism (current pH value), and also after saturation with gas mixtures having different concentrations of CO_2. At these partial pressures of carbon dioxide, its total content and concentration of hydrogen ions also change, so that there is a relationship between the magnitude of the pH shift, the original carbon dioxide content and the total quantity of buffer systems in the blood. Therefore, all of these factors can be calculated using special nomograms.

Other methods of determining the partial pressure of carbon dioxide utilize glass or other types of electrodes that are sensitive to changes in the

concentration of hydrogen ions which are separated from the fluid to be measured by a semipermeable membrane; this membrane allows the carbon dioxide to pass through much better than other substances that affect pH. Therefore, the concentration of hydrogen ions around the electrode will provide some idea of the partial pressure of carbon dioxide.

There are also numerous versions of electrodes for determining the partial pressure of oxygen which give good results under laboratory conditions, but their use is not feasible aboard a spacecraft because it is most efficient to determine the degree of saturation of the blood by means of an instrument that does not use blood — the oximeter. Attempts have also been made to determine the partial pressure of oxygen in the tissues by means of appropriate electrodes. However, the reliability of the results obtained is low: insertion of the electrodes causes damage to the vessels and the formation of a hematoma, then a scar. Therefore, in practice the partial pressure of oxygen is measured not in normally functioning tissues but in those that have been damaged or are granular.

During the last few years, papers have been published that have resulted in a considerable expansion of the possibilities of developing and using the chemical electrodes. This trend in applied science has apparently undergone /9 its broadest development during the last decade [215, 279. 287]. The electrode for determining the concentration of glucose in biological fluids is a solid platinum electrode with a layer of the enzyme glucosidase applied to it, fixed in a gel. Glucose from the surrounding medium diffuses into the gel layer, where it is oxidized by the oxygen in the air to form gluconic acid and hydrogen peroxide. This substance possesses characteristic polarographic properties which make it possible to determine the H_2O_2 concentration by measuring the strength of the current at a certain voltage. Inasmuch as the glucose constantly diffuses from the surrounding space into the gel layer that is located around the electrode and the H_2O_2 moves in the opposite direction, the hydrogen peroxide concentration in the space around the electrode turns out to be proportional to the glucose content in the surrounding fluid (Figure 1).

An electrode sensitive to ammonium ions is made by the firm of Beckman. Its improved version, which makes it possible to determine urea concentration, has been described by Guilbault and Montalvo [215]. It is a glass electrode whose potential is a function of the ammonium ion concentration in the surrounding medium. The electrode is covered with a layer of polyacrylamide gel; the enzyme urease, which breaks down urea to form ammonia, is immobilized in the gel. In a fashion similar to that which occurs in the glucosidase electrode, the urea from the surrounding medium diffuses into the space around the electrode and is converted into ammonia, which then diffuses back out into the medium.

In the steady state the ammonia concentration is proportional to the urea /10 concentration. Inasmuch as there are a great many specific enzymatic reactions which result in the formation of either ammonia or hydrogen peroxide, by

analogy with the two electrodes described above it is possible to develop a number of electrodes for performing various biochemical analyses.

The use of electrodes for biochemical analysis of whole blood and urine has the great advantage that the results can be obtained immediately without going through any of the traditional chemical procedures. For the analysis of blood, it is most convenient to use electrodes in the form of thin tubes, in which the blood sample to be analyzed is drawn up directly from a pricked finger. Such tubular electrodes are used in the microastrup instrument for determining the index of acid-base balance and have been described in a slightly different version by O. G. Belokurov [23]. (Figure 2).

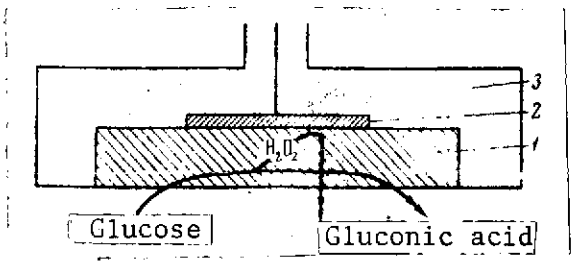


Figure 1. Biochemical Electrode for Determination of Glucose. 1, Gel containing the enzyme glucosidase; 2, platinum electrode; 3, housing.

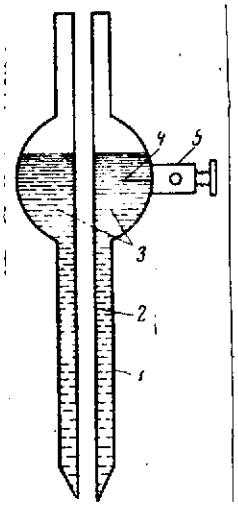


Figure 2. Tubular Electrode for Determination of pH in Small Fluid Volume (According to O. G. Belokurov). 1, External, mechanically strong wall of the electrode; 2, brass tube sensitive to pH changes; 3, space filled with 0.1 N HCl solution; 4, current--conducting electrode; 5, electrical terminal.

Biochemical electrodes make it possible to determine directly the content of the substances under study in the blood plasma without separating it from the erythrocytes, inasmuch as an electrode which is submerged in whole non--hemolyzed blood comes in contact only with the plasma. This is of great significance because the erythrocytes are relatively inert and their content of chemical substances usually does not change so rapidly or to the same degree as it does in the plasma. Therefore, studies of the plasma composition are more informative.

Under spaceflight conditions, collecting plasma or blood serum by ordinary methods is not very feasible because of the tedious nature and difficulty of these procedures. Therefore, it is interesting to consider the possibility of a rapid method of collecting plasma, not after precipitation of the erythrocytes but after their flocculation by the addition of some reagent or other. It has been known for a long time that the addition of a number of substances to the blood — saccharose, gelatin, etc. — leads to the agglutination of the erythrocytes and their rapid separation from the plasma. Winkelman and Pileggi [287] described a special device which consists of a syringe whose walls are wet with a solution of heparin to prevent coagulation of the blood and a solution of beef fibrinogen which agglutinizes the erythrocytes. However, the plasma which is obtained by this means is not suitable for all analyses, particularly since its sodium and protein content has evidently been altered as a result of the wrinkling of the erythrocytes.

Microanalytical Devices

A number of systems have been proposed for microanalysis, including the use of various types of micropipettes, microburettes, microcolorimeters, microbalances, and so on. A description of these instruments can be found in the monographs of Kirk [71], Korneman [76], Alimarin [9] and a number of others. /11
The oldest of the systems that have seen wide application were described by Linderstrom-Lang before World War II. Then the Lowry System appeared [236], used mainly for analysis of tissues from the nervous system, and that of A. A. Pokrovsky [9, 118] for clinical biochemistry and primarily for the determination of enzymes, as well as many others.

The improvement of micromethods of biochemical analysis is following different paths. The most effective is the search for more sensitive and specific and chemical reactions, as well as the use of new physical principles. In this regard, considerable promise is offered by the introduction of enzyme electrodes, determination of hormones by radioimmunological methods and concurrent binding with specific proteins, the use of emission and activation analysis, x-ray fluorescence, atomic absorption spectroscopy and gas chromatography.

Unfortunately, each of these methods, although very sensitive and specific, can only be used for studying a certain class of substances; for certain substances, whose physiological significance is very great (for example, cholestrol and nonesterified fatty acids [NEFA]), none of these methods can be used. The use of new physical principles requires costly and complex apparatus, and the biological and biochemical methods (for example enzyme and immunological

methods) use costly and delicate reagents. Therefore, another so-called classical method of development of microanalysis has not been disregarded — the use of traditional analytical approaches to titration, photometry and so on, but using various devices which make it possible to work with very small quantities of material. The advantage of using this approach is its relative simplicity and availability to small laboratories.

All existing versions of microphotocolorimeters can be divided into three groups: microscopic systems, systems with vertical and horizontal columns of fluid. Microscopic systems (for example those using the decreasing drop [220]) make it possible to measure the absorption of light by negligibly small volumes and thereby increase the sensitivity of the analysis, but it is difficult to work with microdrops — special measures are necessary so that the volume of the fluid under study will not decrease as a result of evaporation. Therefore, microscopic systems for photometry have not been used in clinical biochemistry and it makes no sense to discuss them here.

Colorimetric Systems with a Vertical Column of Fluid /12

All methods of increasing the sensitivity of photocolorimetry boil down to the fact that a smaller quantity of fluid is used for measurement. The usual cuvettes which are used in photocolorimeters or spectrophotometers measure several square centimeters in cross section, and a decrease in the linear dimensions of the cross section of the cuvette, with retention of the same thickness of the layer to be subjected to photometry, results in an error which is proportional to the decrease in area. Therefore, if a cuvette with a cross section measuring 20 × 20 millimeters, let us say, is replaced by a cuvette measuring 2 × 2 millimeters, the loss in the amount of solution necessary for the analysis (i.e., the sensitivity) would be 100. However, the change in the depth of the layer will not cause any loss of sensitivity, so that when working with layers of very small thickness it is necessary to use much more concentrated solutions.

The decrease in the fluid volume to be used for analysis is limited by three factors: (a) the need to have a cross section of the cuvette such that a sufficient amount of light can pass through it for exact measurements; (b) the possibility of reliably and accurately adjusting the position of the cuvette in the light beam and (c) the inconveniences involved in working with a small volume of fluid. The first of these factors has practically no influence inasmuch as modern photomultipliers make it possible to measure very small light flux values. The second is of critical significance because the cuvette must be mounted exactly the same way in the light beam for all measurements. In addition, it must be as cheap as possible and easy to replace. A cuvette whose cross section is reduced to a fraction of a square centimeter must in any case be carefully placed in a beam of light, always in the same way, so that there will be no need for its walls to be strictly parallel. As experience has shown, slight curvature of the optical surface does not make any difference if the light always passes through the same areas (of course, maintaining the same accuracy which is necessary for practical work). This makes it possible /13 to use cylindrical cuvettes with a diameter of several millimeters through which

the light passes in a direction perpendicular to the principal axis of the cylinder. In photometers which are designed for these cuvettes, the light beam usually travels in a horizontal direction and the column of fluid to be subjected to photometry is mounted vertically.

It is convenient to fill fine tubes from above, so that various systems have been proposed to allow drawing the fluid to be investigated up into the microcuvette through the lower opening. Thus, for example, in the Pokrovsky microanalytical system which is widely used in the Soviet Union [9, 118] the cuvette is in the shape of a pipette into which the fluid to be studied is drawn through the lower end (Figure 3).

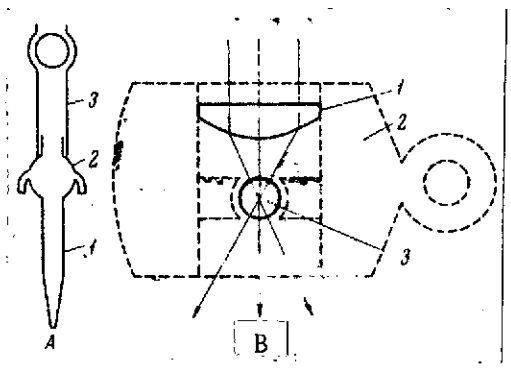


Figure 3. Microcuvette, According to A. A. Polrovsky. A, Side view: 1, cuvette alone; 2, "hooks" for fastening the cuvette in position; 3, rubber tube for drawing in the fluid; B, top view: 1, cylindrical lens; 2, holder; 3, cuvette.

Colorimetric Systems with a Horizontal Fluid Column

The possibilities of certain systems with a vertical column of fluid are limited by the fact that in this volume of fluid the optical density of the layers cannot be increased. This can only be done by passing the light through a column of fluid so that the sensitivity increases by a factor of two. The cross section of such a cuvette is so small that it is very difficult to mount it twice in exactly the same way in a light beam. Therefore, the cuvette is made in the form of a through chamber, i.e., it is mounted in a stationary manner and filled by pumping it full of the fluid to be studied. The glass capillary cuvette of G. V. Troitskiy was designed in this way, described back in 1938 [137], as were others of later design [217, 258, 282]. If photometry involves a fluid whose coefficient of refraction of light is less than that of the walls of the cuvette (as is the case in the overwhelming majority of cases, its internal lumen will function as a light pipe.

Capillary Microcolorimeter with a Lengthwise Beam of Light

Decreasing the volume of fluid to be subjected to photometry in order to increase the sensitivity of the analysis has certain limits, since the sensitivity is governed not by the concentration of the solution to be studied but by the quantity of substance which is present per unit area of the cross section of cuvette. In other words, if we double the length of the cuvette and keep the cross section constant, a solution which is twice as dilute can be used. Concentration of the substance to be studied in small volumes is not always a simple matter; when volumes of fluid are small, losses due to

evaporation are relatively great, and this affects the accuracy of the analysis. A portion of the fluid under study always remains on the walls of the vessel in which the chemical reaction took place as well as in the tube through which it /14 was filled. Decreasing the volume (i.e., the cross section of the cuvette itself) has practically no influence on the magnitude of these losses. Therefore, there is no practical significance to reducing the volume beyond a certain limit (in all experiments, 0.1-0.2 ml). Increasing the sensitivity is achieved by increasing the length of the cuvette. This also has it limits. For example, increasing it from 1 to 5 cm produces a loss of sensitivity which is five times greater, but increasing it another 4 cm (to 9 cm) increases the sensitivity by less than a factor of two.

The internal diameter of the cuvette governs the amount of light which can pass through it in a given system of illumination, i.e., its light transmission. If sensitive photomultipliers are used and the correct portion of the spectrum is isolated by means of light filters, the transmission of the cuvette will almost always be adequate and will not limit the possibilities of the researcher. However, if a prism monochromator is used to split the monochromatic light beam and the detector is a photocell, it is necessary to use cuvettes with greater diameter and accordingly greater transmission.

The method by which the light beam enters the lumen of the cuvette is of great significance. G. V. Troitskiy focused the light on the ground face of a cuvette by means of a microscope objective [137]. Vurok and Bowman [282] suggested feeding the light into the cuvette by means of a light pipe made of glass fibers. The second suggestion, in our opinion, is worthy of greater attention.

In the practical manufacturing of a capillary microcolorimeter, the choice of the optimum dimensions of the cuvette and the optical components of the instrument can only be solved experimentally, so that it is interesting to describe the device although all of its components have been described previously (Figure 4).

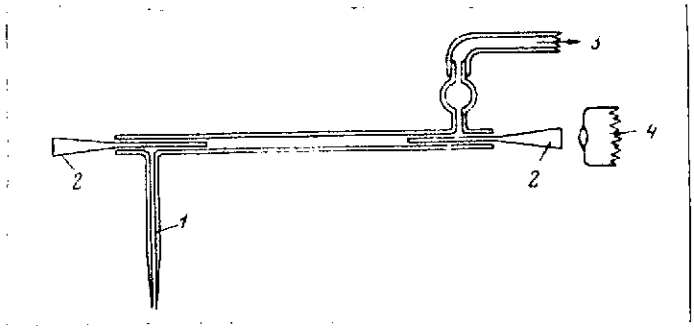


Figure 4. Continuous Cuvette of a Capillary Colorimeter (Version of the Cuvette Designed by G. Z. Troitskiy). 1, Branch for drawing in the fluid to be investigated; 2, light pipe; 3, tube connected to a syringe; 4, illuminator.

The main component of the instrument is the capillary microcuvette, made /15
of glass with an internal diameter on the order of 0.5-1 ml. The fluid to be investigated is drawn into the cuvette through two tubes made of glass of the same or smaller diameter, joined to the main tube at an angle of 90 degrees. The light enters the tube and emerges from it through light pipes which are fastened to the ends of the tube by some transparent material (rubber, tygon gaskets) or simply held in place by epoxy resin or Mendeleyev's cement. When filling the cuvette with fluid it is important for a dense layer of liquid to be between the ends of the light pipes, so that the air bubbles beneath the light pipes will be insignificant. They are even useful, since they prevent contact between the fluid and the sealant.

The light source is a bulb with a power of several watts, powered by a ferroresonance stabilizer. If the bulb is supplied with low voltage (several volts) very small changes of resistance at the contacts will lead to a change in the strength of the current in the circuit and will be reflected in the brightness. It is therefore necessary always to avoid using spring contacts and adjusting rheostats in the low-voltage circuit, and the connections should best be soldered.

The light from the illuminating bulb is focused on the light pipe by means of a microscope objective with a magnification of 20 or 40 X. Passing through the cuvette, it strikes the interference light filter and then the photomultiplier. For this purpose, any photomultiplier is suitable, but the FEU-35 is best because it has a very low dark current and a low anode voltage — 500-600 volts maximum. The FEU-35 has the disadvantage that it is sensitive to light with a wavelength of 400-600 nm, but the majority of clinical biochemical methods require precisely this range.

The photocurrent from the photomultiplier can be measured by a mirror galvanometer or a galvanometer with a shadow indicator, but we usually have used a D.C. amplifier with a pointer galvanometer at the output. As such an amplifier one can use a specially manufactured UIP amplifier or an ordinary pH meter which is used to measure the voltage developed in the output resistor of the photomultiplier directly in millivolts. In this case, the load on the FEU is a resistance of 2-4 milliohms. It is very important that both the load resistor and the power amplifier supplying the FEU be placed in a hermetically sealed housing with a silica gel cartridge to eliminate moisture. A D.C. amplifier with a pointer instrument at the output is convenient since it is compact and may be used with practically any room illumination conditions. Galvanometers with a shadow indicator, particularly the reference type of device (UF) of the MF-2 microphotometer galvanometer are less convenient — in bright light, the light indicator is difficult to see. They are more cumber- /16
some, but at the same time they are more accurate and equipped with an extinction scale which simplifies further calculations.

The FEU obtains its high voltage supply from a VS-22 commercial stabilized rectifier.

Semimicrocuvettes

In those cases where there is no need to determine the quantity of very small amounts of material, but ordinary cuvettes are not suitable for analysis (for example, for a determination of cholesterol and its ethers in the same sample), it is convenient to use the slit cuvettes that were described in the book by Kirk [71] and which were proposed by Lowry [236]. The external dimensions of these cuvettes are the same as the mass-produced cuvettes for the SF-4 spectrophotometer, but their internal dimensions form a slit 1-2 millimeters wide whose optical length is 10 millimeters. The principal difficulty of using this cuvette in a regular spectrophotometer is that the width of the light beam in the SF-6 instrument is 6 millimeters, so that it is necessary to narrow it by focusing it through a lens [16]. Forming that narrow light beam by means of a diaphragm as is sometimes recommended [150] does not make any sense, since it results in a significant loss of strength of the light.

When working with the SF-5 spectrophotometer, the light beam may be made narrower without resorting to an additional lens, since its optical system makes it possible to obtain the same results by shifting the illuminating lamp to an extreme rear position. In this case, it is convenient to use the two-millimeter cuvette from the FEK as the semimicrocuvette, passing the light through it along the long side.

Microfluorometer

In contrast to the situation which exists, in microcolorimetry in the identification of substances on the basis of their fluorescence, any decrease in the volume of the fluid to be studied leads to a corresponding increase in sensitivity. This occurs because in colorimetry the sensitivity is governed by the amount of substance being studied per unit area of cross section of the cuvette, while in fluorometry it is governed by the amount per unit volume. Therefore, although the difficulties in working with small amounts of substance are the same, in fluorometry overcoming them is justified. Any solvent will fluoresce, albeit to a negligible degree, and this glow is combined with that portion of the exciting beam of light which is scattered and reaches the radiation sensor after passing through both the first and second light filters. In order to ensure reliable operation, the concentration of the substance under study must be such that the glow is at least one and a half to two times greater than this background. Microcuvettes for the fluorometer were described in 1954 /17 by Lowry et al. [236], and since then the literature has seen descriptions of several similar cuvettes with volumes from 100 to 200 microliters [225]. Fluorometers with continuous cuvettes have also been described [277]. In practical operation, the limit beyond which it does not make any sense to reduce the volume of the fluid to be studied is governed by the spreading of the solution when it is added to the cuvette and the spots on its walls, which it is difficult to do anything about if the cuvette is small. It is difficult to say which of these factors limits the sensitivity of the method to the greatest degree.

In the majority of mass-produced fluorometers, the separation of both the primary (exciting) light beam and secondary (fluorescent light) beam is accomplished by means of light filters. Such a technical solution is simpler than using monochromators, but sharply reduces the possibility of the experiment since many important substances that are biologically important and are similar in chemical structure will fluoresce in the same range and it is difficult to separate their fluorescence by means of light filters.

In spectrofluorometric analysis, it is theoretically possible either to vary the spectral composition of the exciting light (this is called studying the excitation spectra) or to study the spectra of fluorescence when illuminating the object with a single beam of light. In the majority of cases the second approach has less to offer clinical biochemistry, since the fluorescence spectra are frequently the same for both those substances which are being determined and the disturbing impurities. The method of investigation of the excitation spectra is more promising and it is almost as specific as the study of the absorption spectra, inasmuch as both of these effects have the same physical nature — only the light which is absorbed by the substance under study is capable of exciting fluorescence.

In order to excite fluorescence, frequent use is made of light sources with a linear spectrum, primarily mercury lamps. These sources are convenient because the overwhelming portion of the energy in their spectrum is in the violet and near ultraviolet portion of the spectrum — the 410 and 365 nm lines, which are necessary to determine vitamins and catecholamines. However, these lamps also have a bright 565 nm line which occurs in precisely the region where the substances under study fluoresce. A mercury lamp is not suitable for exciting fluorescence in the determination of steriods on the basis of their glow in solutions of sulfuric acid, since in this case the maximum excitation of fluorescence occurs at 460 nm.

The radiation spectrum of an incandescent lamp practically corresponds to the region of transmission of glass optics, i.e., the region with exciting wavelengths above 400 nm, and it is necessary in studies in the ultraviolet range to use quartz optics and a mercury or xenon lamp as the light source. /18 This immediately complicates the instrument considerably.

I. G. Dlusskaya and others have described a microspectrofluorometer (Figure 5) [52] intended primarily for determining the content of glucocorticoids in the blood, and it was therefore built on the basis of the UM-2 glass monochromator with a high transmission. On the whole, the device is similar to that described by Ya. Popens, E. Sil'nish and I. Vitols [119] but the volume of the cuvette used by those authors was 3 ml, while I. G. Dlusskaya had designed a cuvette with a volume of 0.02 ml, which means that the loss in sensitivity is 150 times. The light source is an incandescent lamp (100 watts, 220 volts), which is powered by a ferroresonance stabilizer. It does not make any sense to use a higher power lamp since an increase in the light flux is usually achieved by increasing the number and the length of the filaments, whose light still cannot be focused on the inlet slit of the instrument. In this respect, even the filament of the bulb being used is too long and a large portion of the

light passes through nonproportionally. The use of this bulb is justified by the fact that its power is convenient to obtain from a mass-produced ferro-resonance voltage stabilizer.

The greatest difficulties are involved in the manufacture of the cuvette. In order to reduce spots it is desirable for the cuvette to have a rectangular cross section and for the exciting light, like the fluorescent light, to pass through the solution-glass interface at right angles. Inasmuch as the dimensions of the cuvette must be minimal, it is necessary to mount it very accurately at the focus of the light beam. Therefore, the cuvette need not be removably /19 but permanently installed, and filled by drawing into it the fluid to be studied. It is difficult to make such a cuvette with straight internal and outside walls. In the instrument a thick-walled capillary has been used as a continuous cuvette for spectrofluorometry, mounted perpendicular to the direction of the light from the monochromator. The solution to be studied is drawn in through the tapered end of the capillary by means of a syringe fastened to the opposite end. In order to reduce the spots on the outer surface of the cuvette, it is mounted in a bar of lucite whose walls are polished. The light beam emerging from the monochromator is focused exactly on the lumen of the capillary. The fluorescent light strikes the radiation receiver through the same bar of lucite, which in this case acts as a light pipe. Since the internal lumen of the capillary is round, it unavoidably develops spots of exciting light which are scattered primarily in a direction perpendicular to the length of the capillary. In this case the fluorescent light will be scattered approximately uniformly in all directions. For these reasons, the light pipe through which the light reaches the FEU is mounted at an angle of 45 degrees to the long axis of the capillary. Experience shows that this installation produces the optimum ratio between the useful signal and the background.

To measure the fluorescent light, as well as to measure the light in microcolorimetry, the FEU-35 photomultiplier uses a pH meter as a D.C. amplifier. A second light filter which transmits only light with a wavelength above 500 m is installed ahead of the FEU; the longwave boundary of the spectral interval is determined by the sensitivity of the photomultiplier.

As when working with the microcolorimeter, an important factor in microfluorometry is the concentration of the substance to be studied in a small volume of fluid. These methods are most conveniently used in those types of biochemical research in which extraction is carried out before photometry or fluorometry, for example, in a determination of corticosteriods. Such extraction is conveniently performed in small cups with conical bottoms, where the drop of extract accumulates in a depression from which it can be conveniently drawn up into the cuvette. The use of a fluid which can be extracted more easily for extraction is practically impossible since such a small drop is very difficult to draw up into the capillary. In those cases when the substance under study cannot be concentrated by an extracting agent, the solution must be evaporated and reagents added to the dry residue.

In fluorescence analysis, the measured light flux values usually turn out to be much less than in photometric analysis. The light flux intensities are

even decreased to a large extent when the excitation spectra of fluorescence or the fluorescence itself are investigated. In these cases, in order to increase the sensitivity of the radiation receiver, a number of rather complicated methods (as far as the apparatus required is concerned) have been proposed. /20

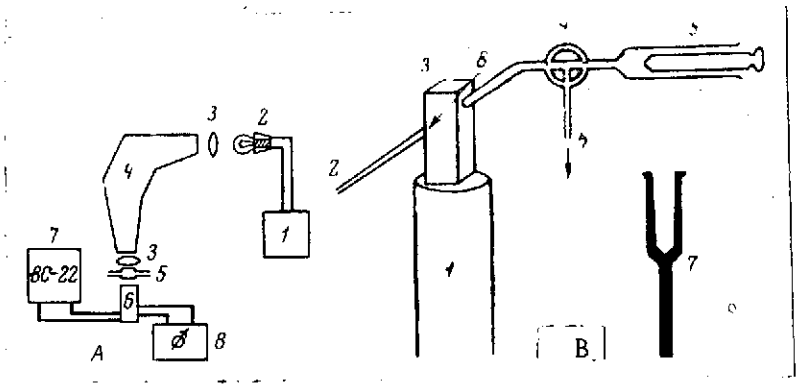


Figure 5. Diagram of Microspectrofluorometer. A, Block diagram; 1, stabilizer of incandescent lamp; 2, illuminating lamp; 3, lens; 4, monochromator; 5, capillary cuvette; 6, photomultiplier; 7, high-voltage power stabilizer for the FEU; 8, measuring device (pH meter). B, diagram of the capillary cuvette block; 1, FEU in its housing; 2, glass capillary (cuvette); 3, light pipe made of lucite; 4, three-way stopcock; 5, syringe for filling cuvette; 6, pump for rinsing and drying cuvette; 7, small vessel with a volume of 4 ml, and a conical bottom, used for filling the cuvette; 8, arrow indicates direction of the optical axis of the monochromator.

As we know, the sensitivity of the FEU is a function of the applied voltage — the higher it is (of course, without going beyond the working limits of the instrument), the higher the sensitivity. At the same time, however, the dark current and noise, i.e., the random chaotic oscillations, increase. The dark current itself causes little disturbance to the measurements, inasmuch as it can be compensated for by electrical means or subtracted from the value of the observed signal. It is a much more complicated affair to get rid of the noise. Its fast component can be eliminated to some extent by connecting a capacitor in parallel with the load resistor, but this increases the inertia of the recording and slows down the working rate of the laboratory technician, and in any case cannot eliminate the slow noise component. The sensitivity of the instrument can be increased by using a more sensitive galvanometer or (what amounts to the same thing) increasing the coefficient of amplification in the DC amplifier which serves to record the photocurrent. However, in this case, as when the FEU voltage is increased, the noise is "pulled out".

The noise of the photomultiplier varies in different sampes of exactly the same series, so that the most suitable of several examples must be selected for operation. The noise can also be reduced by cooling the FEU with dry ice [30].

In order to increase the sensitivity of the photomultiplier, various electronic circuits are used. Thus, for example, modulating the light flux by means of a rotating slotted disc provides an alternating current at the output which is rectified and measured.

The most efficient method of increasing the measurement sensitivity of light flux is the so-called single-electron method. In an ordinary FEU, the photons of the light to be measured, knocking electrons out of the cathode, produce an electron current which has an intensity that increases under the influence of the difference in voltage between the dynodes. The arrival of one photon on the cathode, even if it causes the formation of one electron, will not lead to the development of a photocurrent. In order for such a current to arise from the cathode, it is necessary to knock out a rather high number of electrons simultaneously. When working in the single-electron mode, the arrival of only one photon on the photocathode will lead to the formation of a chain reaction between the dynodes, so that the output of the FEU will record an electrical impulse of a certain intensity. The photomultiplier, operating in the single electron mode, will record the arrival of electrons on the photocathode although not every photon will cause a discharge to develop in it. Picking out the impulses of a certain intensity (in order to avoid noise) and calculating their numbers per unit time by means of a counting circuit, one /21 can measure very small photocurrent values. Not all photomultipliers can operate in the single-electron mode, only a few. Thus, according to the data of A. A. Zaytseva and I. V. Reznikov [57], the FEU-A, FEU-S, FEU-64 and FEU-79 are suitable for this purpose.

Judging by our experience, complicated electronic circuits of this kind are necessary for measurement of photocurrents only when studying fluorescence spectra; when using the fluoroscent method in the clinical biochemistry for solving analytical problems, we have no need for them.

The Microburette

For the microtitration of nonesterified fatty acids, one can use a microburette of any design which makes it possible to titrate acid down to 50 nmoles. For this purpose, we used a comparatively simple device which may easily be manufactured in any laboratory. Its advantage is that it is cheap, so that a necessary supply of microburettes with appropriate characteristics can always be on hand. The basis is a glass tube bent at right angles, whose vertical section terminates in a capillary. The other end of this tube is closed by a polyethylene plug with an opening that corresponds in size to the diameter of a piston and therefore seals it hermetically. The piston is an ordinary steel hypodermic needle with a diameter of about 1 millimeter, whose internal lumen is coated with tin. By means of a micrometer of any design, the needle may be shifted within the tube so that the titrant enters the reaction vessel.

In this microburette, the principal complexity is associated with making the plug. Polyethylene has a high thermal coefficient of expansion, but since it will not adhere to glass, a hermetic joint with the tube can be obtained only when the plug is pushed into the tube in the hot state. When it hardens the polyethylene firmly covers a special bulge in the glass tube, forming a hermetic seal.

In making a plug, it is necessary to use low-pressure polyethylene, as follows. A glass test tube whose internal diameter corresponds to the external dimensions of the future plug is lubricated with a thin layer of vaseline; this is necessary to facilitate extraction of the finished plug. Several drops of polyethylene are placed in the test tube (one can use used test tubes or any other parts) and heated to temperatures of 190 to 200 degrees. This heating is conveniently carried out in an oil or ethylene glycol bath. The end of a glass /22 tube is placed in the molten polyethylene and allowed to harden. A hole slightly smaller in diameter than the piston of the microburette is made in the finished plug providing a good seal.

In order to prepare the burette for operation, the stopper is extracted, and the titrant (0.1-1.3 N solution of alcohol alkali) is added through the opening in the stopper from a syringe fitted with a fine needle until the lumen of the burette is completely filled, after which the stopper is replaced. In microtitration it is very important that the end of the microburette through which the titrant passes into the titrated system is smaller than the diameter is possible; otherwise, there will be an uncontrolled mixing of the titrated and titrating fluids. It is practically impossible to fill the microburette through such a small capillary tip, so that the opposite end is used for this purpose.

During titration, the handle of the micrometer (of any design) makes it possible to move the piston within the cavity of the microburette. The magnitude of its movement is read on a scale on the micrometer and this is used as the basis for the calibration curve. Practically speaking, it is a very complicated matter to ensure that the piston of the microburette follows the backward movement of the micrometer rod, and there is no need that it should do so — its filling in the course of titration, i.e., extraction of the piston, can be conveniently performed manually, and then the rod of the micrometer can be moved to make sure that they are in complete contact and any movement of the micrometer rod will be transmitted to the piston of the microburette.

Titration of microquantities of fatty acids in nonaqueous media involve numerous sources of error due to the spontaneous hydrolysis of dimethylformamide and the penetration of hydrocarbons and moisture from the air. Therefore it is better to titrate automatically, with an electric motor turning the screw of the micrometer at a constant rate, ensuring its gradual forward movement. In this case, the color change at the equivalent point of titration will be very clear.

In the course of titration it is necessary to shift the medium being titrated, simultaneously protecting it from CO_2 and H_2O in the air. This can be done by using any gas stream which is free of these impurities. It is

simplest to use air which can enter either from a microcompressor or a tank from which it is expelled by an alkaline solution. The air is then dried and passes through a layer of silica gel. In order to determine whether or not it is really free of disturbing impurities, it should be passed through a bubbler filled with titration medium containing an indicator until its color changes; one can then be sure that the quality of the gas is good enough for transport.

In microtitration, special attention must be paid to the quality of the glassware, since in the usual method of washing with a chromium mixture and concentrated sulfuric acid, small quantities of acid may remain on the walls after washing. Therefore, test tubes or other glassware in which the titration is to be carried out must be rinsed out in distilled water, checking the pH and adding soda solution if necessary. /23

Studying Dried Blood

Biochemical analysis of whole blood, plasma or serum, dried on filter paper, has two important advantages over the usual biochemical methods employed in the clinic. It makes it possible to do the following:

- to preserve the material to be studied for several days and in many cases for months while awaiting analysis;

- to do without centrifuging to obtain a protein-free filtrate.

The first of these characteristics is important primarily for processing materials that have been collected under field conditions, when there is no possibility of immediately carrying out the necessary studies and the results are not required immediately. Drying the blood samples on filter paper considerably facilitates their shipment by mail.

Usually blood protein or sera are precipitated when determining water soluble substances such as sugar, urea, amino acids and so on; the precipitate is removed by centrifuging and the protein-free fluid (deproteinized fluid) is used for analysis. This procedure, although it is simple for manual operations, is difficult to automate. Therefore, in devising various automatic systems, much effort has been devoted to avoiding this stage and replacing precipitation by dialysis.

This principle is the basis for the function of the widely used automatic devices for biochemical analysis made by the firm of "Technicon" [81]. However, the dialysis method is relatively complicated and so the instruments that use it cannot be simple and cheap. In this regard, the use of dried blood is of considerable advantage, inasmuch as the water-soluble components are very easily extracted from the blood and dried on the paper, which is then placed in a solution of a fluid which denatures the proteins. The latter remain fixed in the paper and the water-soluble components pass into the solution. This method considerably simplifies automation of the analyses.

In clinical biochemistry, the use of analytical methods for working with blood that has been dried on paper was proposed by I. Bang [20] who did his work at the beginning of the present century. In his book entitled "Micromethods for Studying Blood," he described a number of methodological approaches which would make it possible to analyze dried blood for sugar, urea, amino acids, chlorides, iodine, compounds, alkilinity, cholesterol, neutral fat, phosphatides, /24 and protein fractions. Many of these methods, from the standpoint of contemporary analytical approaches, appear primitive, but they were an important step forward in their time.

Later, various authors returned to the study of dried blood on numerous occasions. This method is very important in forensic medicine for finding blood stains and classifying them. Manova, Dolezhal and Kayal proposed that urea should be determined in this fashion [245], and Svirskiy suggested that dried serum be used to determine the antigen structure, since such studies were required in centralization and the transmission of material in dry form was much simpler than in the fluid form [125]. Methods of analysis of lipid components [25, 107, 155, 194, 227] and amino acids [178, 253] have also been published.

Desiccation is one of the most effective methods of preservation of material while awaiting analysis, but not all components of the blood are preserved equally well. According to the studies of T. A. Orlova, sugar is lost much sooner than urea or the lipid components — cholesterol and phospholipids.

The following are the data expressed in per cent for the original level of substances in dried blood.

Conditions	Urea	Sugar	Cholesterol	Phospholipids
Content in liquid blood	104	102	-	-
Content in dried blood				
Immediately	100	100	100	100
After 2 weeks	97	99	100,8	99
After 2 months	100	93	100	102

According to the data of F. B. Levin and T. T. Berezov, dried blood serum is suitable for the determination of esterase, although the enzyme is partially denatured when dried. The authors feel that the losses can be taken into account in calculations which employ appropriate empirical coefficients [91]. This problem was studied in greater detail by Augustinson and Holmstead [157] who showed that if 0.05 ml of blood is dried on filter paper for 30 minutes, the sample can be stored for several weeks at 20-30° and several months at 4°. In order to preserve the material and especially the sugar, the rate at which the blood is dried and the possibility of its contamination by microbes are very important.

Complex Method of Analyzing Blood Dried on Paper /25

In order to get an idea of the composition of the blood of cosmonauts during flight, two approaches are theoretically possible: the first is the development of high-speed methods which will make it possible to analyze it directly aboard the spacecraft and the second involves the preservation of collected samples, transportation of the latter to Earth and their analysis under laboratory conditions. It is most convenient to preserve them by drying, since this method is simple and the dried sample requires no special measures for preservation. The second approach, generally a better one, is freezing, but it requires a very complicated, heavy and (what is most important) energy--consuming device, since in order to freeze the sample it is necessary to have additional energy expenditure. Processing by using various preservatives — alcohol, acetone, and so on — unavoidably has some effect on the composition of the blood, thus complicating further analysis.

Inasmuch as the collection of every blood sample under flight conditions and (what it most important) its transport to the laboratory involves certain difficulties, it is advantageous to have available a method of complex microanalysis which would make it possible to determine several components in a given batch of dried blood. Among the many systems of methods of microanalysis that have been described in the literature, none can be used under space medicine conditions without special modifications. Therefore, a complex method of microchemical analysis of blood has been worked out whose function is described below:

Extraction of dried material with a 5% solution of trichloroacetic acid	Extract	Glucose, urea, creatinine, inorganic phosphorus, acid-soluble phosphorus, galactic acid
Residue on paper		
Dissolution in alkali and dissociation in the dichloromethane--methanol-water system	Water layer	Lipid phosphorus, triglycerides (as in glycerine) iron
	Dichloromethane layer	Cholesterol, total lipids (as the total fatty acids)

Provision is made for subsequent extraction of various classes of substances from the blood which has been dried on the filter paper. /26

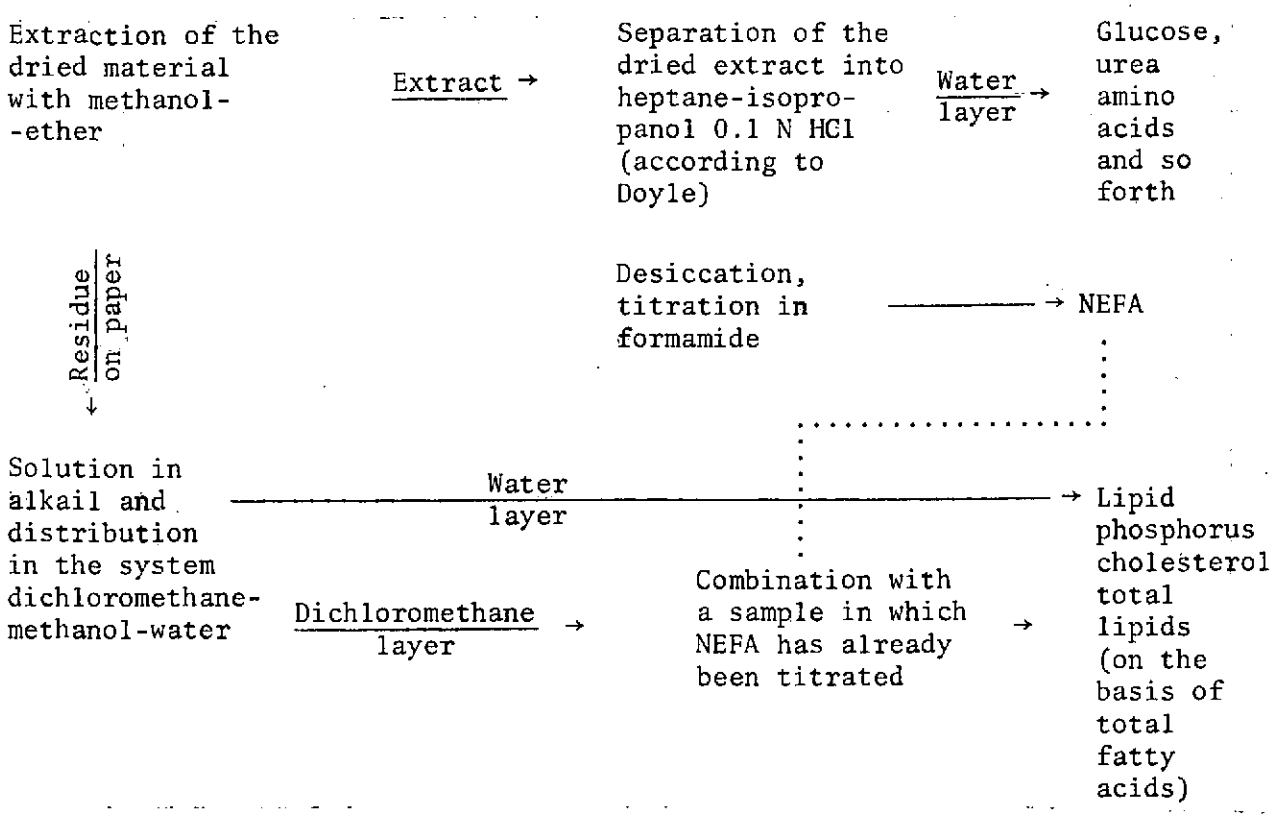
Depending on the problem being studied, it is possible to use various methods of analysis which differ in the amount of material which is used for the study, the difficulty involved and the number of substances to be investigated. When necessary, it is also possible to combine various versions

of methods of collecting samples and other approaches to processing them. As a result, a flexible system has been developed which can easily be adapted to the specific conditions of a given experiment or medical investigation.

In accordance with an abbreviated version, the dried blood, plasma or serum is initially extracted with a 5% solution of trichloroacetic acid, into which all of the water-soluble substances are transferred, while the proteins and lipids remain adsorbed on the paper. In this extract, by means of appropriate specific reactions, glucose, urea, creatinine, acid-soluble and inorganic phosphorus are determined. The clot remaining on the paper, which contains all the proteins and lipids, is dissolved in alkali, and the content of cholesterol, phospholipids, and (if necessary) total protein and iron are determined (in order that the content of hemoglobin and serum protein can be calculated on the basis of these data). This system will make it possible to utilize the collected material for investigation more economically.

A number of solutions have been tested for extracting water-soluble substances, but only trichloroacetic and hydrochloric acid have been found to be suitable. The use of alcohol, as was done by I. Bang [20], was ruled out since in addition to the water-soluble substances it extracts some of the lipids, complicating the cholesterol determination. Precipitation of proteins according to the Folin and Wu method is carried out in two stages: the blood is initially processed with sodium tungstate, then with sulfuric acid. In the first step, a considerable portion of the dried blood is rinsed away and the determination of cholesterol and phospholipids is practically impossible in a precipitate consisting of proteins and lipids. Extraction of dried blood with a solution of phosphoromolybdic acid interferes with the determination of phospholipids on the basis of phosphorus. The solution of zinc sulfate makes it possible to extract satisfactorily all of the water-soluble substances but the protein-lipid clot turns out to be too firmly attached, so that it is practically impossible to extract cholesterol from it.

In the system of analysis which begins with extraction of dried blood, plasma or serum with a solution of trichloroacetic acid, it is very difficult to determine the nonesterified fatty acids (NEFA) and amino acids. Therefore, in those cases when these substances must be studied in the same sample, as well as other ingredients, it is necessary to resort to a more complicated system of analysis.



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This boils down to the fact that the NEFA and all of the water-soluble substances are extracted at 35 to 40° by a mixture of methanol and alcohol in the proportion 3:1, and the extraction is carried out twice with batches of 5 to 10 ml each. The combined extracts are evaporated in a stream of air, avoiding overheating, and the dry residue is dissolved in a mixture of heptane and isopropanol and 0.1 N HCl hydrochloric acid (a version of the Dole system) and then stratified by adding water. The layer of water is used to determine glucose, urea and amino acids, while the heptane layer is evaporated, dissolved in a medium containing formamide, and the NEFA is determined titrimetrically. Inasmuch as a portion of the cholesterol present in the material under study has entered as well, the solution that remains after titration is combined with the extract from that part of the serum which was retained on the filter paper after extraction with methanol-ether.

For this purpose, the liproprotein clot dried on the paper is dissolved in 0.1 N NaOH, and the phospholipids are hydrolyzed. Dichloromethane and methanol are added to the alkaline rinse, after which it is stratified with water, the phosphorus changes to the aqueous phase and the cholesterol enters the dichloromethane, which did not enter the methanol-ether in the first extraction. The dichloromethane phase is combined with the formamide solution which remains after titration of NEFA; evaporation is carried out and the cholesterol is determined. Which of the systems of analysis is to be preferred is a function of the specific purposes of the investigation, what parameters must be studied, and whether whole blood or serum is used for the determination. Sugar, urea, and amino acids are distributed uniformly between the plasma and the erythrocytes,

and they may therefore be determined with equal success in whole blood and in serum. Determination of cholesterol, NEFA and lipid phosphorus in whole blood is not very informative.

The conditions for performing the work depend to a large extent on the methods of collecting the blood or other material for investigation, and this /28 in turn affects the further course of the analysis. The following is a description of the four basic versions, each of which has its own advantages and disadvantages.

The First Method of Collecting Blood Samples. 0.1 ml (rarely 0.2) of blood is expelled from a pipette onto filter paper, which has previously been cleaned by a special processing method. The piece of paper may be of any shape, but its size must not be less than 10 cm^2. The blood must be added as slowly as possible since it is important to be sure that it is completely absorbed by the paper and dried in air; the samples are kept in a desiccator over calcium chloride or another desiccator. The advantage of this method lies in the fact that the blood is applied properly, dried rapidly, can be stored conveniently and transported easily. The disadvantage is that water-soluble fractions can be extracted from such a piece of paper only by using a volume of fluid of at least 5 ml, so that the extract is not very concentrated and it is necessary to use relatively sensitive methods for its analysis. In this version, the trichloroacetic extract is used to determine glucose on the basis of the reaction with anthrone or the glucosidase method, urea is determined on the basis of the reaction with diacetylmonoxime or (after being broken down with urease) on the basis of the phenol hypochloride reaction. However, this same extract can be used for microdetermination of creatinine on the basis of the reaction with picric acid (the Jaffe reaction); the concrete details of these methods are given below.

Following extraction of water-soluble substances with trichloroacetic acid, a protein-lipid clot remains on the filter paper, which is dissolved in alkali; methanol and dichloromethane are then added to the solution. The homogeneous mixture which is formed is diluted by adding water. This procedure is similar to that suggested by Folch, a system of ethyl alcohol and chloroform, which is widely employed for separation of lipids [199]. We have used it not only because it gives the most complete separation of cholesterol in comparison with all other systems, but because when a solution of blood alkali is separated in it, all of the color produced by the products of hemoglobin breakdown change to the aqueous phase which does not disturb further determination. When using other extraction methods, it is not possible to eliminate all of the color from the solution containing cholesterol. The precipitate which is formed when the blood proteins are precipitated by trichloroacetic acid will contain the phospholipids as well as proteins, as was pointed out by King [230] and Zilversmit and Davis [291]. Since the nonlipid phosphorus passed into the TKHU solution together with the water-soluble components, the rinse from the paper contains only the phosphorus of the phospholipids.

Second Method of Collecting Samples. The blood or serum is applied to a small circular piece of paper which is placed on the bottom of a penicillin vial. If the material is more than the paper can absorb, it will not be lost, since it is inside a vessel. Due to the fact that the size of the paper is /29 much greater than in the first system, after the blood has dried it can be extracted not with 5 ml of a solution of trichloroacetic acid but with 1 ml, and the concentration of water-soluble substances will be much higher. As soon as the trichloroacetic extract has been obtained, alkali is added to the vial, which dissolves the protein-lipid clot and hydrolyzes the phospholipids. The rinse is diluted with a mixture of methanol and water. The water layer is used for the determination of the phosphorus in the phospholipids and the dichloromethanol layer is used for cholesterol determination. The advantage of this method lies in the fact that one can use the less sensitive (but more specific) orthotoluidine method of determining sugar and also in the fact that fewer reagents are consumed and more of the analysis can be carried out in the same vessel.

Third Method for Collecting Blood Samples. If it is not necessary to perform a complex blood analysis but it is necessary to determine the presence of only one substance, for example sugar or urea, it is sufficient to use 0.02 ml of blood for analysis, which is applied by a pipette from the Sali hemometer and applied to the filter paper. The spot dries rapidly, is cut out, stored in a solution of hydrochloric or trichloroacetic acid. Sugar is determined by the anthrone or glucosidase method, and urea is determined by the diacetylmonoxime or urease method.

The Fourth Method of Collecting Samples During Flight. Under spaceflight /30 conditions, collection of blood samples for biochemical analysis by ordinary methods is complicated or even impossible, since we do not know whether it would be possible to draw a fluid into a pipette without bubbles in the absence of gravity. Working with glass laboratory ware is inconvenient in the cabin of a spacecraft. Therefore, another approach is to be preferred: the blood which flows out of a wound made by pricking a finger is blotted up with filter paper which is immediately placed in a special hermetically sealed chamber that contains a desiccator (Figure 6). After return to Earth, the container is weighed together with the desiccator, paper and blood. Judging by the increase in its weight relative to preflight values, one can determine how much blood was collected. In practice, if the blood is collected so that it saturates paper of a certain size, the amount of material collected will vary slightly. At the same time that the blood is collected on the filter paper for biochemical analysis, smears are prepared on a slide for counting the components by the leucocytic formula in the usual fashion.

For convenience in carrying out all these procedures involved with the collection of blood samples during flight, the necessary equipment is kept in a single container — the self-contained blood microanalyzer. It is a metal box in which 10 hermetically sealed containers for blood samples, 10 containers with disinfectant, slides for making blood smears, plastic discs for making smears, scarifier needles (Frank needles) and a linen cloth are contained. The basic

element of the kit is the containers for blood samples. The kit is put together so that each container can be removed for weighing without disturbing its seal.

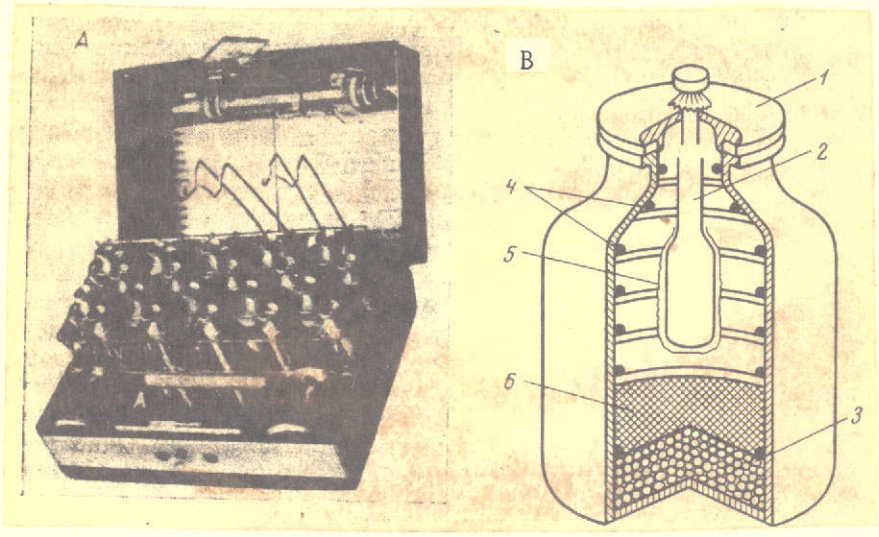


Figure 6. General View of Box (A) and Diagram of Container (B) for Collection and Storage of Dried Blood Samples During Flight. 1, Hermetically sealed cover; 2, rod to which paper is fastened for collecting blood samples; 3, desiccator (silica gel); 4, spring; 5, paper for collecting samples; 6, screen.

Description of Methods for Microchemical Analysis of Blood

Preparation of Small Pieces of Paper for Collecting Blood

Filter paper, including ash-free filters, as well as paper for chromatography frequently contain various impurities which disturb the determination of individual substances in dried blood, so that such paper must be purified. It is very important to use varieties of paper that do not lose their mechanical strength when cleaned; otherwise, the paper fibers entering solution used for determination of sugar by the anthrone method will considerably distort the results of the analysis. In addition, lipid impurities are also very important because they disturb the determination of cholesterol. The quality of the paper is less significant for other methods, especially the fermentative ones.

In order to get rid of disturbing water-soluble substances, a packet of appropriately cut paper is washed with a 3-5% solution of acetic acid and kept in it at 60° for 2 to 3 hours; then the acid is wiped off and the paper is /31 rinsed twice more. Under these conditions, the paper is washed 3 times with distilled water then extracted in a Buchner funnel and dried at a temperature of 40 to 50°. If it is desirable to determine the cholesterol in the blood, the paper must also be processed in a Soxhlet apparatus: initially it is

extracted with methanol for the duration of 1 workday and then for 2 workdays with dichloromethane. It is then dried in air.

The amount of impurities which remains after such treatment may be a function of many factors, so that the purity of the paper must be checked periodically by conducting control (blank) experiments in which the analysis is performed on a strip of paper without any blood on it.

Application of Material to Paper and its Storage

Blood, serum or plasma are applied to paper filters by blowing the material out of a pipette. Inasmuch as viscous fluids are being applied, it is necessary to blow them out as slowly as possible so that the minimum quantity of material left behind on the walls, where the blood frequently dries in the form of patterns on the filters. If the blood is weightless, as it is anticipated will be the case during spaceflights, it will saturate all of the paper. If the material is expelled from a pipette, it is usually convenient for complete analysis to collect 0.1 ml but if it is planned to carry out only one study of some kind, for example determination of sugar or urea, 0.02 ml of blood will suffice for analysis. Such a small amount of blood is easy to collect and the spot that it forms will dry more rapidly. The latter fact is of great significance, since rapid drying of the blood promotes better storage of its components.

Notes can be made with a pencil on the filter paper containing the blood and the paper can be kept in a hermetically sealed container above a desiccator (silica gel, calcium chloride).

Analysis of Blood Samples Collected on Large Filters

Obtaining TCA Extract for Determining Water-Soluble Substances

Filter paper with blood dried on it, plasma or serum, in the amount of 0.1 ml is moistened in a conical flask with 5 ml of 5% TCA. If the blood spot is smaller than the size of the paper and small areas remain around the edges, they can be cut off to advantage. If not 0.1, but let us say 0.05 ml of blood has been collected for analysis, not 5 but 2.5 ml are extracted. In those /32
cases when the blood is collected by weight and the amount collected is not large (let us say 88 mg) it is still moistened with 5 ml of solution and this simplifies further calculations. Our experience shows that it is convenient to collect either 0.1 ml of fluid to be studied, where the extract will suffice for repeated determination of both urea and sugar, or 0.02 ml, when 1 ml of TCA solution is used for extraction and the extract wull suffice for only one determination.

The paper together with the blood is kept in the TCA solution for 15 minutes, and all of the water-soluble substances can be extracted in this period of time, it is then removed and dried in air.

Determination of Sugar by the Anthrone Method

Principle. The glucose contained in the trichloroacetic filtrate, when heated, reacts with the anthrone solution in sulfuric acid to form a green color.

Reagents: (1) 5% solution of trichloroacetic acid (TCA);
(2) 0.2% solution of anthrone in 95% sulfuric acid (by volume).

This is prepared on the day of the determination;

(3) standard solution of glucose, at a concentration of 100 mg%. It is kept in a refrigerator. On the day of the analysis, it is diluted with a 10 times greater amount of 5% TCA.

Method of Determination. To 1 ml of TCA extract of dried blood, we add 2 ml of anthrone solution in sulfuric acid, shake vigorously and place in a boiling water bath for 5 minutes. It is necessary to check carefully to be sure that no water enters the test tube, since this would cloud the solutions. In order to protect them, the test tubes are covered with pear-shaped glass stoppers. After heating is over, they are cooled in cold water and subjected to photometry at a wavelength of 625 nm.

The results are plotted on a calibration curve; to plot this curve solutions of glucose are treated in a similar fashion in trichloroacetic acid, which contains 10, 20 and 30 micrograms of glucose per milliliter. These solutions are prepared by adding 0.1, 0.2 and 0.3 ml of standard solution, respectively to 1 ml 5% TCA.

Determination of Galactic Acid

Principle. In processing the solution of galactic acid with concentrated sulfuric acid in the presence of copper salt and orthophosphoric acid, an acetic aldehyde is formed which reacts with paraoxydiphenyl ($C_6H_5 \cdot C_6H_4 \cdot OH$) with development of a violet color. The reaction is very sensitive but capricious, so that it is important to maintain all of the conditions of the analysis strictly during determination.

Reagents: (1) Mixture of 3 g copper sulfate ($CuSO_4 \cdot 7H_2O$) and 9 ml orthophosphoric acid. A homogeneous solution must be prepared, but this requires some time;

(2) 1.5% solution of paraoxydiphenyl in dimethylformamide. 15 ml paraoxydiphenyl are dissolved in 1 ml diphenylformamide. /33

Method of Determination. The paper with the dried blood is soaked with a 5% solution of trichloroacetic acid at the rate of 5 ml per 0.1 ml of blood. To the 0.2 ml of trichloroacetic extract we add 0.1 ml of a mixture of copper sulfate and orthophosphoric acid and 2.5 ml concentrated sulfuric acid. Extraction is carried out vigorously; precisely 3 minutes later the container

is placed in a boiling water bath, then cooled for 3 minutes in an ice bath. One drop of a 1.5% solution of paraoxydiphenyl in dimethyl formamide is added; this drop must fall precisely in the center of the test tube, after which extraction is carried out vigorously and the solution is allowed to stand for 10 minutes at room temperature. It is then heated in a boiling water bath for one and a half minutes, cooled in water and subjected to photometry at a wavelength of 565 nm.

At the same time, a blank test is run in which a piece of paper without blood (but of the same size) is moistened in the solution of trichloroacetic acid and the remainder of the determination is carried out as above. To plot the calibration curve, solutions of galactic acid or lithium galactate are prepared in 5% trichloroacetic acid, containing 0.2-2.0 micrograms in 0.2 ml, processed like the experimental samples, but subjected to photometry against their own blind test in which only the solution of trichloroacetic acid was used. In order to obtain the extraction data, it is necessary to maintain all of the conditions of determination very strictly and measure the time with a stop watch, carefully agitating the samples after adding the reagents.

Determination of Urea on the Basis of the Reaction with Diacetylmonoxime

Principle. The urea contained in the trichloroacetic extract reacts when heated with diacetylmonoxime to form a solution which is colored yellow.

Reagents: (1) 1% solution of diacetylmonoxime in 5% acetic acid; (2) 0.5% solution of chloramine, diluted 10 times before use; (3) mixture of two volumes 95% sulfuric acid and one volume 42% hydrochloric acid ($HClO_4$), prepared immediately before use;

(4) a standard solution of urea at a concentration of 100 mg%, diluted 100 times before use with a 5% solution of TCA.

Microvariant of the Determination. To 0.5 ml of TCA extract we add 0.15 ml of a mixture of sulfuric and hydrochloric acids and 0.25 ml of a solution of diacetylmonoxime in acetic acid, agitating carefully. The solution is heated in a boiling water bath for 10 minutes, then cooled for 2 to 3 minutes in cold water; then 0.125 ml of a solution of chloramine is added to stabilize the color. The samples are subjected to photometry at a wavelength of 475 nm against a control in semimicrocuvettes with an optical path length of 1 cm. The reaction is photosensitive, so that all of the operations must be carried out while /34 avoiding direct illumination and covering the samples with lightproof caps during the time they are being heated in the water bath.

The calculation is carried out using a calibration curve, plotted by using a solution of urea in 5% trichloroacetic acid, each 0.5 ml of solution containing 2, 3 or 4 micrograms of urea (calculated on the basis of the total amount of substance and not the nitrogen, as is done in some methods). The calibration curve is usually bent upward.

Macrovariant of the Determination. Series operation with quantities of solutions on the order of one tenth of a milliliter is inconvenient, so if there is no need to make parallel determinations from a given filter, it is more practical to work in the macrovariant which uses 2 ml of TCA filtrate to which are added 0.6 ml of a mixture of sulfuric and hydrochloric acids, 0.5 ml of a solution of diacetylmonoxime and 0.5 ml of a solution of chloramine for stabilization of the color. In this case further determination proceeds as in the macrovariant but the total quantity of solution subjected to photometry is 3.6 ml and it is possible to work with mass-produced rectangular cuvettes for the spectrophotometer.

Determination of Creatinine

Principle. Creatinine in an alkaline solution reacts with picric acid to form an orange color. Since the breakdown products disturb this reaction, they are removed by washing with an ether solution.

Reagents: (1) 0.1% solution picric acid;
(2) 3.3% NaOH solution. Prior to using these solutions, they are mixed in equal parts;
(3) ether;
(4) liquid nitrogen.

Method of Determination. One ml of trichloroacetic filtrate is placed in a flask with a conical bottom and an external ground-glass joint, with a capacity of 40-50 ml (Figure 7). Five ml are added to this and extracted. The ether is drawn off and the extraction is repeated once more. Most of the trichloroacetic acid and impurities are removed thereby. The flask containing the solution is connected to a device for lyophilic drying by means of a ground-glass joint the device is shown in Figure 7. Liquid nitrogen is poured into it. The flask must have an external ground-glass joint so that the lubricant, without which it would be impossible to seal the joint between the flask and the device for vacuum drying, does not enter the solution.

The air is drawn out of the system by means of an oil forevacuum pump and the liquid nitrogen is caught in the trap. The water and the remainder of the trichloroacetic acid are evaporated in vacuum and precipitated on the walls of the trap. To speed total evaporation of the sample under study it is heated in water at 30-40°. The evaporation time is a function of the degree of rarefaction in the system which in turn is determined by the quality of the ground-glass joints and the performance of the pump. An indication of a poor /35 vacuum is rapid evaporation of the nitrogen from the trap. If the vacuum is a good one, as soon as all the filtrate has been distilled off, the nitrogen will not necessarily boil.

To the dry residue, we add 0.1 ml of a mixture of equal parts 0.1% solution of picric acid and 3.3% solution of NaOH. It is used carefully to wash off the walls of the flask, which is allowed to stand 15 minutes, after which 0.4 ml distilled water is added and photometry is performed in a capillary cuvette (see Figure 4) with a light filter that transmits light with a wavelength of 490 nm.

At the same time, a control flask is prepared in which 0.3 ml of a mixture of reagent (picric acid and alkali) is mixed with 1.2 ml of water. This sample is used to fill a capillary microcuvette and the apparatus is set to zero extinction or 100% transmission (depending on the design of the instrument). Then the cuvette is filled with the solution under study and the quantity of transmitted light is measured. After the series of measurements has been concluded, the 0 setting is checked by using the control sample.

Calculation is performed on the basis of a calibration curve, for whose preparation the flasks used for vacuum drying are filled with 0.125, 0.25, 0.5, 0.75 and 1.0 ml of a creatinine solution containing this substance in a concentration of 1 microgram per ml and 0.5 ml each of a 5% solution of trichloroacetic acid, treated twice with batches of 5 ml each of ethyl alcohol, and then evaporated as in the case of the experimental samples. The performance of the color reaction and the photometry are the same as in the experimental samples.

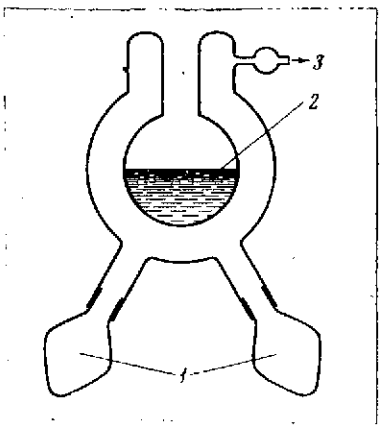


Figure 7. Device for Eliminating Trichloroacetic Acid by Vacuum Distillation. 1, Flask with solution to be studied; 2, liquid nitrogen; 3, outlet to vacuum pump.

Obtaining an Extract for Determination of Fat-Soluble Substances

The filter containing the blood, after extraction in trichloroacetic acid, is dried in air; this usually requires about an hour; it is then cut into small pieces with scissors and moistened with 1.5 ml of a 0.3 N solution of caustic soda. The material must remain in this solution /36 until the blood is completely washed out of the paper (if convenient, leave overnight). 0.5 ml of the rinse is taken for mineralization with subsequent determination of lipid phosphorus, iron and if necessary total nitrogen, and the remaining 1.0 ml of rinse, together with the piece of paper, is covered with 15 ml of a mixture of dichloromethane and methanol (2:1). After careful stirring, the mixture must be homogeneous and should not divide into two phases. If this separation occurs, it means that the ratio between the quantities of alkaline solution, methanol and dichloromethane has been disturbed, for example, because methanol containing water was used or because some of the alkaline rinse was lost.

The homogeneous mixture formed after alcohol and dichloromethane are added is stratified, adding exactly 3 ml of water, to form 2 phases: an upper colored phase which is the water phase and a lower colorless one which contains no water. In order for the stratification to occur rapidly, the solution must be agitated; however, excessively vigorous agitation will lead to the formation

of an emulsion and the phases can be separated only by centrifuging. It is therefore very important to choose the intensity of agitation at which the most rapid and complete separation of the phases takes place. However, even under the most optimum conditions, the lower layer will be cloudy immediately after separation and must be allowed to stand for several hours so that all of the drops of the water phase can rise. When centrifuging at 2000 rpm, clearing is accomplished in 10 minutes. To determine cholesterol, one can use only the clear solution in the lower phase, since even a small quantity of the upper colored phase will affect the result of the color reaction.

Determination of Cholesterol

Principle. Under the influence of a mixture of concentrated sulfuric acid and acetic anhydride, cholesterol is converted into a colored compound (the Lieberman-Burkhard reaction).

Reagents: A mixture of concentrated sulfuric acid, acetic anhydride and dichloromethane in the proportion 0.5:5:10 (by volume). It is prepared on the day of the determination.

Method of determination. 4 ml of the lower transparent layer is drawn with a pipette with a tapered tip and placed in a dry test tube, then evaporated in a stream of air. To accelerate the evaporation, it is convenient to heat the test tube containing the sample by means of a reflector: when analyzing individual samples, they may be evaporated in a boiling water bath, carefully immersing the tubes and agitating them constantly.

0.9 ml of a mixture of sulfuric acid, acetic anhydride and dichloromethane is added to the dry residue, left in darkness for 30 minutes and the measured spectrometrically on 3 wavelengths — 525, 625 and 725 nm. In calculating the half-sums of the extinction values measured at wavelengths of 525 and 725 nm, /37 calculations are made on the basis of the extinction value at a wavelength of 625 nm. The value which is obtained is proportional to the cholesterol content.

To plot the calibration curve, a series of standards is prepared: 2 ml of cholesterol are dissolved in 10 ml dichloromethane and measured into flasks in amounts of 0.5, 1.0 and 1.5 ml; these solutions are then evaporated. Each sample contains 100, 200 and 300 micrograms of cholesterol, respectively. They are dissolved in 15 ml of a mixture of dichloromethane-methanol (2:1), adding 1.0 ml of a 0.3 N solution of caustic soda and 3 ml of water. As in the experimental samples, 4 ml of the lower layer are taken, dried and subjected to the color reaction. The resultant extinctions correspond to blood cholesterol contents of 100, 200 and 300 mg%.

Determination of Lipid Phosphorus

Reagents: (1) A mixture of 4 parts 95% sulfuric acid and 1 part 42% hydrochloric acid;
(2) a molybdenum reagent is prepared by mixing equal parts of 6.5% solution ammonium molybdenate in water and 13 N sulfuric acid;

(3) a solution of eiconogen (1-amino-2-naphthol-4-sulfonic acid) in bisulfite buffer. A basic solution is prepared, for which 15 g sodium acid sulfate (sodium bisulfite) is dissolved in 50 ml of water together with 0.5 g sodium sulfite, after which 0.25 g of purified eiconogen is added to this solution and dissolved. Then water is added up to 100 ml. Instead of sodium bisulfite, one can use an equal quantity of sodium pyrosulfite, which when dissolved in water hydrolyzes to form bisulfite. The purity of the eiconogen preparation can be determined on the basis of its external appearance — it should be colorless or slightly pink. If the preparation is colored, it must be recrystallized from the bisulfite buffer, drawn into a Buchner funnel and washed in it with cold water and alcohol;
(4) isobutyl alcohol.

Method of determination. Lipid phosphorus is transferred to the alkaline blood smear on the paper. For analysis, 0.5 ml of this rinse (the remainder is used to determine cholesterol) is transferred into a flask for combustion with a volume of 10 ml, adding 0.1 ml of a mixture of sulfuric and hydrochloric acids and heating on a hot plate with an enclosed coil. The first time, heating is carried out carefully until the water boils; then, when heavy white vapor appears, the flasks are sealed with glass stoppers and heating is continued until the solution is completely colorless. This usually requires 5 to 6 hours.

The mineralizate is dissolved in 5.0 ml of water, a sample of 0.5 ml is taken for determination of iron, 1.0 ml of molybdenum reagent is added with 0.5 ml of eiconogen and the solution is placed in a water bath at 38° for 20 minutes. The color which is formed is extracted with 2 ml of isobutanol. The alcohol layer is separated by centrifuging and its color is measured in a semimicrocuvette at a wavelength of 680 nm. /38

In plotting the calibration curve, solutions are prepared which contain (in 0.4 ml) 1.5, 2.0 and 2.5 micrograms of phosphorus in the form of its potassium salt KH_2PO_4, to which is added 0.1 ml of a mixture of acids and a determination is performed as in the experiment. In preparing the standard solution, this salt is preferable to the others, since it does not contain water of crystallization. 4.383 g of this salt contains 1 g of pure phosphorus.

Determination of Iron

Reagents: (1) 25% solution of ammonia in water;
(2) 2% solution of hydroquinone in water;
(3) 1% solution of orthophenanthroline in water.

Method of Determination. 0.5 ml of the mineralizate are collected in a test tube to which 0.08 ml of a 25% solution of ammonia is added; then the mixture is evaporated over the flame of a gas burner until the smell of ammonia disappears. The residue is dissolved in 0.5 ml of doubly distilled water, to which is added 0.05 ml of a 2% solution of hydroquinone and 0.05 ml of a 1% solution of orthophenanthroline. After 7 to 10 minutes, photometry is performed in semimicrocuvettes at a wavelength of 510 nm.

Analysis of Blood Samples Collected on Small Filters in Vials

The version of analysis described above, in which the blood is placed on large pieces of filter paper, is not always convenient since the material cannot be extracted with small volumes of solvent, so that it is impossible to use the less sensitive but chemically more specific orthotoluidine method of determining blood sugar, the consumption of reagent is increased, and it is necessary to wait until the blood has dried. In order to avoid these shortcomings, another method of analysis has been devised in which the blood or serum is placed on comparatively small pieces of filter paper which are located in flasks (of the penicillin type) with a volume of 10 ml; in this case the blood does not run off and so it saturates a thicker layer. The material collected in this fashion can be transported immediately after collection; extractions are carried out in the same vial, making it possible to save glassware. The disadvantage of this version is the fact that the blood dries slowly and some of the components may be lost. In addition, it is less convenient to transport the filters containing the blood in the vials than it is to transport the dried filters.

Pieces of filter paper that have been purified in the manner described for the previous method are cut into small circles about 2 cm in diameter and placed on the bottoms of flasks.

The blood, plasma or serum (0.1 ml) is blown out of a pipette onto the bottom of the flask, onto the paper, where it is dried in air.

To a small flask with dried serum or plasma, we add 1.0 ml of a 5.0 solution of TCA from a pipette and allow it to stand for 30 minutes. 0.5 ml of the extract is removed for sugar determination by the orthotoluidine method and 0.25 ml for urea determination. /39

Sugar Determination

Principle. Orthotoluidine, heated when it contains glucose in a trichloroacetic extract, gives a color which is very specific.

Reagents: 6% solution of orthotoluidine in glacial acetic acid, containing 0.15% thiourea.

Method of Determination. To 0.5 ml of TCA extract, we add 2 ml orthotoluidine reagent and place the container in a boiling water bath for 10 minutes, cool and measure light absorption at a wavelength of 640 nm.

Determination of Urea

Principle and reagents. Same as in the determination of urea in the previous method.

Method of Determination. To 0.25 ml of TCA extract we add 1.5 ml of a 5% solution of trichloroacetic acid, 0.45 of a mixture of sulfuric and hydrochloric acid (2:1) and 0.4 ml of a 1% solution of diacetylmonoxime in 5% acetic

acid. This is placed in a boiling water bath for 10 minutes, cooled and then 0.4 ml of a 0.05% solution of chloramine is added, after which light absorption is measured at a wavelength of 475 nm.

Obtaining An Extract for Determination of Lipids

When the samples have been taken from the filter for determining sugar and urea, using the same flask containing the paper and the blood in which a small amount of trichloroacetic filtrate remains, we add 0.25 ml of a 1 N solution of caustic soda and allow it to stand for several hours until all of the blood or serum is dissolved in the alkali. It is best to leave the sample with the alkali overnight, but if this is not possible, it is frequently sufficient to allow it to stand for 4 to 5 hours. If the blood is being studied, the time when it has all washed out of the paper is clearly evident if the serum or plasma has dried; however, it is difficult to be sure that all of it has gone into solution, so incubation must be prolonged.

As soon as the dried material has been dissolved in the alkali, we add 2.5 ml methanol and 5 ml dichloromethane to the same flask, agitate carefully and add 1.5 ml of water to the homogeneous mixture that is formed; the mixture then separates out into 2 phases, an upper aqueous one and a lower dichloromethane one. As in the case of the separation of the phases in the previous method, the nature of their separation is largely a function of how vigorous the agitation was — it should be carried out so that the phases succeed in /40 separating but not so a permanent emulsion is formed. It is best to do this by means of a glass stirring rod which is rotated rapidly at the interface between the two phases. If the agitation is successful, after 30 to 40 minutes of standing the phases will separate clearly and the lower dichloromethane phase will be completely clear. In the opposite case, it will be necessary to resort to centrifuging at 2 to 2.5 thousand rpm, which can be carried out using the same vials.

As a result of contact with the alkali, all of the phospholipids are hydrolyzed, and the inorganic phosphorus which is formed changes to the aqueous phase, while the cholesterol is all found in the lower phase.

Determination of Cholesterol

Principle and reagents. Same as in the previous method.

The lower phase obtained by the method described above (4 ml) is evaporated in a stream of air, heating the test tubes gently with an electrical reflector. To the dry residue we add 2.5 ml of a mixture of sulfuric acid, acid anhydride and dichloromethane, 0.5:5:10 (by volume), allow this to stand in darkness for 30 minutes and subject it to photometry at a wavelength of 625 nm. If for some reason contaminants have entered the evaporated residue of the lower phase, it will be necessary to perform photometry as was done in the previous method on 3 wavelengths (525 [sic] and 725 nm) and to calculate the correction, whose existence has made it necessary to perform photometry on 3 wavelengths, consists of the substances that are contained in the paper, which it is difficult to

remove. Since in this version the size of the paper is much smaller, the contaminants introduced are correspondingly fewer.

Determination of Phosphorus

Principle. In determining inorganic, acid-soluble and lipid phosphorus, the same reagents are used. The ability of eiconogen (2-amino-2-naphthol-4--sulfate) to reduce phosphoromolybdic acid to form molybdenum blue is used. The sensitivity of the reaction can be increased considerably if the reduction is performed during boiling, and photometry is carried out at a wavelength of 830 nm; in this case it will be possible to determine the amount of phosphorus on the order of 1 microgram in the sample.

Reagents: (1) molybdenum reagent — 1 g of ammonium molybdenate $(NH_4)_6 \cdot Mo_7 O_{24} \cdot 4H_2O$ are dissolved in 500 ml of 0.5 N sulfuric acid;

(2) A solution of eiconogen in bisulfite buffer — 150 mg of sodium sulfate are dissolved in 30 ml of water $(NaSO_3)$ and 4.5 g of sodium acid sulfate $(NaHSO_3)$ and 75 mg of eiconogen are added to the solution. Instead of $NaHSO_3$, one can /41 use a corresponding amount of $Na_2S_2O_5$;

(3) 42% hydrochloric acid $(HClO_4)$, this acid can be used in another concentration, but it is then necessary to change the volume appropriately, inasmuch as the amount of acid added must be constant.

Method of Determining Inorganic Phosphorus. The quantity of trichloroacetic extract corresponding to 0.02 ml of blood is used, i.e., if the serum was extracted in small flasks with 1 ml TCA solution, 0.2 ml is used but if 0.1 ml of dried blood was extracted with 5 ml TCA solution, 1 ml of extract is used. A 5% solution of TCA is added to the solution, so that the total volume is 1 ml; 3 ml of the molybdenum reagent and 1 drop of eiconogen solution. This is mixed and placed for 7 minutes in a boiling water bath, then cooled and subjected to photometry at a wavelength of 830 nm, against a blank sample, in which a trichloroacetic extract has been prepared in a similar fashion from pure filter paper.

Method of Determining Acid-Soluble Phosphorus. Trichloroacetic extract, corresponding to 0.01 ml whole blood or 0.02 ml serum, is transferred to a large test tube (20 × 200 mm) to which 0.5 ml 42% hydrochloric acid is added and heated at 180-200° until the sample is completely evaporated and does not fluoresce. In order to avoid overheating the samples, it is convenient to heat them in an ethylene glycol bath, whose boiling point corresponds to the optimum conditions of mineralization in hydrochloric acid. It is also possible to heat them on an electric plate or in a sand bath, adjusting conditions accordingly. Heating must be such that the water contained in the sample is evaporated but the hydrochloric acid whose dihydrate $HClO_4 \cdot 2H_2O$ boils at 200° condenses on the walls of the test tube and trickles to the bottom.

To the cooled mineralizate we add 3 ml of molybdenum reagent and 1 drop of eiconogen solution, boil for 7 minutes in a water bath to develop the color, and perform photometry at a wavelength of 830 nm against a blank prepared in the same fashion.

Method of Determination of Lipid Phosphorus. From the layer of water which is left behind after the alkaline rinse is separated in the methanol-dichloromethane-water system, a portion is selected which corresponds to 0.02 ml of blood or serum, and mineralized with 0.5 ml 42% hydrochloric acid, as the acid-dissolving phosphorus is mineralized. To the cooled mineralizate we add 3 ml of molybdenum reagent and 1 drop of eiconogen solution, heat in a boiling water bath for 7 minutes, and subject to photometry at a wavelength of 830 nm against a blank.

Plotting the Calibration Curve. The calibration curve is plotted in the same way whether working with mineralized or nonmineralized material, so that in practice it is sufficient to have only one curve for all 3 determinations. /42
To plot it, a standard solution is prepared, containing 438.3 mg KH_2PO_4 in 100 ml water; 1 ml contains 1 mg phosphorus. Before using it, it is diluted 100 times with water to obtain a working standard solution that contains 10 micrograms of phosphorus per ml. To prepare the calibration curve, 0.5-3.0 micrograms of phosphorus are added to the sample (to 0.5-0.3 ml of a working standard solution, respectively); the volume is then brought up to 1 ml with a 5% solution of trichloroacetic acid or mineralized with hydrochloric acid in the same way as the experimental samples, the molybdenum reagent is added, then the eiconogen; the whole is heated in a boiling water bath and subjected to photometry.

Titrimetric Determinations of Nonesterified Fatty Acids (NEFA)

Principle. NEFA are extracted from dried serum or plasma by a mixture of methanol and ether, purified in a heptane-isopropanol-water system, transferred to a medium containing dimethylformamide and titrated with alcohol alkali.

Reagents: Organic solvents of at least C.P. grade are used.

(1) Mixture for extraction of NEFA. Three parts methyl alcohol and 1 part ethyl alcohol are mixed;

(2) Mixture for extraction of NEFA according to Dole. On the day of the determination, 8.0 ml isopropanol, 2.0 ml heptane, 2.0 ml water and 0.2 ml 1 N sulfuric acid are mixed (1 N HCl can be used instead of the sulfuric acid).

(3) Indicator solution. Ten mg of thymol blue are dissolved in 0.5 ml dimethylformamide;

(4) Dimethylformamide medium for titration of NEFA. Before use, 3.5 ml dimethylformamide, 1 ml ethyl alcohol, 0.5 ml chloroform and 0.025 ml thymol blue solution are mixed in dimethylformamide;

(5) Alcohol solution of alkali. Initially a saturated solution of KOH in water is prepared, with approximately 15 g of alkali being required for each 10 ml of water. On the day of the determination this solution is diluted 100 to 300 times with alcohol.

Method of Determination. 0.1 ml of serum or blood plasma is dried on the bottom of a penicillin flask on paper. Extraction is carried out twice, using batches of 4 ml each of the 3:1 methanol-ether mixture, with each extraction lasting 30 minutes at 50° (in a water bath), after which the flasks are covered with cork stoppers wrapped in aluminum foil. Both extracts are combined and evaporated in a stream of air while being heated by an electrical reflector. Initially evaporation was performed in large test tubes that were capable of holding all 8 ml of the combined extract. Then, when very little fluid was left, they were decanted into small test tubes with conical bottoms and a volume of about 2 ml, after which evaporation to dryness was performed.

Then 0.6 ml of Dole mixture was added to the dry residue together with 0.2 ml water and 0.3 ml heptane; the mixture was centrifuged, the top layer decanted into a second test tube, evaporated to dryness and dissolved in 0.05 ml of medium for titration, containing formamide and thymol blue. The test tubes were covered, allowed to stand for 20 minutes until all of the NEFA was dissolved, then titrated with alcohol alkali. /43

During titration, air bubbles must be passed through the medium continuously, so the tip of the burette must also be submerged in the medium. The end of titration is clearly evident — the medium changes color, from yellow to blue through green. Due to the instability of dimethylformamide in an alkaline medium, samples that have already been titrated lose their color quite rapidly, so that it is very important to add the titrant at a constant rate.

Calibration Curve. Usually the titres are checked in acid-alkaline titration on the basis of a standard solution of strong acids. In determining NEFA this is only necessary when adjusting the method for checking the operation of the microburette. The specific titre of the solution is checked by titration of standard amounts of fatty acids (0.02-0.2 micromole). It is most convenient for this purpose to use stearic acid, whose standard solution is prepared in chloroform. To plot the calibration curve, 7, 14 and 28 micrograms of acid are used, placed in small test tubes in a chloroform solution; the chloroform is evaporated, the acid is purified in the Dole system and titration is carried out as with the experimental samples. Simultaneously with titration of the experimental samples and standards, it is necessary to titrate the medium as well. These values were calculated from the results of titration of experiments and standards.

Determination of Glucose by the Fermentative Method

Principle. Glucose is oxidized by the enzyme glucosidase to form hydrogen peroxide; the amount of the latter is a function of its ability to oxidize phenolphthaline to phenolphthalein in the presence of copper ions. Inasmuch as glucosidase is active only in a neutral medium, and phenolphthalein is colored in an alkaline medium, the trichloroacetic filtrate is initially neutralized

with dibasic sodium phosphate; the fermentation reaction is then checked and an alkaline reaction is produced by adding a solution of caustic soda.

Reagents. (1) 0.25 M solution of secondary sodium phosphate, 9.7 g $Na_2HPO_4 \cdot H_2O$ or 18 g $Na_2HPO_4 \cdot 12\ H_2O$ are dissolved in 200 ml of water;

(2) Glucosidase solution: 3 mg of enzyme, with an activity of about 10,000 units per mg, is dissolved in 10 ml of water;

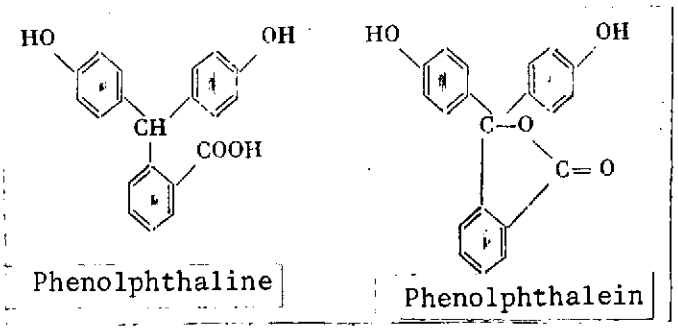
(3) 0.5% solution of phenolphthaline: 75 mg of substance are dissolved in 15 ml of 0.1 N NaOH; the solution should remain colorless or slightly pink, since colored solutions are not suitable for the operation. In order to make the solution more stable, 3 mg of trylon can be added to it; this substance, /44 by binding ions of heavy metals, prevents oxidation of phenolphthaline by the oxygen in the air. Larger amounts of trylon are unacceptable since they disturb the oxidation of the phenolphthaline by hydrogen peroxide.

(4) The working alkaline solution of phenophthaline is unstable, so that it is prepared immediately prior to use. 30 parts of 0.3 N caustic soda, 2 parts 0.5% phenolphthaline solution and 1 part 0.12% copper sulfate solution (120 mg $CuSO_4 \cdot 5\ H_2O$ per 100 ml water) are mixed.

Method of Determination. 1 ml of trichloroacetic extract, corresponding to 0.02 ml of blood (if it is more concentrated, 5% trichloroacetic acid is diluted accordingly) are added to 2 ml of a 0.25 M solution of secondary sodium phosphate and 0.1 ml (2 drops) glucosidase solution. The mixture is allowed to stand 1 hour at room temperature; then 2 ml of the working alkaline solution of phenolphthaline are added and the solution is again allowed to stand 30 minutes. When this time has elapsed, photometry is carried out in cuvettes with an optical path length of 1 cm at a wavelength of 520 nm against a blank sample. If it turns out that the blood glucose level is above 200 mg%, the analysis must be repeated, using 1 ml of extract diluted twice with a 5% solution of trichloroacetic acid for the determination.

The calculations are made on the basis of a calibration curve; the latter is plotted by using 10, 20 and 40 micrograms of glucose for analysis, which is dissolved in 1 ml 5% trichloroacetic acid. These samples correspond to blood glucose levels of 50, 100 and 200 mg%.

Synthesis of phenolphthaline. Phenolphthaline is not a chemically stable compound and is readily oxidized by the oxygen in the air, with the oxidation being particularly rapid in aqueous solutions in the presence of salts of heavy metals. For this reason, phenolphthaline is not sold but may be easily synthesized in the laboratory from the widely available substance known as phenolphthalein.



For this purpose, 5 g of phenolphthalein, 250 ml of a 20% KOH solution and 2.5 g of zinc dust are placed in a liter flask with a reflux cooler. The solution, which is dark red in color, is boiled until it is colorless; this usually requires 5 to 8 hours. There is practically no need to add solution prior to complete decolorization; it is sufficient that it merely becomes /45 slightly pink in color. During boiling the zinc dust slowly reacts with the alkali and the hydrogen thus formed reduces the phenolphthalein.

As soon as the solution has been decolored, the zinc dust residue is separated by filtering the still hot solution through a glass filter; the filtrate is transferred to a large beaker and allowed to cool. While stirring constantly, small amounts of concentrated hydrochloric acid diluted twice with water are added. This must be added until the reaction of the medium becomes strongly acid, which can be determined by using Congo red indicator paper or universal indicator. Approximately 200 ml hydrochloric acid is required. The white precipitate which settles out is filtered in a Buchner funnel by means of a paper filter and rinsed with water. An effort is then made to draw off as much of the water as possible; the residue is removed from the filter and dried either in a vacuum or in an inert gas medium (N_2 or CO_2). The finished preparation must also be stored in an anaerobic medium if possible.

Possibilities of Complex Microchemical Analysis of Dried Blood

Sugar. When using a solution of trichloroacetic acid for the extraction of blood that has been dried on filter paper, both glucose and the majority of substances that disturb its determination pass over into the extract. In theory, other solutions can be used for extraction in which proteins dissolve poorly, for example dilute hydrochloric acid or a solution of zinc sulfate, but their use complicates or even renders impossible further determination of lipid substances, so that in the present version of the method it is precisely trichloroacetic acid that has been chosen. In the TCA extract, the glucose can be determined by any of the three methods currently most widely used in clinical biochemistry: glucosidase, anthrone and orthotoluidine. The enzymatic glucosidase method proposed by Nelson [247] is rightly considered to be the simplest, most sensitive and most specific method of determining blood sugar

[82, 95]. In specificity and sensitivity, it is comparable only with other enzymatic methods based on the hexokinase reaction of the phosphorylization of glucose by ATP. The glucose-6-phosphate which is formed is determined on the basis of its ability to reduce NADF in the presence of the enzyme glucose-6--phosphate dehydrogenase. The reduced form of NADF H_2 which is thus formed is determined spectrophotometrically or fluorometrically. Since more expensive reagents are required for performing the hexokinase method of determination of glucose than are required for the glucosidase method, it has received relatively less publicity. The use of this method in series work is complicated by the fact that the reduced form of NADP is determined by spectrometry in ultraviolet light. The possibility of the enzymatic method of determining sugar in a solution of trichloroacetic acid was initially the subject of dispute because this acid denatures the proteins; it was found however that if a weak solution of TCA was neutralized, the activity of the glucosidase that was added as well as that of the peroxidase would be satisfactorily retained. This is indicated by the results of the work of T. A. Orlova who compared the results of the determination of blood glucose and standard solutions by the glucosidase method following precipitation of the proteins with hydrochloric and trichloroacetic acid (in mg%, M ± m). /46

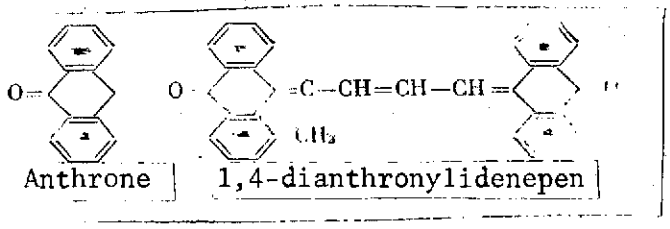
Indirect support for the possibility of enzymatic determination in a trichloroacetic extract is provided by the results of work that show that leech muscle in a neutralized TCA solution reacts to acetylcholine in the same way as in a saline solution [31].

The comparison made by T. A. Orlova of the results of blood sugar determination in healthy persons by various methods confirmed the relatively good degree of their agreement:

	Results of Determination (M ± m, mg%)		
No. of studies	23	21	63
Anthrone method			
Liquid blood	85 ± 2.6	-	-
Dried blood	86 ± 2.0	90 ± 3.8	82 ± 2.2
Orthotoluidine method			
Dried blood	-	71 ± 4.3	-
Glucosidase method			
Dried blood, extraction with 5% TCA	-	-	64 ± 1.7

V. K. Goroditskiy and I. L. Selivanov [47] suggested the determination of the hydrogen peroxide which is formed in the glucosidase reaction by a chemical method based on its ability to oxidize colorless phenolphthaline to brightly colored (in the alkaline pH region) phenolphthalein. This principle can be used successfully to determine glucose in the TCA extract from dried blood.

In clinical biochemistry, the anthrone method of blood sugar determination, based on the ability of 10-dehydro-9-ketoanthracene (anthrone) to produce a green color with hydrocarbons has been widely used.



According to the studies of Helmit and Tattanhalli [221], the colored substance has the structure of 1,4-dianthronylidenepentene (2). This method was introduced into the practice of biochemical analyses by Roe in 1955 [256] and has been widely used since then. Its sensitivity is almost the same as that of the glucosidase method but the specificity is much less, since anthrone reacts with all hydrocarbons and a number of their derivatives, so that with sugar loads, when the phosphoric ethers of hexoses appear in the blood, the results turn out to be overestimated. This method was also used to determine the high-molecular polysaccharides, for example polyglucine, which enter the circulatory system as blood substitutes. As it applies to the analysis of blood that has been dried on paper, the anthrone method has the same potential error: contamination by individual fibers of cellulose could lead to the development of a bright color which is equivalent to very high blood sugar levels. /47

In terms of specificity, the orthotoluidine method is practically the same as the enzymatic methods of sugar determination [191, 120], and it is based on the ability of aromatic amines in an acid medium to react with furfurol derivatives which are formed when sugars are heated in the presence of strong acids. The specificity of the method is governed by the fact that orthotoluidine produces a reaction only with aldosugars and ketosugars; ethers of sugars and high-polymer carbohydrates do not react with it. Since the method is relatively less sensitive than the enzymatic or anthrone methods, it can only be used for analyzing blood or serum which has been dried on small pieces of filter paper in penicillin flasks when the extract is obtained in a more concentrated form. Another fact which limits the use of this method is the need to heat the concentrated solutions of acetic acid, which have a very sharp odor that makes the tasks of the laboratory technician unpleasant.

In addition to the three basic and most promising methods discussed above [122], which are recommended by the Society of Medical Laboratory Technicians as unified, everyday clinical practice uses many other sugar determination methods. For one reason or another they cannot be used for blood analysis when that substance has been dried on filters. This includes all methods in which the proteins are precipitated by zinc, since this involves the formation of very strong lipoprotein clots from which it is impossible to extract cholesterol. The picrate method recommended by A. S. Krinitskiy [85] is also not taken into account, since it is less specific and less sensitive than anthrone.

In determining sugar in dried blood, the most probable source of error is breakdown of glucose during storage. The probability of this is increased if the blood spot is dried slowly or if yeasts or microbes fall upon it, so that it is very important not to touch the pieces of paper on which the blood has /48 been placed. The probability of breakdown of the sugar is also relatively greater when the blood is dried on small filters in penicillin flasks, where the evaporation of water is slower. However, if the necessary precautions are taken, the filter containing the blood can be stored to await analysis without a noticeable loss of sugar (p. 24) [of the Russian text].

Urea

To evaluate the function or state of the kidneys, as well as the intensity of tissue catabolism in clinical biochemistry, extensive use is made of the determination of residual nitrogen and its components. By residual or nonprotein nitrogen, we mean all of the nitrogenous substances in the blood which remain after the proteins have been precipitated. The quantity of these substances is a function of the method of precipitation of the protein: if a stronger precipitator is used, for example tungstic acid according to Folin and Wu, there will be fewer of these substances than if the proteins were precipitated by using trichloroacetic acid. The basic component of residual nitrogen is urea, whose nitrogen amounts to 10-15 mg% in the blood of a healthy human being, followed in magnitude by the nitrogen component from amino acids and oligopeptides (5-8 mg%), and the nitrogen from all the other nonprotein substances — creatinine, creatine, uric acid, and so on, which amount to less than 5 mg%. In practical clinical operations, the determination of the residual nitrogen is carried out more frequently than the determination of the rest of the components, particularly urea, obviously because until recently there were no sufficiently simple and reliable methods for determining them. To detect the initial levels of pathology of the kidneys, the change in residual nitrogen and urea content is of definitive importance.

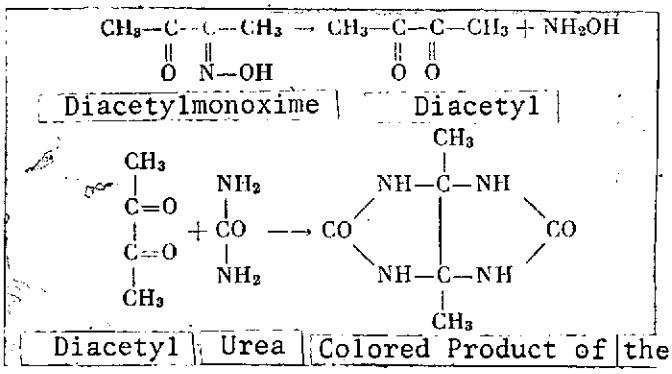
With respect to problems involving observation of cosmonauts during flight, analysis of urea turned out to be more convenient, since it can be determined in the same TCA filtrate as the sugar and its determination is not tedious. At the same time, however, the interpretation of the results of the analyses is simpler, since we are talking about an individual substance. Urea is the final product of protein metabolism and its accumulation in the blood may indicate either a slowing down of its excretion from the kidneys or an intensification of processes of protein catabolism. The amino acid level in the blood is only slightly dependent on kidney function and is determined by the rate of their utilization by the tissues as well as the intake with food and breakdown of tissue proteins. Therefore the change in the total content of certain substances, i.e., residual nitrogen, may be the result of highly diverse processes which is not simple to distinguish in nature.

There are several methods of determining urea in the blood, each of which in turn has numerous modifications. The most accurate and convenient method is /49 obviously the enzymatic urease method which is based on the decomposition of urea by the enzyme urease. The ammonia which is formed is determined on the

basis of the phenol hypochloride reaction. Since there is practically no endogenous ammonia in the blood, mistakes can occur only as a result of losses during fermentation or inaccuracy of the chemical reaction.

Clinical biochemistry makes extensive use of various versions of the determination of the components of residual nitrogen on the basis of the reaction with hypobromide which, depending on the conditions under which the reaction occurs, are referred to either as the determination of urea according to Borodin [33] or the determination of residual nitrogen according to Rapoport [131]. These methods are based on the ability of hypobromide in an alkaline medium to oxidize the amino groups and ammonia to form molecular nitrogen. The hypobromide which remains undecomposed oxidizes the iodine which is determined either by titrimetric means or by colorimetry. Certain modifications of this method are very sensitive but unreliable. An important shortcoming of this reaction is the fact that it makes it possible to determine not the individual substance but a number of compounds that contain amino groups.

Many chemical methods have also been described which are used for determining urea and employ its ability to produce color reactions with various diketones and their derivatives, as well as with phenol hypochloride reagent. Of all these methods, the ones most widely used are those based on the reaction with diacetylmonoxime proposed in 1939 by Firon for determination of citrullin. According to his studies, as well as those of Cotte et al. and Sienst [183, 196, 265], the chemism of this reaction is as follows: in a heated acid medium, the diacetylmonoxime goes to form diketone-diacetyl, which is condensed with the urea.



The reaction proceeds well in the presence of oxidizers which are needed to remove the hydroxylamine which is formed, since its accumulation prevents color development. In order for the substance which is forming not to break down when exposed to light, various stabilizers are added to the reaction mixture: antipyrine or glucironalacetone, usually thiosemicarbazide [145]. This method is highly specific: only citrullin, allantoin and alloxan disturb the determination, but their concentrations in the blood are so small that they make practically no impression on the results of the analysis. From the practical viewpoint, the shortcoming of the method is its unreliability, the lack of a linear relationship between urea concentration and the optical density of the solution (calibration graphs have the shape of curves that are bent upward) and the need to work in a dark location due to the high photosensitivity of the reaction. When all the necessary conditions are met, the method is completely reliable. /50

One proof of the specificity of the method of blood urea determination is the fact that following treatment of the paper containing the dried blood with a solution of urease, the color reaction with diacetylmonoxime does not occur. To check the accuracy of the method, the urea content was determined simultaneously in 16 samples using the method described and the urease method. For this purpose, 0.2 ml of blood were added to a solution of crystalline urease in a phosphorus buffer with a pH of 4.85; the ammonia which was formed after incubation was isothermally distilled in penicillin flasks on a rotator [3] and determination was carried out on the basis of the reaction with Nessler solution. The mean square deviation between each pair of determinations was 0.75 mg%, and the results of the urease method were 2 mg% higher on the average than the diacetylmonoxime results. This difference is insignificant as far as practical operations are concerned. This follows from the data from a comparison of the results of urea determination in the blood of healthy individuals by various methods (M ± m, in mg%):

	Results of Determination (M ± m, mg%)	
No. of determinations	27	16
Diacetylmonoxime method		
Dried blood	27 ± 1.3	33 ± 1.2
Liquid blood	28 ± 1.3	-
Urease method		
Liquid blood	-	35 ± 1.5
Residual nitrogen		
Liquid blood	-	39 ± 1.0

Creatinine

The study of the creatinine content in the blood is of considerable interest. This is because this substance is used for determining the level of renal clearance. Unfortunately, clinical biochemistry does not currently have available a reliable method for determining it, which is comparable in sensitivity and specificity to the methods used for determination of glucose and urea.

Usually creatinine is determined on the basis of the color reaction with picric acid (the Jaffe reaction); color reaction with alpha-naphthol (Sakagushi reaction) is also used, which makes is possible to determine arginine and all of the substances which contain the $NH\text{-}CHNH_2\text{-}NH_2$ group. This method is less specific one and is therefore more frequently used for enzymological studies /51

when it is not the endogenic creatinine which is being determined but that which is formed from a substrate which has been added [64, 136].

In order to increase the specificity of the determination on the basis of the color reaction with an alkaline solution of picric acid, numerous improvements have been proposed which boil down to finding a method of eliminating the proteins in which the amount of substances which disturb the determination would be minimal. A protein-free extract is also purified by removing the disturbing substances with ether, oxidizing them with cerium salts or by chromatography [69, 97]. A specific method has also been proposed for obtaining creatinine by extracting it with tetraphenyl borate [164].

In order to increase the sensitivity of the method, colorimetry must be performed in a capillary microcuvette, following concentration of the trichloroacetic extract by evaporation under vacuum in a system for lyophilic drying, since more severe conditions may destroy the substances being studied. Trichloroacetic acid present in the solution disturbs this evaporation and so must be removed, even partially, by extraction with ether. Most of the disturbing substances are removed as well.

Many clinical biochemists prefer the method of creatinine determination proposed by Popper et al. [254], which uses the fact that serum or plasma proteins are precipitated by picric acid, which is a color reagent for determining creatinine. Most of the substances which disturb the determination are eliminated. This method was recommended by the All-Union Society of Medical Laboratory Technicians as being a unified method [122] and its microvariant, based on the analysis of 0.1 ml of the liquid portion of the blood, may be carried out in a mass-produced continuous cuvette in the BIAN apparatus [105]. This method cannot be used for analyzing serum or plasma with considerable hemolysis, as in whole blood.

Cholesterol

In developing the method, the greatest difficulty was faced in determining cholesterol and lipid phosphorus. The erythrocytes contain less cholesterol than the plasma and they are more inert in the physiological sense, so that the diagnostic value of the determination of this substance in whole blood is relatively less than in its fluid part. This position is illustrated by Figure 8 in which the results are given for a determination of total cholesterol by /52 our method in the blood and serum in comparison with the results of a determination in the serum by the direct method of Il'k. Nevertheless, in those cases when it is not possible to obtain plasma or serum, determination of cholesterol and phospholipids in whole blood may be valuable. Inasmuch as cholesterol is chemically stable and is not destroyed when blood is dried, it is convenient to use a version of the method for its determination in the plasma or serum in which the material being studied is dried in 10 ml flasks.

The literature on methods of cholesterol determination in the blood and its component parts is voluminous and there is practically no way of analyzing and classifying even the works dealing with the most widely employed methods of its determination, which are covered in surveys [46, 130]. All of the methods

of determining cholesterol can be divided into two groups — the direct ones in which reagents for performing color reactions are added directly to the plasma or serum being studied, and the extraction methods, in which the cholesterol is initially extracted and then determined using some version of the color reaction. Direct methods were previously not suited for the analysis of whole blood, since the hemoglobin in the latter produces a color with the reagent for determining cholesterol.

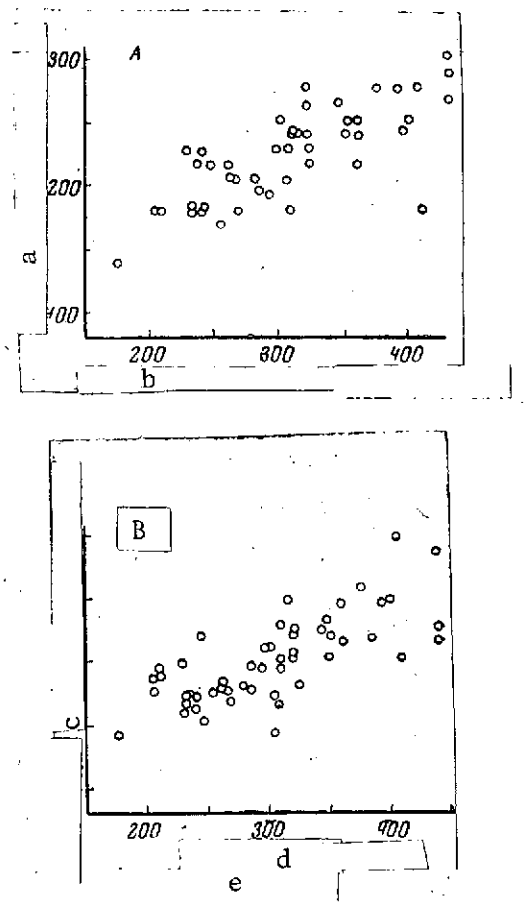


Figure 8. Relationship Between the Results of Determination of Blood and Serum Cholesterol by Various Methods. A, Comparison of results obtained in the analysis of serum by the method of Il'k and by our method; B, comparison of the blood and serum levels with both analyses being performed by our method. a, Cholesterol according to Il'k, mg%; b, cholesterol by an original method, mg%; c, blood cholesterol level, mg%; d, serum, mg%; e, serum cholesterol level, mg%.

The overwhelming majority of extraction methods are also unsuited for whole blood inasmuch as the stained products which are formed when hemoglobin breaks down cross over into the non-aqueous phase and subsequently disturb the performance of the color reaction. Cholesterol determination is complicated further by the fact that after extraction of the water-soluble substances, a dense precipitate of lipoproteins forms on the paper and it is very difficult to extract the lipids from the precipitate. For this reason, it has been found to be unsatisfactory to extract water-soluble substances with zinc oxide hydrate: even prolonged boiling in methanol with a reflux cooler, ether or dichloromethane did not produce anything like total extraction of cholesterol. Processing the pieces of paper with the residual lipoprotein clot by means of ether, alcohol or dichloromethane as well as various combinations of these failed to give the desired result: on the one hand, it was not possible to extract the cholesterol completely; on the other hand, under more severe conditions the color passed into the non-aqueous layer, subsequently disturbing the determination. The only suitable method was a slightly modified method of Folch [199]: after TCA extraction the protein and lipoprotein complex was rinsed with a weak solution of alkali, /53

to which methanol and dichloromethane had been added, and the resultant hemogenic mixture was divided by adding water. This was the only one of the numerous methods of extraction in which all of the color crossed over into the water layer. Good phase separation and total color transfer is a function of the correct ratio between the selected components and the alkalinity of the medium. Strange as it may seem at first glance, the addition of a large amount of water during stratification makes the non-aqueous layer cloudy. All of the color crosses over to the water layer only if the reaction in the medium is alkaline; in an acid reaction, the dichloromethane layer is also colored. An increase in alkali concentration means that after methanol and dichloromethane are added in ordinary proportions there is no formation of a homogeneous system but it immediately breaks down into two phases instead.

A comparison of this version of the extraction of the lipids from serum according to Folch with the results of extraction by other solvents or their combinations has shown that this method makes it possible to separate larger amounts of cholesterol than any other of the tested and widely used methods. This casts some doubt on the correctness and reliability of the overwhelming majority of published papers, whose results were obtained by means of methods which were unable to determine all of the cholesterol contained in the blood.

In the following we have presented the results of a determination of the cholesterol content in the blood serum following its extraction by various methods. The results are given in percentages of the values obtained in the extraction of cholesterol from an alkaline solution of blood using our modification of the Folch method:

No. of serum	Isopropyl alcohol	Ethyl-acetate-ethanol (3:1)	Alcohol-acetone (1:1)	Alcohol-ether (3:1) according to Bloor	Alcohol alkali-petroleum ether according to Abel
1	86	75	78	83	91,5
2	73	64	67	70	89,5
3	64	70	79	75	87,5
4	72,5	73	73,5	73,5	91

Commas indicate decimal points.

There is a danger that this result may have been obtained not because the extraction of the mixture of methanol-dichloromethane of the alkaline solution of blood was a more effective method of extracting the cholesterol than the methods used earlier, but because some other substances were also extracted which were not cholesterol but gave a color reaction with acetic anhydride in the presence of sulfuric acid. In order to check this theory, a solution of the lipid extract of serum in alkaline methanol was heated in a reflux cooler for hydrolysis of the ethers of cholesterol; the cholesterol in the hydrolysate was purified according to Folch. It was found that after this kind of treatment all of the substance that yielded the Lieberman-Buchard reaction was precipitated by digitonin, and the infrared spectra of this extract correspond to the infrared spectra of the cholesterol standards. The slight differences, particularly the high value of the peak at 2660 cm^{-1} in the experiment in comparison with the standard preparations, was obviously caused by contamination (Figure 9). This / 54

shows that the substance which was found was really cholesterol. Since repeated extraction of the water layer left after the initial separation according to Folch cannot be used to extract any noticeable amount of substance which yields a color reaction according to Lieberman-Buchard, it would appear that with our method of extraction all of the cholesterol has actually been extracted. In order to perform the color reaction for cholesterol, many different reagents have been suggested. The color reaction used in the present version of the method, with acetic anhydride in the presence of sulfuric acid according to Lieberman-Buchard, is not the most sensitive but its advantage is a high degree of reliability and good linearity over a wide range of concentrations. According to the studies of certain authors, the color develops more strongly in the presence of cholesterol ethers than if only the free substance itself were present. According to our data, this difference is not great enough to have a significant influence on the results of the determination.

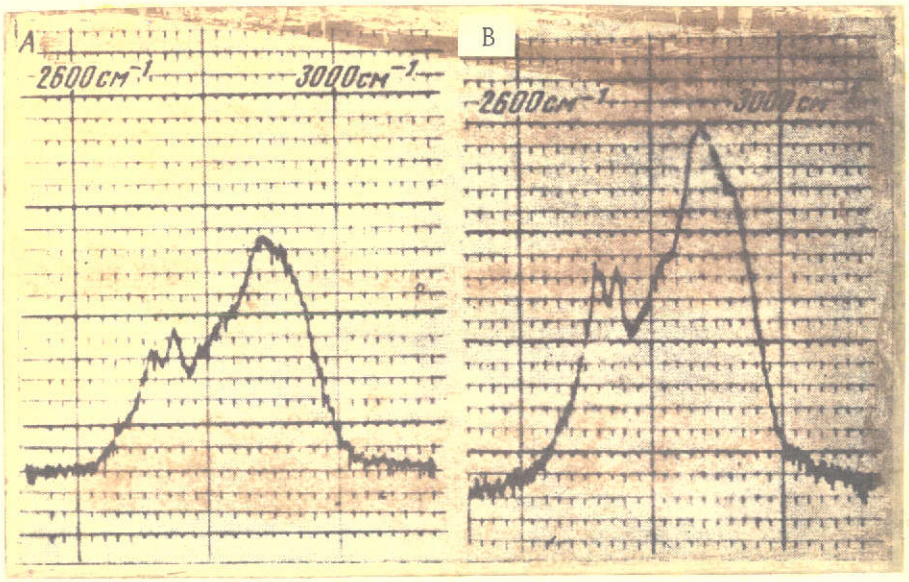


Figure 9. Infrared Spectra of Absorption of Standard Solutions of Cholesterol in Carbon Tetrachloride (A) and an Experimental Sample (B).

In processing blood with 0.3 N NaOH, a considerable part of the fatty acid ethers are broken down; this applies to a large part of the ethers of cholesterol — even after several hours, their content has decreased noticeably. /56
Subsequently this process slows down, and even after 24 hours there is still a considerable quantity of ether-bound cholesterol (Figure 10). In various blood samples, hydrolysis proceeds at different rates. It is obvious that this irregularity is caused by differences in the composition of the fatty-acid residues of the cholesterol ethers. According to the survey of Goodman [212], about half of all the esterified blood serum cholesterol is bound with linoleic acid, and there are also ethers of oleic, stearic, palmitic and other fatty acids; the quantitative relationships between them vary considerably (Table 1).

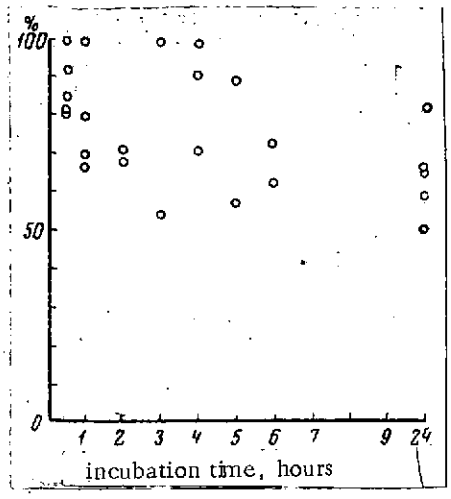


Figure 10. Quantity of Ether-Bound Cholesterol As A Function of the Incubation Time of Serum in an 0.3 N NaOH Solution (in % of Original Content).

Lipid Phosphorus

In the complex method, a version of the determination of lipid phosphorus is employed which was proposed in his time by King [230], which amounted to the inorganic phosphorus being extracted by trichloroacetic acid. The phosphorus that remains in the composition of the lipid clot is considered to be lipid phosphorus. This approach differs from the more widely used method in which the lipids are extracted from the plasma or serum by some fat solvent, usually a mixture of alcohol and ether according to Bloor [166]. King's approach to this problem appears to be more correct, since almost all of the compounds that belong in the category of nonlipid phosphorus are soluble to some extent in those substances which are used for the extraction of lipids. Rather, it is something else which is surprising — why is it that in the extraction of plasma or serum with a mixture of alcohol and ether according to Bloor, that so much acid-soluble phosphorus passes over into the extract? It is obvious that the reason lies in the fact that a considerable number of the compounds that resemble acid-soluble phosphorus — inorganic phosphorus (orthophosphates), phosphoric ethers of sugars and nucleosides — are absorbed on the protein clot and only a small part reaches the extract.

According to the method of King, an error can occur when the proteins are precipitated by trichloroacetic acid; the material which is studied remains for some time in a strong acid, and a portion of the phospholipids may break down. On the other hand, the lipid phosphorus contains all of the phosphorus that goes to make up the proteins and it is possible that a part of it may be categorized as phosphoproteins. Hence, neither method can be regarded as satisfactory from the purely chemical standpoint.

The amount of phosphorus extracted by the Bloor mixture is always greater than that which is precipitated by the lipoproteins, and this may be an indirect argument to support the fact that the latter method is more exact. The Bloor method is not suitable for the complex analysis of dried blood, since the alcohol-ether extract may contain a considerable quantity of water-soluble substances. The determination of phosphorus in the precipitate that remains after extraction of the dried blood by trichloroacetic acid serves as a natural continuation of the method described above for determining water-soluble substances. /57

By comparing these approaches we must keep in mind that the determination of phospholipids on the basis of the phosphorus content in the precipitate which is formed when TCA is added to the blood serum was recommended by the All-Union Society of Medical Laboratory Technicians as a unified method [122].

TABLE 1. FATTY ACIDS (IN %), INCLUDED IN THE COMPOSITION OF ETHERS OF BLOOD PLASMA CHOLESTEROL [212]

Number of observations	Data	Composition of fatty acids of cholesterol ethers					
		Palmitic 16:0	16:1	Stearic 18:1	Oleic 18:1	Linoleic 18:2	20:4
15	Healthy persons, 18-24 years	11,7	6,0	2,7	18,7	48,4	5,0
15	Healthy persons, 46-71 years	12,0	8,4	3,1	20,7	44,9	3,9
20	Atherosclerosis	16,6	10,7	2,9	20,9	38,6	2,8
17	Hyperlipemia	19,8	10,6	2,5	24,1	33,5	2,2
20	Healthy persons, 18-40 years	12,4	6,1	2,4	18,4	47,8	5,3
20	Healthy persons, 45-67 years	12,3	8,1	2,7	21,0	44,5	4,2
17	Hypercholesterolemia	17,4	4,2	3,5	24,5	41,9	4,2

Commas indicate decimal points.

It is obvious that not all of the substances containing phosphorus that make up the lipoprotein clot can be considered phospholipids. Thus, if the precipitate which is obtained when 5% TCA is added to blood serum is then dissolved in an alkali and separated in a mixture of methanol-dichloromethane according to Folch, the nonpolar phase will attract only that part of the phosphorus and the rest will remain in the aqueous phase. This is partially inorganic phosphorus, separated from the phospholipids during the dissolution in the alkali, but another part is bound up with the proteins, so that it can be precipitated by adding new batches of TCA. These experiments still do not prove the existence of phosphoproteins in the blood serum, but they do allow the question to be raised.

The phospholipids, particularly in an alkaline medium, are labile, so that it is not a simple matter to extract them in an unchanged form. It has been shown that some can even be reesterified by separation in columns [174]. In the case of dissolution in alkali of blood which was previously dried on paper and treated with TCA solution, some of the phospholipids break down, which can be seen by virtue of the fact that the corresponding spots on the thin-layer

chromatograms become smaller, and when the alkaline smear is stratified in the mixture of methanol-dichloromethane a portion of the phosphorus crosses over into the water layer. If the lipoprotein clot is dissolved in 0.3 N caustic soda and left overnight, most of the phosphorus will cross over into the aqueous layer in stratification according to Folch. Inasmuch as cholesterol thus crosses over into the nonpolar phase, this approach makes it possible to determine both of these substances in the same blood sample.

For the determination of phosphorus, one usually uses colorimetric methods that are based on the reduction of phosphoric-molybdenic acid to form molybdenum blue. Various versions of this method differ only in the reducer used and the temperature conditions under which the reaction is performed. To reduce, one usually uses eiconogen (sodium salt of 1-amino-2-naphthol-4-sulfonic acid), hydroquinone, stannous chloride and ascorbic acid. In addition, it has also been proposed to use amidol, methol, hydrazine, semicarbazide and thiourea [49, 51], as well as other aromatic amines and combinations of these substances [52]. According to Bartlett [53], the greatest sensitivity is obtained when measuring the absorption of light at the 830 nm wavelength as soon as the phosphoric--molybdenic acid has been reduced by eiconogen while heating. The increase in /58 sensitivity may also be achieved if the molybdenum blue which is formed is extracted by some solvent which does not mix with water, for example butyl alcohol or isoamyl alcohol. Inasmuch as its amount is quite high in the determination of total phosphorus and there is no need to achieve high sensitivity with the method, one can select the one with the greatest sensitivity. From these considerations, a method was chosen involving reduction by eiconogen according to Bartlett [160] because it possessed good linearity and reliability.

Nonesterified Fatty Acids (NEFA)

All methods of determination of the total amount of nonesterified fatty acids can be divided into two groups — one of them is based on the titration of the lipid extract of blood serum according to Dole [37, 38] while the others are based on the ability of fatty acids in alkaline pH to transport copper ions from the aqueous to the lipid phase, for example the method of Barrete and Mano [159]. In addition to these 2 methods, which have received maximum attention in clinical studies, there are a number of others in which individual fatty acids are identified by means of chromatography [5, 70]. The total concentration of nonesterified fatty acids changes following the administration of various hormones, physical exercise, hunger [110] and so on, so that its investigation in many instances provides more for evaluating the state of physiological processes than the study of the composition of individual fatty acids, which gives more of an idea of the characteristics of their synthesis or their intake into the organism with food.

The titrimetric method of Dole is really a further development of an idea of Whedon and Orskov [251, 252, 285] who extracted serum with ether and then titrated the acids that had crossed over. Dole introduced extraction of the fatty acids with long chains, diluting the binary mixture of heptane-isopropyl alcohol by adding excess water. He managed to show that under these conditions only the molecules of the higher fatty acids cross over into the heptane and

the substances which disturb the determination (phospholipids, lactic acid and so on) remain in the aqueous phase [190].

Non-ionized NEFA are quite soluble in nonaqueous solvents, but their salts are more soluble in aqueous solutions, so that extraction according to Dole amounts to carrying it out in a highly acid phase so that the acids will be in a non-ionized form. This method is completed by titration of the heptane layer with alcohol alkali in the presence of thymol blue indicator. The alcohol is not mixed with the heptane, so that titration takes place in a dual phase system, which indicates its accuracy. Therefore the sensitivity of the method cannot be increased significantly in comparison with our version, in which 1 ml of serum /59 is used, although such attempts have been undertaken [77].

The various versions of the colorimetric method also do not completely satisfy the requirements of microanalysis, inasmuch as when working with small amounts of fatty acids they carry over into the nonaqueous solution small amounts of copper that are commensurate with those substances which enter as a result of contamination by droplets of the aqueous phase and which it is very difficult to get rid of. Therefore, A. Ya. Kuus and Kh. Ya. Kuus [87] feel that the colorimetric method of determination according to Duncombe [192] has some important shortcomings that are difficult to eliminate.

A very important factor in the analysis of NEFA in blood and serum is its extraction. In the Dole method, a mixture of heptane-isopropanol and sulfuric acid is used for this purpose, while in the method of Barrete and Mano it is a mixture of alcohol and ether. Unfortunately, the authors did not check to see whether or not the procedures they proposed really ensured extraction of all the fatty acids.

As a matter of fact, to determine which of these methods gives the best results is not a simple affair, inasmuch as the free fatty acids form complexes with proteins in blood serum which sometimes are very strong [180, 270]. It is natural that acids added from outside are more easily attracted, since the proteins are already saturated by the endogenic acids. On the other hand, many ethers of fatty acids are labile compounds, so that when extracting under relatively harsh conditions, for example in the presence of an alkali, they are hydrolyzed and it becomes impossible to determine whence came the additional amount of NEFA: these are the acids which were firmly bound with the proteins and which previously could not be extracted, or those which were formed as a result of hydrolysis of complex ethers. This problem may be solved in part by studying the excretion of radioactive acids added to the serum or the blood. It is assumed that the indicators which are added will be mixed uniformly both with those endogenic fatty acids firmly bound with the proteins and those which are simply dissolved in the droplets of the lipoproteids or loosely bound with the proteins, but that some of them will not go to make up the chemical compounds as a result of reesterification. Such an assumption, generally speaking, is quite arbitrary, since reesterification of certain lipids may even occur in the process of chromatography using columns [174] and we cannot exclude the possibility that the strength of their bonds with the proteins in many instances may be greater than in the composition of complex ethers.

Studies performed by T. A. Orlova [110] have shown that radioactive fatty acids added to serum and blood can be extracted only by a mixture of alcohol and ether in the ratio of 3:1 (Table 2). Other extraction agents that have been tested, including Dole's mixture, extracted only some of the radioactivity that had been added. Therefore, in developing a micromethod of determination of /60 NEFA in dried blood or serum, it was precisely this reagent that was chosen. When added to native serum or plasma, a very fine suspension of protein precipitates is formed which is difficult to remove by filtration, so it is better to extract material which has previously been dried on filter paper; then the precipitate is automatically eliminated from it.

It is practically impossible to extract liquid blood in this fashion, since in the process of its treatment colored products of the breakdown of hemoglobin are formed which cross over into the nonaqueous phase. In the extraction of dried blood, if the reagents and the blood are properly dehydrated, no color will be formed that will disturb the determination.

Titration of fatty acids belongs to the category of nonaqueous titration, whose practice has been developed in considerable detail; the theory is largely still unclear, however. The main problem is the determination of the concept of acid and alkali which acquires a different and at the same time broader meaning in connection with nonaqueous solutions than when it is applied only to aqueous solutions. Without going into the theory of the problem involved, which was discussed in detail in a number of monographs [84, 113, 149], we shall merely /61 mention that the dissociation constant of acid, the transition point of the indicator and its color depend upon the choice of solvent. By changing it, it is possible to make a weak (i.e., one which dissociates with difficulty) acid into a strong one, i.e., one which dissociates readily. Therefore, if we have correctly chosen the system of solvents, we can make the point of transition of the indicator (i.e., the end of titration) sharper and make the titration itself more reliable.

Very roughly, all solvents can be divided into two classes: those that are protogenic or acid, whence come the glycols, and the acceptors, which include the amides, for example dimethylformamide. In protogenic solutions, the acids dissociate worse than they do in aqueous ones. They become weaker and can even react like bases. In acceptors, on the other hand, the acids dissociate better, i.e., they become stronger.

In conjunction with the determination of fatty acids, at least two methods of nonaqueous titration are possible. The first is titration of acid in a protogenic glycol medium and the second is titration with alkali in a medium of an acceptor solvent of formamide. Both of these versions have been tested and found suitable for biochemical studies, but the second system is more sensitive and simple, so that it will be given preference.

In a glycol medium the fatty acids behave like weak alkalis, i.e., they bind the acids which are added. Therefore, if we dissolve the salt of a fatty acid in a medium which contains a mixture of ethylene glycol, chloroform and butyl alcohol, it may be titrated quantitatively with a weak solution of hydrochloric acid in the presence of thymol blue indicator. The chloroform is added to the

medium so that both the original products, the salts of the fatty acids and the nondissociated fatty acids that are formed during titration, will be soluble in it. Since the chloroform and the ethylene glycol will not mix, isobutanol is added to the medium, which makes them miscible.

TABLE 2. AMOUNT OF RADIOACTIVE FATTY ACIDS WHICH CAN BE EXTRACTED BY VARIOUS METHODS (IN % OF THE AMOUNT ADDED TO THE SOLUTION), ACCORDING TO THE DATA OF T. A. ORLOVA [110].

Method of extraction	Extracted material		
	Aqueous solution of salt of fatty acid	Liquid blood or plasma	Dried blood
Heptane-isopropyl alcohol-water	88—90	8—20	—
Methanol-dichloromethane-water (5:10:3)	28—48	—	—
Methanol-dichloromethane-0.3 N NaOH (5:10:3)	16—20	20—21	7—24
Methanol-dichloromethane-0.25 N NH_4OH (5:10:3)	29—47	—	—
Methanol-dichloromethane-copper-ammonia reagent (5:10:3)	22—47	27	32
Ether during heating	—	42—50	7—12
Ethanol-ethyl acetate	—	—	48
Benzene-ethyl acetate	—	—	12—14
Ethanol-ether (3:1)	—	—	62—85
Methanol-ether (3:1)	—	—	78—88

In practice, the procedure for the titration of NEFA in a glycol medium is as follows: the material to be studied is dissolved in a mixture which contains four parts ethylene glycol, three parts chloroform and one part isobutanol, and 10 ml of this mixture contains 1.5 mg phenolphthalein and 1.5 mg thymol blue. Titration is initially carried out with a 0.05 N alcohol solution of NaOH until a red color appears, indicating that all of the fatty acids that have been added have become salts. Then the solution is titrated again with 0.02-0.04 N hydrochloric acid until a blue color appears because of the transition of the thymol blue. The amount of acid used for titration is also an indicator of the fatty acid content in the solution.

In practical work, the method of glycol titration of fatty acids has been found to be of the same order of accuracy as the Dole method. Practically speaking, it cannot be used to determine an amount of acid less than one micromole. Obviously, the accuracy of the determination can be seen from the fact that it is necessary to work with two indicators and especially the fact that at the beginning of titration it is necessary to convert all the acids to their /62 salts, so that the pH of the solution must be alkaline. Under these conditions, absorption of carbon dioxide from the air takes place and this may subsequently be titrated like the fatty acids.

The variety of direct titration of fatty acids in formamide developed by T. A. Orlova [112] gives better results. As in the case of the glycol medium, here a mixture is used which consists of seven parts dimethylformamide, one part chloroform and two parts ethyl alcohol, which is necessary so that both the fatty acids (which dissolve only in lipid solvents and are insoluble in water) and their salts will dissolve well in it; these salts, on the other hand, will dissolve only in water. The color change of the indicator is largely dependent on small changes in the composition of the medium, particularly the presence of traces of moisture, so that when working with a new batch of reagents, if a good indicator change is not obtained, it is necessary to change the composition of the medium slightly. Since carbon dioxide and moisture from the air disturb the determination considerably in glycol titration, special measures must be adopted so that their infleunce can be excluded. The proposed method for determining NEFA boils down to their extraction by a mixture of methanol and ether while heating to 45°, evaporation of the extracts, repeated dissolution in a mixture of heptane, isopropyl alcohol and sulfuric acid as proposed by Dole, stratification of this mixture by adding water, evaporation of the heptane layer, its dissolution in dimethylformamide and titration with alcohol alkali.

A comparison of the results of NEFA determination by the method described with the data given in the literature for analyses according to the original method of Dole, as well as the direct comparison of these two methods performed in our laboratory, has shown that in many instances the values are of the same order, and this is surprising, since in our method we used a more drastic extraction of NEFA, as indicated by the results of experiments involving addition of radioactive palmitic acid.

A direct comparison of the results of titration of standard solutions of stearic acid in these two media has shown that the titration itself is very

effective. The possibility remains that by using the method of Dole in addition to fatty acids with long chains, some other substances are revealed as well. To check this assumption, experiments were performed in which the extract of serum obtained by the original method of Dole, i.e., by mixing it with heptane, isopropanol and sulfuric acid with subsequent separation by adding water, is divided into two parts. One of them was immediately titrated with alcohol alkali while the other was evaporated to dryness prior to titration and the dry residue dissolved in a mixture of dimethylformamide, alcohol and chloroform. At the same time, in the second batch of the same serum, the NEFA were determined by our method. The results of these experiments are shown in Table 3, from which /63 it is apparent that in all cases involving evaporation to dryness of the extract obtained by the Dole method, from 30 to 50% of all the acids was lost. From these experiments it may be concluded that, by using the mixture of heptane-isopropanol and sulfuric acid, we extract from the serum, in addition to the nonvolatile NEFA with long carbon chains, a somewhat larger amount of certain volatile acids as well. This is supported by experiments in which the extract obtained by the Dole method was distilled under vacuum in a closed vessel as shown in Figure 11. The extract was placed in one of the extensions; the flask was then covered by a lid and evacuated while cooling with liquid nitrogen. Being at a vacuum, the extension containing the extract froze while the other was cooled and the solvent and the volatile acids were distilled in it. All of the liquid condensed in the second extension of the vessel and could be titrated by alcohol alkali. The dry residue remaining after the distillation was dissolved in dimethylformamide and its nonvolatile acid content determined. The results of the experiments are shown in Table 3. As we can see from this table, during distillation under vacuum, in all cases some part of the acidity was distilled off together with the solvent. At the same time, the acidity remaining /64 after evaporation of the solvent in many instances was higher if the titration was performed in dimethylformamide than when it was performed in a mixture of heptane-isopropanol-sulfuric acid. Obviously this has to do with the fact that titration according to Dole cannot be carried out exactly as was done in the single-phase system.

Determination of Glucocorticoids in the Blood

There are many methods of determining adrenocortical hormones in the blood, most of which are based on certain chemical reactions.

In evaluating the functional condition of the human organism under intensive working conditions, considerable interest surrounds the determination of those compounds which display the glucocorticoid function of the so-called 11-oxycorticosteriods. These include the substances that occur in the 11 position of the -OH group: hydrocortisone (cortisol, substance F, corticosterone (substance E), as well as cortisone (substance B), which is distinguished from hydrocortisone by the fact that it has =0 in the 11 position instead of -OH. Inasmuch as both of these substances are easily converted into one another in the organism, they have the same biological effect.

From the chemical standpoint, the most precise method of determining steroid hormones is the so-called method of dual isotope labeling, but which cannot be used on a large scale since it is very tedious and requires a great deal of

material for study. The method of concurrent bonding with protein is comparatively easy and very sensitive, but was devised recently and has not had sufficiently wide approval. In one sense, its shortcomings consist in the fact that all of the glucocorticoids are determined simultaneously and progesterone and certain of its derivates are determined as well, which are likewise bound with the transcortin.

TABLE 3. RESULTS OF TITRATION OF NEFA EXTRACTED FROM SERUM ACCORDING TO THE DOLE METHOD (HEPTANE-ISOPROPYL ALCOHOL-0.1 N H_2SO_4) AND PROCESSED BY DIFFERENT METHODS

Sample	By the original Dole method	Following evaporation and repeated dissolution of the dry residue		Distillation of the heptane layer under vacuum	
		In the heptane layer of the Dole medium	In a medium containing dimethylformamide	Distillate	Dry residue in dimethylformamide
1	1720	1300	980	220	750
2	530	320	550	130	550
3	300	160	450	0	450
4	320	400	950	—	—
5	300	450	750	—	—
6	330	450	750	—	—
7	700	480	400	250	750

Commas indicate decimal points.

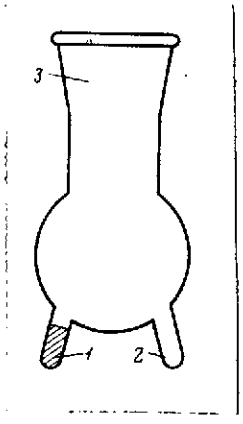


Figure 11. Vessel for Distillation of Extracts Under Vacuum; the Extracts Contain Fatty Acids. 1, Extensions containing distilled extracts; 2, extension into which the extract is distilled; 3, ground--glass stopper.

The fluorometric method of determination of the 11-oxycorticosteroids is somewhat inferior to the other two in terms of accuracy and specificity, but if the necessary precautions are taken it can be quite accurate and simple. According to the studies of a number of authors, the blood of healthy human beings contains practically only one glucocorticoid — hydrocortisone, since the concentrations of corticosterone and cortisone are comparatively low. Therefore the results of determinations, carried out by the method of a dual isotope label, concurrently bound with protein, and by fluorometric methods agree in adult men without pronounced pathology [224, 232].

Any method of determining adrenocortical hormones must begin with their extraction. To do this, we use chloroform, dichloromethane, ethylacetate and so on. Depending on the solvent which has been selected, one can extract more /65 or less of the various substances of a steroid nature accompanied by other lipids which interfere with the determination. From our standpoint, it is best to extract blood or serum with dichloromethane, which collects all of the glucocorticoids quite actively and has a low boiling point. This means that evaporation in the course of further purification will involve less heating of the solution and the consequent threat of destruction of the substances being studied.

Cornell, Moore and Jones [231], studying the distribution of labelled hydrocortisone injected intravenously, have shown that most of the hormone present in the blood and its indirect metabolites are dissolved in the plasma while 14 to 20% is bound up with the erythrocytes. A smaller portion of this amount is loosely adsorbed on the surfaces of the red blood cells and can be washed off with physiological saline. Most of the "free" (i.e., not conjugated with glucuronic or sulfuric acid) corticosteroids in the plasma or the erythrocytes form strong complexes with the proteins, which break down, however, during extraction with dichloromethane. In the plasma, these complexes are strongest when they are with transcortin, although hydrocortisone will also form bonds with other proteins. According to the cited work [231], the strength of the complexes with the proteins of erythrocytes is higher than with plasmatic ones.

An example of the distribution by fractions of hydrocortisone and its nearest metabolites can be seen in Table 4.

Fluorometric methods of determination of 11-oxycorticosteroids have a brief but stormy history. The fact that many substances glow when treated with sulfuric acid has been known for a long time. Corresponding historical reports can be found in the monographs of Jayle [226], M. A. Yudayev [150], M. A. Konstantinova-Shlezinger [74] and Yudenfrend [152], as well as in the survey of E. Z. Naugol'nyye [106]. An important step in the development of the present version of this method was the suggestion by Moore [246] to use a reagent containing 20% alcohol and 20% sulfuric acid, in which all substances containing the oxy group in the 11 position and the keto group in the 3rd position will fluoresce. In our experience, this reagent gives better results than a mixture of sulfuric acid and acetic acids [119]. Progesterone and cortisone in the amounts in which they occur in the blood have practically no influence on the results of the determination of the 11-oxycorticosteroids, while cholesterol and

neutral fats may have an effect, since although they only fluoresce weakly, they are present in the blood in large amounts. These compounds are less polar than the oxycorticosteroids and can be eliminated from the material to be studied by washing it with hexane prior to the extraction of the hormones. This method has found applications because it is simple, but it gives results which are worse by comparison with the preliminary extraction of all of the substances, from /66 which the glucocorticoids are then extracted. They can be extracted by chromatography using a column with silica gel or silicic acid according to McLaughlin, Kanieka and Grey or according to O. K. Botvin'yev and Yu. V. Vel'tischev [28]. According to our experience, this approach is not acceptable in micromethods, since small amounts of corticosteroids are crushed in the chromatographic medium and contaminants unavoidably occurring in them disturb the determination.

In the extraction of plasma or rinsings from erythrocytes with dichloromethane, some of the substances which disturb determination do not cross over into the true solution and form a thin suspension whose droplets slowly rise upward when the solution is allowed to stand. Therefore, by allowing the extract to stand, one can reduce the disturbance caused by secondary lipid impurities to a certain degree.

Experience in determining a number of substances indicates that extraction of one fluid with another usually is not very effective: in order for it to be complete, it is necessary to repeat the extraction several times and to use a considerable excess of the extracting agent. Stratification of a single-phase system gives better results. To do this, the material to be separated is initially dissolved in a mixture of 2 liquids, one of which is poorly miscible in a third, in a mixture of hexane and alcohol to which water has been added. Then 2 phases are formed — a water-alcohol phase and a hexane-alcohol phase; the more polar substances will be found in the water-alcohol part while the less /67 polar ones will be in the hexane-alcohol phase. The theory of fluid extraction has been well developed and described in many works, particularly a book by L. Alders [7]. This approach was used by Folch [199] to isolate lipids from tissues, while Dole [190] used it to isolate nonesterified fatty acids from blood serum.

In developing a system for purification of materials by phase separation, a so-called phase diagram is initially constructed (Figure 12) which has the appearance of an equilaterial triangle. Each side of this triangle corresponds to different mixtures composed of only 2 components. Thus, the bottom side reflects all possible combinations of hexane and alcohol, and point A located on it corresponds to a mixture of 80 parts hexane and 20 parts alcohol. The points inside the triangle correspond to 3-component mixtures; for example, point B corresponds to a mixture of hexane-alcohol-water in proportions of 72:18:10, which is obtained by mixing one part of water with 9 parts of a mixture of hexane and alcohol (80:20).

The shaded parts of the diagram correspond to those combinations of water, alcohol and hexane which are mutually miscible and form one phase. On the other hand, the unshaded areas represent those proportions in which the components in which we are interested are not mutually miscible and form 2 phases — one nonpolar and one polar.

TABLE 4. SAMPLE DISTRIBUTION BY FRACTIONS OF HYDROCORTISONE CONTAINED IN THE BLOOD AND ITS NEAREST METABOLITES (ON THE BASIS OF THE DATA OF CORNELL, MOORE AND JONES [231])

Fraction	Specific extraction agent	Identified substances contained in a given fraction	Sample content relative to the total substances,%	
			In the plasma or elutrient from erythrocytes	In erythrocytes
"Free"	Dichloromethane	F, E, THF	35	72
Polar "free"	Ethyl acetate	6-β-OH-	10	7
Conjugated				
With glucuronic acid	Mixture of alcohol and ether	THF, THE β-cortol	42	3
With sulfuric acid	Ditto	F, THF	8	5
Unidentified conjugate	Ditto	unknown	5	13

Note: Most of the substances that come from under the "free" fractions form compounds of varying strength with proteins: F, hydrocortisone (cortisol); E, cortisone; THF, tetrahydrocortazol; THE, tetrahydrocortisone; 6-β-OH-F-6-β-hydrocortisol.

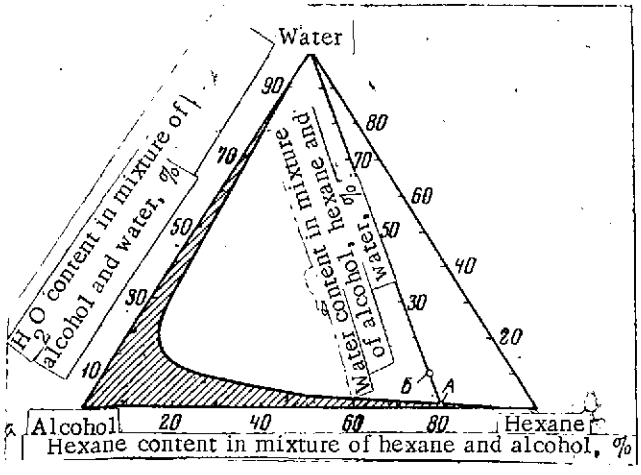


Figure 12. Triangular Phase Diagram of the Water-Hexane--Alcohol System.

For purification of the materials, we proceed as follows: initially the material to be separated is dissolved in a mixture of 2 mutually miscible fluids, in this case hexane-alcohol (80:20); then water is added so that the system stratifies, forming 2 phases — a more polar water-alcohol one into which the 11--oxycorticosteroids pass and a less polar one, the hexane-alcohol mixture, where cholesterol and neutral fats are found. The triangular phase diagram /68 facilitates the choice of the quantities of fluids to be mixed. They must be such that the original proportions correspond to the zone of mutual miscibility (see point A) and the final proportions occur in the zone of nonmiscibility (see point B) (Figure 16).

Since spectrophotometry is more specific than colorimetry, replacement of measurement of fluorescence at one previously selected wavelength by its radiation over a wide wavelength range, i.e., by spectrofluorescence, makes it possible to increase the selectivity of the method. Two approaches are possible here. In the first case, the luminance is stimulated by light at a constant wavelength and the spectral composition of the emitted light is studied (the so-called fluorescence spectrum). In the second case, only the wavelength of the exciting light is changed, and the integral value of all the fluorescence is recorded simultaneously. This is known as study of the excitation spectra of fluorescence. In the overwhelming majority of cases, the study of excitation spectra of fluorescence yields more information since the spectral composition of the secondary radiation is the same for a number of related compounds and the fluorescence excitation spectrum is usually characteristic. Practically speaking, this is the absorption spectrum of the substance under study. Indeed, the luminance of molecules can be caused only by the light which they absorb!

The fluorescence excitation spectra of solutions of certain steroids in a sulfur-alcohol reagent are shown in Figure 13. The measurements were made on a /69 microspectrofluorometer, which is described in the section headed "Apparatus" on page 21 [sic]. In all cases, the total amounts of light from fluorescence was measured in the range from 500 to 580 nm, and the wavelength of the exciting light was varied. As we can see from the figure, hydrocortisone and corticosterone have characteristic peaks in the 460-470 nm range, while the spectra of these substances differ slightly; this is evidently because different products are formed under the influence of processing of the sulfuric acid. Cholesterol and cholic acid do not have peaks in this area.

It must be pointed out that the chemical reaction of conversion of steroids into fluorescent substances in the presence of sulfuric acid is rather capricious. Therefore, according to our experience, it is impossible to utilize the development of luminance with time to differentiate cortisol from corticosterone, as has been proposed by various authors.

The excitation spectra of solutions of various steroids in a mixture of sulfuric acid and alcohol are nonadditive, i.e., if two substances are present simultaneously in the solution (let us say, hydrocortisone and cholic acid), the total spectrum will not be a simple sum of the spectra of each of these substances in the same concentration. Obviously, in the course of the chemical reaction these substances will react with each other to form new fluorescent

products. Therefore, large amounts of byproducts prevent the fluorometric determination of corticosteroids. However, if there are only a few disturbing substances their influence can be excluded by using an approach similar to that which is used in spectrophotometry and is called "the Van Allen three-wave method": fluorescence is measured 3 times, each time using light with a new wavelength for excitation, in our case 462, 472 and 483 nm. The luminance of cholesterol or bile acid, dissolved in sulfuric acid, when excited by light with a wavelength of 472 nm, is equal to the halfsum of the values of the fluorescences for excitation with light having a wavelength of 462 and 483 nm. In the case of hydrocortisone, this difference has a positive value. Therefore, when carrying out measurements at these wavelengths and processing the results of the measurements by using the formula $\Phi_{472} - \frac{\Phi_{462} + \Phi_{483}}{2}$ where Φ_{462}, Φ_{472} and Φ_{483} are the intensities of the corresponding fluorescences, we obtain a value which is proportional to the content of 11-oxycorticosteroids in the solution and in the first approximation is independent of the impurities present in the solution. This correction makes it possible to exclude the mechanical superposition of the luminance of one substance on another, but does not take into account possible influences of impurities on the formation of fluorescent products of hydrocortisone on its processing with sulfur-alcohol reagent. /70

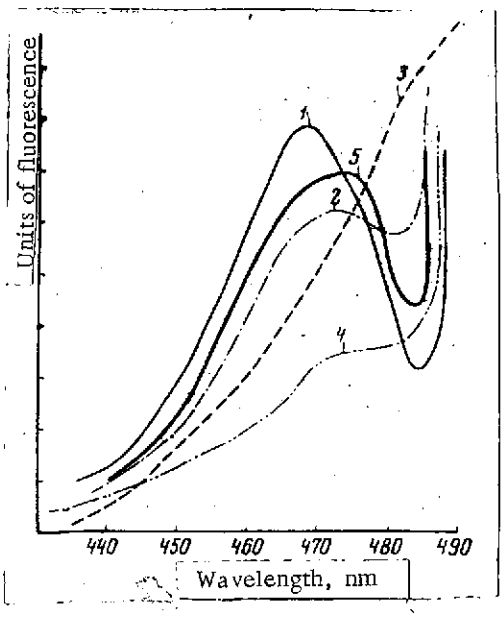


Figure 13. Spectra of Stimulation of Fluorescence of Solutions of Certain Steroids in a Sulfur--Alcohol Reagent. 1, Corticosterone; 2, hydrocortisone; 3, cholesterol; 4, cholic acid; 5, extract from 0.3 ml plasma.

In order to make the method of determination of 11-oxycorticosteroids more sensitive, measurements are carried out in a microspectrofluorometer which requires only 0.02 ml of fluid to fill the cuvette. This makes it possible to determine hydrocortisone reliably in 0.3 ml of serum or plasma when its content is low or in 0.1-0.2 ml when it is higher. Taking the sensitivity of the method into account, we must keep in mind that thanks to the improved purification of the extract and the introduction of a correction for nonspecific fluorescence, the content of 11-oxycorticosteroids in the blood turns out to be lower than for the usual method of measurement, since the material used for the investigation must be relatively greater.

If it is necessary to carry out a series of tests, particularly with small laboratory animals, the acquisition of large quantities of serum will become difficult, so that it is better to make the measurements directly in the blood. In this case, the following approach has proven to be reliable: the blood sample to be investigated, immediately after being collected, is placed in a small quantity of physiological solution and centrifuged. The erythrocytes are precipitated and the steroids which are dissolved in the plasma and loosely attached to the surfaces of the blood cells go into solution, where they are measured by the usual method.

Fluorometric Determination of 11-Oxycorticosteroids

Reagents. All of the reagents used for microdetermination of oxycorticosteroids must be well purified, so that several practical hints for handling this problem are given below.

Sulfuric acid. In methodological handbooks on the determination of steroids, it is frequently written that sulfuric acid must pass the Saval test. In fact, this requirement is satisfied by acids that are rated C.P. and A.P. (All-Union State Standard 4204-66). The Saval test is used to determine the quality of alcohol and consists in the following: when a mixture of equal parts of these substances is heated, if both products are sufficiently pure the solution will remain colorless and transparent. This test is in reality a preparation of sulfur-alcohol reagent for fluorometry.

Dichloromethane. Glucocorticoids can be extracted from biological material by means of dichloromethane (CH_2Cl_2), methylene chloride, freon (30) or chloroform ($CHCl_3$). The first of these substances is preferable since it has a lower boiling point and a lower specific gravity. This facilitates evaporation, decreasing the threat of destruction of the hormones by heating. It is convenient to transfer the glucocorticoids from the dichloromethane to small amounts of sulfur-alcohol solution, in which they fluoresce, so that this solution is heavier and collects in the bottom of the extraction vessel. In working with /71 chloroform, the proportions are reversed — a drop of fluorescent reagent floats on it in the form of a thin ring which is difficult to collect and transfer quantitatively into the microcuvette of a spectrofluorometer.

To purify dichloromethane, one or two liters of the product are placed in a conical flask containing 50 ml of concentrated sulfuric acid and set on a magnetic

stirrer for five to six hours; if the acid darkens rapidly, it is replaced. During purification, the flask is covered with a lightproof hood. The sulfuric acid is drawn off and 50-100 ml of water are added. The flask is then shaken several times with a circular motion and the water decanted. Additional rinsing with concentrated solutions of ammonia and soda is carried out and the material is dried with anhydrous sodium sulfate or sodium carbonate. The dichloromethane is distilled in a glass apparatus.

Benzene. The benzene fraction, with a boiling point of 70-100 degrees, is rinsed out with a solution of potassium permanganate, sulfuric acid and then water. Distillation is carried out in a water bath. There should be no open flames about (danger of explosion).

Method of Determination. The material to be investigated is 0.3 ml of plasma, serum or corresponding amount of rinse with erythrocytes is extracted with 3 to 4 ml dichloromethane. This can be conveniently done in large test tubes (20 by 200 mm), shaking them manually for 1-2 minutes to form a uniform suspension. As soon as the suspension has been allowed to stand sufficiently long (2 hours is enough), the lower layer can be easily removed so that the serum remains on the walls. The extract is dried in small test tubes, allowing them to stay overnight uncovered but protected against the entry of dust.

The dry extract is dissolved in 0.6 ml of a mixture of benzene or hexane--ethyl alcohol (4:1 parts by volume) and stratified by adding 0.01 ml of water. The lower alcohol layer is transferred with a fine Pasteur pipette to a dish with a conical bottom and evaporated in a dessicator where a vacuum is produced by means of an oil pump. Evaporation in air, even with only gentle heating, causes destruction of some steroids.

The dry residue is dissolved in approximately 0.5 ml of dichloromethane, then 0.02 ml of fluorescent reagent is added, prepared by mixing one part ethyl alcohol and four parts sulfuric acid. This is mixed with a glass rod and allowed to stand for 30 minutes. After the half-hour has passed, the fluorescent reagent on the bottom of the test tube is drawn off from beneath the layer of dichloromethane into the capillary of the microspectrofluorometer and the fluorescence is measured by exciting it with light at wavelengths of 462, 472 and 483 nm.

The calculation is performed using a calibration curve constructed by using standards containing 0.01 to 0.05 microgram of hydrocortisone in the form of an aqueous solution, proceeding as with the experimental samples. In preparing the calibration curve, it is necessary to keep in mind that one can use only chemically pure preparations as standards. Inasmuch as they dissolve poorly in water the hydrocortisone is initially dissolved in a small amount of alcohol /72 and then water is added up to the necessary volume. Pharmaceutical preparations for intravenous injection contained in ampoules, although they are called hydrocortisone, are in reality a hemisuccinate of hydrocortisone — in them, the hydroxyl on the 21st atom is linked by ether with one of the carboxyl groups of succinic acid. This substance does not pass from the aqueous solution into the dichloromethane and is unsuitable as a standard.

Determination of the Mass of Circulated Blood and the Rate of its Movement

There are many methods of determining the mass of circulating blood and its components — plasma volume and erythrocyte mass. For this purpose, it has been proposed that various dyes or blood substitutes be injected into the vascular tree as well as proteins labeled with radioactive iodine or chromium. The size of the erythrocyte mass has been estimated on the basis of the dilution of the labeled erythrocytes or by using foreign but compatible red blood cells or by labeling them with radioactive iron or chromium. The total amount of hemoglobin in the organism can only be determined by means of carbon monoxide.

The staining methods of determining the plasma volume are based on the fact that a dye is added to the blood and slowly eliminated by the vascular tree. Since Evans blue was synthesized, other dyes have practically never been used for this purpose, since this substance is easily determined in small concentrations and is lost slowly from the blood stream because it is bound to the protein [201]. However, its use suffers from several inaccuracies and shortcomings; in the first place, Evans blue still can escape from the plasma during investigation, although in small amounts, and secondly it must not be used very frequently since the dye is retained in the organism and colors the sclera of the subject. In order to determine the magnitude of the erythrocyte mass using this method (and this is sometimes more important than the determination of the plasma volume), it is necessary to know the hematocrit value, which is usually measured in the peripheral blood, while it may be quite different in the blood in the internal organs.

The methods of determining the plasma volume on the basis of dilution of various blood substitutes is convenient because the pharmacopenic preparation is administered intravenously and which definitely causes no harm to the patient. Their disadvantages lie in the fact that there are no reliable chemical methods of determining the concentration of blood substitutes in the blood and the turbidometric methods proposed for this [21, 90, 96] are unfortunately insufficiently accurate.

Isotopic methods of determining the blood mass were proposed by Heresy [144] who is rightly considered to be the father of radioisotopic methods in chemistry and medicine. At the present time, they are widely employed and /73 described in detail in a number of handbooks, including the book by Laitha [89]. Chromium isotopes are most frequently used to determine the erythrocyte mass. To determine the plasma volume, proteins labeled with radioactive iodine or chromium are administered intravenously. These methods have all of the shortcomings and advantages associated with isotopic methods of diagnosis when the radioactive substance is administered into the organism of a patient. The actual performance of the tests is simple from the technical standpoint, since no preliminary chemical procedures are required which can always be a source of error. At the same time, however, the investigator is faced with a dilemma on each occasion — if he selects a preparation with a longer halflife, the radiation dose to which the patient is exposed will increase accordingly since the preparation will be retained longer in his system. However, when working with shortlived isotopes, the necessary preparation cannot be obtained in

advance (in our case, plasma proteins or erythrocytes), and then used as necessary. As far as use aboard spacecraft is concerned, intravenous administration of such preparations does not appear to be very realistic.

In 1882, Grecant and Quinquad [214] determined the total hemoglobin in the organism by using the ability of this substance to bind itself to carbon monoxide. This method enjoyed a certain degree of popularity in the 1920s [28, 117], but was then abandoned because the analytical techniques which existed at that time did not make it possible to work with small quantities of blood and to perform accurate measurements. The latest achievements of spectroscopy make it possible to turn to this method once again and to make it just as accurate as the rest. The principle of determination consists in the fact that the subject breathes air to which a certain quantity of carbon monoxide has been added. If we know how much of this gas has been breathed and its concentration in the blood, we can calculate the total hemoglobin in the organism.

From the standpoint of the interests of aerospace medicine, the determination of the total hemoglobin by means of CO has the important advantage that the indicator substance is administered through the lungs, eliminating the need for venepuncture. In addition, one can obtain a good deal of interesting data on the status of the circulation of the blood and the respiration.

Recently, two versions of the method have been developed, each with its own shortcomings and advantages. One was proposed by the Swedish researcher Sjostrand [266, 267]: the carboxyhemoglobin concentration in the blood is determined by a calculation based on its partial pressure in the alveolar air, eliminating taking blood samples. In our laboratory, a second version has been developed wherein the compositions of the air and blood are investigated simultaneously. In the following we shall give the general outlines of the determination of CO in the air and blood, then deal with concrete methods of determining mass of blood and the information which can be obtained in this manner. /74

The progress of the methods of determination of carbon monoxide in the air is closely linked to the development of infrared technology. Other methods of gas analysis have been found to be either insufficiently accurate or too costly. A molecule of CO has the same mass (28) as a molecule of N_2, so that they can be distinguished only with a mass spectrometer with double focusing, which, today, at least, cannot be considered a piece of ordinary laboratory apparatus. Gas chromatography, like mass spectrometry, is required for analyzing very small amounts of substance, and this is its advantage over infrared techniques, but the accuracy is less.

As we know, all gases whose molecules consist of only two different atoms have very characteristic absorption spectra in the infrared. Not only those gases whose molecules are symmetrical, such as nitrogen, oxygen, and hydrogen are absorbed there, but also helium, whose molecule consists of only one atom. The infrared spectra of certain gases which are used for medical research are shown in Figure 14 and it is evident that they are so characteristic that it is not difficult to identify the corresponding substance. The situation is

somewhat worse for quantitative analysis, since the bands are narrow and it is difficult to record them on an ordinary instrument without distortion; the width and height of each band is a function of the quality of the design of the instrument and the total gas pressure. Therefore, when working with a universal infrared spectrophotometer, the calculations may be carried out only by means of a calibration curve which is plotted specifically for the concrete conditions of operation of that particular instrument.

To determine the gas content on the basis of the absorption of infrared rays, so-called optical-acoustical gas analyzers have come into wide use. Their operation is based on the following principles. The gas is heated only by the light which it itself absorbs, so that a pressure rise in a cuvette filled with some chemically pure gas serves as a very sensitive indicator of the intensity of the light at those wavelengths which are specifically absorbed by that gas. All of the remaining light passes through such a cuvette without heating it and, of course, without increasing the pressure. This makes it possible to eliminate complex monochromatic devices.

Practically speaking, instruments of this kind operate as follows: a beam of light from a source is directed alternately by means of a rotating mirror, first through the cuvette containing the gas to be studied and then through a cuvette filled with a standard of the same gas (whose concentration is known precisely). Both light beams are then projected by a system of mirrors onto the same cuvette, also filled only with the gas whose concentration is to be determined. If the light is absorbed differently in the experimental and standard /76 cuvettes, the indicator cuvette is heated non-uniformly, the pressure in it pulsates, these pulsations are picked up by a sensitive sensor and amplified. By blocking one of the light beams with a diaphragm, these pulsations can be made to disappear and thus the degree of coverage of the diaphragm is an indicator of the gas concentration in the cuvette being studied. In industrial instruments, the mechanism that covers the diaphragm is usually linked to an automatic recorder. This method is good because it does not require a costly monochromator but the optical-acoustical analyzer makes it possible to detect only one gas.

In our laboratory, we use the Zeiss UR-10 infrared spectrophotometer for gas analysis; it is good because it makes it possible to study light absorption over a broad spectral range and simultaneously to detect not only carbon monoxide but certain other gases, although this instrument is inferior to the best examples of optical-acoustical analyzers in terms of sensitivity and accuracy.

The accurate determination of small concentrations of carboxyhemoglobin in the blood is a difficult problem and, regardless of the extent of the literature on this subject, it has been insufficiently treated on the whole [54, 135]. Existing methods can be broken down into several groups: (1) spectroscopic ones, where the basis of the determination is the measurement of the absorption of light by the hemoglobin and carboxyhemoglobin; (2) chemical methods whereby using the relatively high resistance of carboxyhemoglobin to the action of denaturing agents, the hemoglobin is broken down and COHb remains in the solution; (3) gasometric, where the CO is removed from the blood and the volume either measured directly, chemically on the basis of a reaction with palladium oxide,

or by gas chromatography. In determining the total hemoglobin in the organism, the accuracy of the method is of critical importance. Thus, let us say that instead of 5.0%, 6.0% carboxyhemoglobin has been found in the blood. From the standpoint of evaluating the toxic action of this substance, this difference is insignificant but it can lead to an error of 20% in the determination of the blood volume.

Of all the methods of determining the COHb concentration in the blood, the most promising one as far as accuracy is concerned at small concentrations is the spectroscopic method, since the substance under study is not subjected to chemical conversions which can always be a source of error.

The spectral characteristics of oxyhemoglobin and carboxyhemoglobin are very similar and in the ranges in which we are interested they form sharp peaks. In order to obtain reliable results, the light absorption measurements must be carried out at high accuracy on an instrument which makes it possible precisely to determine the spectral intervals at a width of 1-2 nm, since even the slightest inaccuracy in this regard is equivalent to a rather considerable error /77 in the carboxyhemoglobin level. To do this kind of work, it is desirable to select that part of the spectrum where not only the difference in the light absorption of these substances is maximal, but where the absolute values of the molar coefficients of extinction are great. This makes it possible to do an analysis in the micro version by performing measurements on thin layers of highly dilute blood.

Practically speaking, there are two spectral ranges which can be used for spectroscopic determination of carboxyhemoglobin against the background of oxyhemoglobin — they are the green (wavelengths 540-580 nm) and blue wavelengths 420-435 nm). Both areas have already been proposed on many occasions for this purpose by various authors. From our standpoint, the wavelength of 420 nm is preferable, since in this region the ratio $\frac{EHbCO - EHbO_2}{EHb_2O}$ is greater than in the green. The absolute values are higher, so that smaller quantities of blood will suffice for analysis.

In the blood, in addition to carboxyhemoglobin and oxyhemoglobin, there are always small quantities of its non-oxygenated form, metahemoglobin, and there may possibly be other derivatives whose composition is not known precisely. In order to simplify the conditions of analysis, it is necessary to convert if possible all of the hemoglobin which is not bound up with the CO into the oxygenated form, without affecting thereby the quantity of carboxyhemoglobin. This is accomplished most simply by diluting the blood with a solution saturated with air which simultaneously hemolizes the erythrocytes and oxygenates the hemoglobin. Aqueous solutions saturated with air contains 0.6% oxygen and the oxygen capacity of the blood (the maximum quantity of oxygen which can bind all of the hemoglobin contained in it), is 20-25%. This means that if the blood is diluted 50 times, there will be sufficient dissolved oxygen to saturate all of

the hemoglobin but this amount is insufficient to expel the CO from the carboxyhemoglobin.[1]

Human erythrocytes contain respiratory enzymes which retain their activity even after hemolysis, so that when hemolyzed dilute blood is stored without access to air, only after a few hours the portion of the hemoglobin which has been determined loses its oxygen. This process does not begin immediately but only after several hours of storage, and then all of the dissolved oxygen has been consumed. It is possible to decrease the respiration of the hemolysis by adding a respiration inhibitor to it, for example, streptomycin. A more powerful inhibitor — cyanide — is unsuitable because it can bind with the hemoglobin and change its spectral properties. Streptomycin is also suitable because it prevents the development of microbes. /78

In order to be able to measure with sufficient accuracy the concentration of COHb, its presence must increase the optical density of a solution by at least 0.3-0.4 unit of optical density. It is found that this can occur only when the total value of extinction is about 2, when the light transmission is about 1%. Such large values can only be measured by the compensation method, comparing the light absorption of the dilute blood with a calibrated light filter.

The purpose of the study is to determine not the absolute but the relative content of carboxyhemoglobin, i.e., the ratio $\frac{[HbCO]}{[HbO_2] + [HbCO]}$. To do this, it is necessary to know the concentration of both of these substances in the dilute blood, measuring the light absorption on two wavelengths simultaneously. This method, which has been called the two-wave method, was studied in detail by Milleken [83, 24] and Chance [147] and is widely used in biochemistry. It has the advantages that the optical properties of a given portion of the solution are measured on two wavelengths and so the various sources of error — spots, cloudiness, etc., affect both values simultaneously. This makes it possible to achieve high accuracy. Unfortunately, in the general case the calculations associated with the two-wave method are rather cumbersome; the calibration graph is curved, which considerably complicates working with it. Therefore, it is necessary to resort to various stratagems, particularly choosing for measurement those wavelengths on which certain coefficients drop out, which simplifies the computations.

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[1]The ratio between the amounts of oxide and carboxyhemoglobin and the partial pressure of O_2 CO in the solution is determined by the equation $\frac{[HbO_2]}{[HbCO]} \cdot 215 = \frac{[O_2]}{[CO]}$, from which we obtain $CO = \frac{1}{215} [HbCO] \frac{[O_2]}{[HbO_2]}$, where the subscripts in brackets represent the concentrations of the corresponding substances (for more detail, see the following).

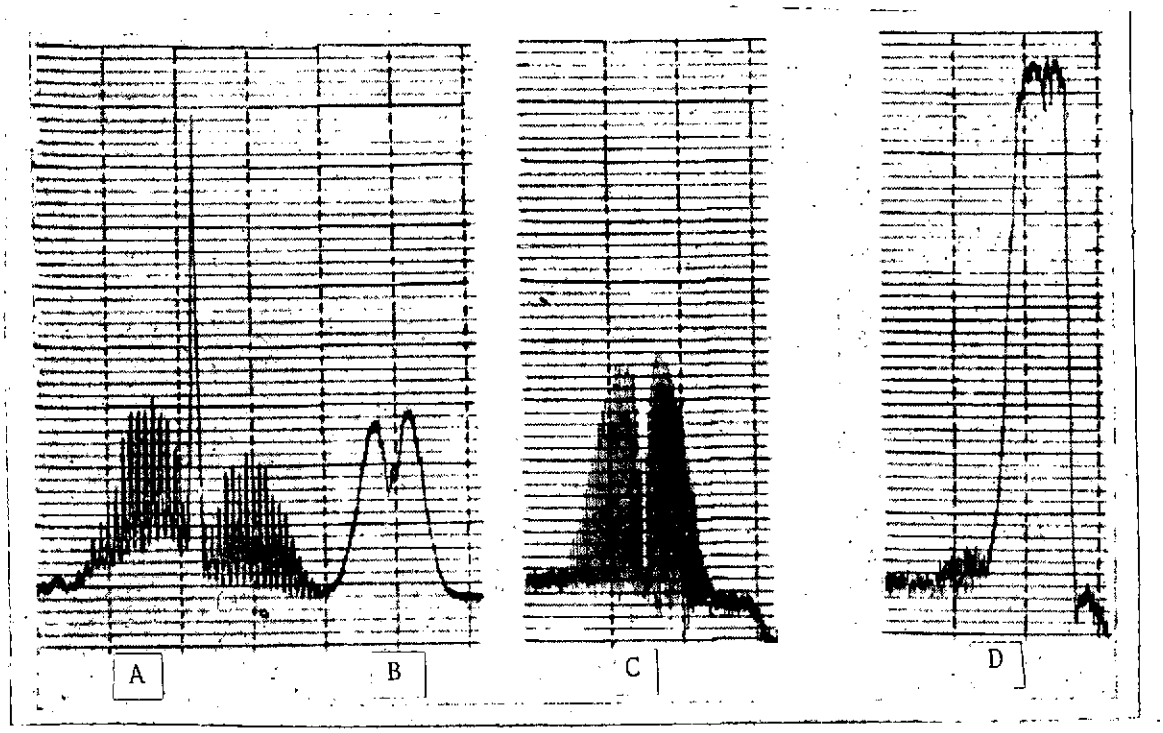

Figure 14. Infrared Absorption Spectra of Several Gases. A, Methane (2,800-3,200 cm^{-1}); B, acetylene (3,200-3,400 cm^{-1}); C, carbon monoxide (2,000-2,300 cm^{-1}); D, nitrous oxide (2,000-2,300 cm^{-1}).

The light absorption of dilute solutions of blood obeys the Lambert-Behr Law, so that we can write the following equations,

$$\begin{aligned} I_1 &= I_{01} e^{-[HbO_2]k_{11}-[HbCO]k_{12}}, \\ I_2 &= I_{02} e^{-[HbO_2]k_{21}-[HbCO]k_{22}}, \end{aligned} \tag{1}$$

where I_{01} and I_{02} represent the intensity of light with wavelengths λ_1 and λ_2 striking the cuvette containing the solution to be studied; I_1 and I_2 are the intensities of the same light beams after passage through the solution: K_{11} and K_{12} are the absorption coefficients of HbO_2 and HbCO at wavelength λ_1; K_{21} and K_{22} are the same on wavelength λ_2; $[HbO_2]$ and [HbCO] are the total amounts of HbO_2 and HbCO per unit area of the cuvette, i.e., the products of the concentrations of these substances and the lengths of the cuvettes.

The same equations in logarithmic form are as follows: /79

$$\begin{aligned} \ln I_1 &= \ln I_{01} - [HbO_2]\, k_{11} - [HbCO]\, k_{12} \\ \ln I_2 &= \ln I_{02} - [HbO_2]\, k_{21} - [HbCO]\, k_{22} \end{aligned}$$

or assuming that $\frac{I_1}{I_{01}} = -E_1; \quad \ln \frac{I_2}{I_2} = -E_2,$ we will have

$$\begin{aligned} E_1 - [HbO_2]\, k_{11} - [HbCO]\, k_{12} &= 0 \\ E_2 - [HbO_2]\, k_{21} - [HbCO]\, k_{22} &= 0 \end{aligned}$$

The solution to these equations is as follows:

$$[HbO_2] = \frac{-k_{12}E_2 + k_{22}E_1}{k_{11}k_{22} - k_{21}k_{12}} \quad \text{and} \quad [HbCO] = \frac{-k_{21}E_1 + k_{11}E_2}{k_{11}k_{22} - k_{21}k_{12}};$$

we are interested in the relative carboxyhemoglobin content equal to the following:

$$\frac{[HbO]}{[HbO_2] + [HbCO]} = \frac{k_{21}E_1 - k_{11}E_2}{k_{12}E_2 - k_{22}E_1 + k_{21}E_1 - k_{11}E_2}. \tag{2}$$

From (2) we can see that the relationship between the degree of saturation of the blood with carboxyhemoglobin and the experimentally measured values of optical densities E_1 and E_2 in the general case have a rather complicated nature, but in particular cases this equation may be simplified. Two versions are possible.

First: If the light absorption of HbO_2 and HbCO is the same on one of the wavelengths, then K_{21} equals K_{22} and in this case (2) will be considerably simplified:

$$\frac{[HbCO]}{[HbO_2] + [HbCO]} = \frac{k_{21}E_1 - k_{11}E_2}{(k_{12} - k_{11})E_2} = -\frac{k_{11}}{k_{12} - k_{11}} + \frac{k_{21}}{k_{12} - k_{11}} \cdot \frac{E_1}{E_2}.$$

The degree of saturation in the blood is proportional to the ratio of the extinctions at the two wavelengths.

Second case: If the wavelengths have been chosen so that the light absorption of the oxyhemoglobin in them is the same and the carboxyhemoglobin value is different, (2) is also simplified. In this case we will have $K_{11} = K_{21} = K$. Now:

$$\frac{[HbCO]}{[HbCO] + [HbO_2]} = \frac{k(E_1 - E_2)}{(k_{12} - k)E_2 + (k - k_{22})E_1}. \quad (3)$$

If the wavelengths are selected so that $K_{12} - K$ equals $K - K_{22}$ (we will show below that this is possible), (3) will assume the form:

$$\frac{[HbCO]}{[HbCO] + [HbO_2]} = \frac{k}{(k_{12} - k)} \cdot \frac{E_1 - E_2}{E_1 + E_2},$$

i.e., the difference between the measured extinction values, divided by their sum, is directly proportional to the percentile content of carboxyhemoglobin in the blood. The calibration graph, plotted in appropriate coordinates, in /80 this case has the shape of a straight line, which simplifies the calculations considerably. In theory, when developing a method of determining HbCO in the blood, we can use either the first or second method, but the second is more convenient. In this case, at low carboxyhemoglobin concentrations, light absorption on both wavelengths is about the same so that it is easier to achieve higher measurement accuracy than when working with the first method, when the absolute value of light absorption on a given wavelength is several times less than in the second.

In 1949, Sjöstrand [267] proposed a method of determining the total hemoglobin in the organism which does not involve the analysis of blood; the carboxyhemoglobin content of the blood is calculated on the basis of data from an analysis of the alveolar air.

The reaction of carbon monoxide with oxyhemoglobin:

$$HbO_2 + CO \rightleftarrows HbCO + O_2,$$

like all reversible chemical reactions, obeys the laws of acting masses so that

$$\frac{[HbCO] \cdot pO_2}{[HbO_2] \cdot pCO} = K_e, \quad (4)$$

where the values in brackets are the concentrations of oxyhemoglobin and carboxyhemoglobin in the blood and pO_2 and pCO_2 are the partial pressures of these gases in the blood. The dimensionality (i.e., the units in which the values are measured which go into these equations) have no significance since similar perimeters are used in the numerator and denominator. It is therefore important only that the content of carboxyhemoglobin and oxyhemoglobin in a given medium be expressed in the same units, for example in relative percent, and in the gaseous phase in fractions of a normal atmosphere. Under equilibrium conditions

the ratio of the partial pressures of the gases in the blood is equal to the ratio of their partial pressures in the alveolar air, so that if we know its composition, the percentage of saturation of the blood by oxygen, and the value of the equilibrium constant Ke, there will be no difficulty in calculating the carboxyhemoglobin content in the blood. It will be equal to:

$$[HbCO] = K_e [HbO_2] \cdot \frac{p\,CO}{p\,O_2}.$$

During the study, the hemoglobin concentration will vary within small limits (95-97%) so that the product of K_e and HbO_2 is a constant; it is called the Sojstrand factor or constant K.

According to Katchman et al. [229], who studied the possibilities of this method in detail, the value of K_e is equal to 215 on the average and K = 21,000. In these experiments, before beginning the study, the subject breathed pure oxygen for 30 minutes to remove endogenic CO from the organism. Then he switched to breathing from a closed system (spirometer) with pure oxygen, to /81 which 25 ml of carbon monoxide had been added. The expired carbon monoxide was absorbed by a special absorber and the oxygen that was used up was constantly replaced by new. Approximately 12 minutes after the beginning of respiration the concentration of carbon monoxide in the air had attained a constant level and the stability of this state was checked for a short time, so that in general the subject was breathing from the system for about a half hour. According to the statement of the authors themselves, the method yields good results only when studying persons who do not smoke, since in smokers the original content of carboxyhemoglobin in the blood is high. A comparison of the results obtained with the volume of circulating plasma, measured by means of administration of albumin labeled with radioactive iodine, showed good agreement on the whole. The Sjöstrand method usually yielded somewhat higher figures but the difference in most cases did not exceed 10%. Taking into account the fact that in one case it is the plasma volume that is determined and in the other the quantity of hemoglobin and that the hematocrit value in the entire organism may be determined only approximately, the agreement of the results must be considered very good.

For determining the carboxyhemoglobin of the blood on the basis of the composition of the air the duration of reflux breathing is of great importance. In the cited work of Katchman et al., the duration of this period was about a half hour following the time the subject began breathing oxygen, containing the trace amount of carbon monoxide. In this case, at least 15 minutes were required for the CO to be absorbed by the blood and be distributed uniformly through the entire organism. However, if the process runs in the opposite direction and there is already carboxyhemoglobin in the organism of the subject (for example as a result of industrial poisoning), the time required to establish equilibrium is much less. Thus, according to the data of Cuihandu et al. [181], 3 to 5 minutes is sufficient.

Methods based on the principal proposed by Sjöstrand are very convenient because they make it possible to eliminate collecting blood samples; the entire procedure is technically simple, but its information content is less and the

examination itself requires much time, more than if the quantity of carbon monoxide absorbed and its concentration in the blood were being studied separately. The sudden introduction of an indicator solution of CO into the blood stream in the course of several inspirations makes it possible rapidly to create a relatively high concentration of carboxyhemoglobin in the blood, "labeling" that blood which actively circulates through the lungs and also observing the change in its concentration so that one can get an idea of how rapidly the blood moves through the organism. The possibilities of such an interpretation of the dynamics of the concentration of carboxyhemoglobin can be seen from the example of the action of the negative pressure on the lower half of the body (NPLB) [13]. /82 This test involves the lower half of the body of the subject being placed in a container which is sealed at the level of the waist, in which it is possible to create rarefaction of several tenths of a milliliter of mercury. As a result, the blood is deposited in the lower extremities where there is a slight analogy to the effect produced by acceleration or blood loss on the organism. In Figure 15, we can see the dynamics of the concentration of carboxyhemoglobin in the blood after a subject has been breathing a gas mixture containing CO for 20 seconds and during a test in which he was also subjected to the effects of the NPLB. As we can see, in the second case the concentration of carboxyhemoglobin immediately after the effect of breathing of the mixture was much higher and gradually fell. The second sharp drop occurred when the pressure was reduced and the labeled CO in the blood could mix with that which was previously deposited.

If we know how much CO has been absorbed in a unit time of breathing a gas mixture, we can get some idea of the diffusion capacity of the lungs. The information obtained in this fashion concerning the volume of the residual air in the lungs is of interest in itself. Finally, in theory the possibilities of the method may be considerably expanded by adding to the gas mixture still another indicator solution which would dissolve well in the blood by passing through the lungs, for example acetylene or nitrous oxide. These substances have characteristic absorption bands in the infrared near the absorption pole of carbon monoxide and methane and their determination on the same universal instrument does not pose any difficulties. This approach has all of the advantages provided by simultaneous recording of several physiological parameters.

A method worked out by O. A. Virovets and later by R. K. Kiselev for determining the total hemoglobin in the blood involves the subject breathing a /83 mixture of carbon monoxide and methane for twenty to forty seconds; before and after this, blood samples are collected to determine the carboxyhemoglobin content. The gas mixture is analyzed on an infrared spectrophotometer before and after breathing. On the basis of these data, the amount of CO in the functional dead space is calculated (methane is required for this) as well as the amount absorbed by the blood. Dividing it into the carboxyhemoglobin content of the blood, we can find the total amount of hemoglobin in the organism.

Determination of the Carboxyhemoglobin Content in the Blood

Reagents. One per cent solution saponin in boraye buffer, pH 7.8.

System for Measuring Absorption of Light. This device was constructed on the basis of a DFS-13 spectrograph with a diffraction grating, but it is also

possible to use simpler spectral instruments. The arrangement is shown in Figure 16. The light from a stabilized light source, an ordinary incandescent bulb (127 V, 100 W) which was powered through a ferroresonance voltage stabilizer, was projected by means of a lens on the entrance slit of the instrument. Instead of a cassette containing film, a plate was installed with two openings in the form of vertical slits, so that the difference between the wavelengths of the light beams which struck these slits would be approximately 11 nm. One of these light beams, the one with the shorter wavelength, strikes the entrance slit of the measuring device directly while the other reaches it only through a system of mirrors. By this means it is possible alternately to illuminate the solution being measured, first with one and then the other light beam. The intensity of the light was measured by means of a FEU-14B photomultiplier, connected to a mirro galvanometer with a logarithmic scale, making it possible to perform calculations directly in extinction units. /84

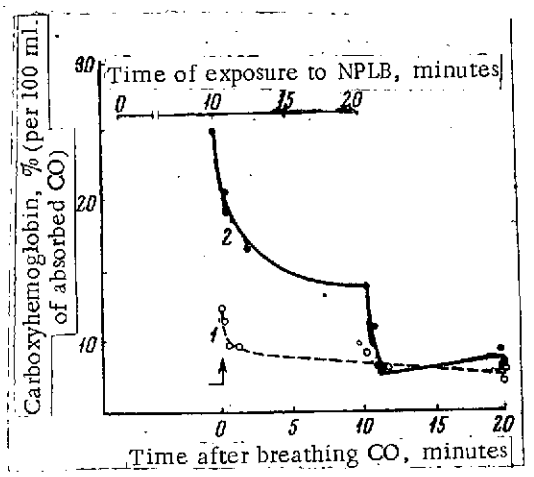

Figure 15. Change in the Carboxyhemoglobin Content in the Blood Following Breathing of Measured Quantities of Carbon Monoxide. 1, Control experiment; 2, experiment. Arrow indicates moment of inspiration of CO.

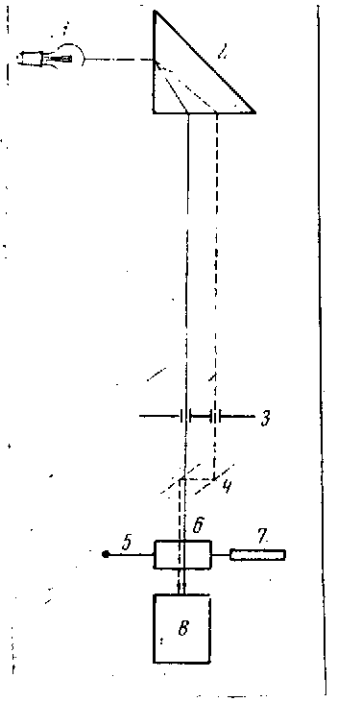

Figure 16. Device for Determining Small Quantities of Carboxyhemoglobin. L, Light source; 2, monochromator; 3, slits; 4, system of movable mirrors; 5, device for rapid replacement of the cuvette with the solution being measured by a standard light filter; 6, cuvette containing blood diluted 50 to 100 times; 7, standard light filter; 8, photomultiplier.

Method of Determination. 0.02 ml of blood is collected from the finger tip and dissolved in 2 ml of saponin-borate buffer. If the solution appears opalescent or cloudy it is centrifuged, but this is rarely necessary. If it is not possible to perform the measurements immediately

0.1 ml of blood is dissolved in 10 ml of buffer and this solution is placed in a glass flask with a tight cover so that it can be filled to the top. The sample is stored for a day in this fashion without losing the carboxyhemoglobin. In some blood samples, after they have been diluted with saponin-borate buffer, the consumption of oxygen proceeds so intensively that after several hours of being in a hermetically sealed vessel at room temperature all of the dissolved oxygen us used up and the oxygen that is bound with the hemoglobin begins to break down. Such samples can easily be distinguished by their color. Prior to the start of determination of COhb, they must be oxygenated again by introducing air bubbles beneath the cover of the flask and shaking the solution.

In the measuring unit of the device, instead of a cuvette containing blood, there is a calibrating light filter whose optical density is known exactly; it may be (for example) 1.5. The measuring galvanometer is set so that when light with a wavelength of 419 nm passes through the light filter, the galvanometer will undergo maximum deflection, i.e., the extinction will be equal to 0. The cuvette, with an optical path length of 1 ml, is filled with dilute blood and then substituted for the calibrating light filter. The galvanometer shows the magnitude of the difference between the optical density of the light filter and the sample. In order to determine the true value of extinction of the blood solution, it is necessary to add to this value the extinction of the calibrating light filter and thus zero the instrument.

As soon as the light absorption has been measured at the wavelength of 419 nm, the mirror system is used to illuminate the cuvette with the solution to be studied, using light with a wavelength of 412 nm. The alternation of the light beams is repeated several times so that it is possible to measure more exactly the difference between the optical densities of the blood solution at 2 wavelengths. During these measurements, the position of the cuvette containing the blood and the diffraction grating are not changed. Adding the corresponding amounts to the optical density of the calibrating light filter, the true optical densities of the blood solution are calculated on two wavelengths.

Zeroing the instrument and plotting the calibration curve. The instrument allows smoothly changing the position of the diffraction grating, so that the position of the openings through which the light beams emerge from the instrument provide a constant value for the difference in wavelength between these beams, but not their absolute value. Finding the position of the diffraction grating at which the necessary wavelengths fall on the slits onvolves a certain amount of /85 difficulty since the mechanical measuring devices of the instrument do not provide the necessary accuracy. However, an error in setting the wavelength is equivalent to increasing the original carboxyhemoglobin content. Therefore, the problems of adjusting the instrument and zeroing it are the same. For this purpose the cuvette is filled with dilute blood in which the carboxyhemoglobin content is considered minimal (collected from non-smokers). The light absorption of this solution is measured alternately in both light beams and, by slowly shifting the position of the diffraction grating within very narrow limits, it will be possible to make the light absorption in both beams the same, i.e., to make the difference in extinctions equal to zero.

Since it is very difficult to prepare standard solutions of carboxyhemoglobin of a specific concentration, in order to plot the calibration curve one cuvette is filled with a dilute solution of carboxyhemoglobin in the appropriate fashion, i.e., with blood which has been completely saturated with carbon monoxide, while another is filled with a solution of oxyhemoglobin; they are lined up in the measuring device so that the light passes simultaneously through both cuvettes. In this case, absorption proceeds as if both substances were in the same solution. Convenient dilute blood values for plotting calibration curves are given in Table 5.

TABLE 5. COMPOSITION OF MIXTURES FOR PLOTTING CALIBRATION CURVE (IN ML)

Carboxyhemoglobin, %	1st cuvette		2nd cuvette		Carboxyhemoglobin, %	1st cuvette		2nd cuvette	
	Blood saturated with CO	Borate buffer	Blood saturated with O_2	Borate buffer		Blood saturated with CO	Borate buffer	Blood saturated with O_2	Borate buffer
1	0,02	1,98	0,02	1,98	5	0,1	1,90	0,10	1,90
2	0,04	1,96	0,04	1,96	6	0,12	1,88	0,12	1,88
3	0,06	1,94	0,06	1,94	8	0,16	1,84	0,16	1,84
4	0,08	1,94	0,08	1,92	10	0,2	1,8	0,2	1,8

Commas indicate decimal points.

In the previous section it was shown that if the light absorption of oxyhemoglobin is the same at both wavelengths, the calculations are considerably simplified and the carboxyhemoglobin concentration will be proportional to the difference between the extinctions at 2 wavelengths, divided by their sum. Therefore we can calculate the value:

$$\frac{E_{419} - E_{412}}{E_{419} + E_{412}}$$

where E_{419} and E_{412} are the extinctions of the solutions being studied, prepared /86 according to the table. In plotting the calibration curve, these data are plotted as a function of the percentile content of carboxyhemoglobin (first graph in the table).

Determination of the Amount of Absorbed CO

In order to determine the amount of carbon monoxide which the subject has absorbed from the gas mixture, it is analyzed twice on the infrared spectrophotometer — once before and again after the end of respiration. The first test was carried out so that we could check the composition of the mixture. The sample for analysis was collected in a cuvette with an optical path length of 10 cm, and the optical surfaces of the cuvette (front and back walls) had to be made of materials that would transmit infrared rays with a length of 4-5 microns. Discs of halogen salts which are hygroscopic are frequently used as such materials. In this case, the gas samples must be passed through a dryer beforehand. The carbon monoxide concentration is determined on the basis of the size of the peak with a wavelength of 2160 cm^{-1}, and the methane concentration must be determined on the basis of the peak with the wavelength of 3030 cm^{-1}. In these calculations it is necessary to calculate the magnitude of the optical density of the gas at maximum absorption. After finishing breathing the gas mixture, a second sample is taken and analyzed under the same conditions. For calculating the concentrations, a calibration curve is plotted for which a series of mixtures is prepared which contain increasing concentrations of carbon monoxide and methane.

Calculation. Initially we determine how many times the original volume of gas mixture (2 liters) was diluted by the residual air in the lungs. This value is equal to the ratio of the concentrations of methane prior to the start of breathing of the mixture and after its conclusion $[CH_4]_{final}/[CH_4]_{init}$. If the original volume was 2 liters, the final colume will be $2[CH_4]_{final}/[CH_4]_{init}$ CO_{final}. Since the total amount of CO in the mixture (in liters) prior to the start of respiration was 2 $[CO]_{init}$, the amount of CO absorbed is

$$2[CO]_{init} - 2\frac{[CH_4]_{init}}{[CH]_{final}}[CO]_{final} = [CO]_{abs}.$$

Since 1 g of hemoglobin can bind 1.34 ml of CO (Hufner coefficient), in order to be able to determine the quantity of hemoglobin which was bound with the carbon monoxide that was absorbed, this value is divided by 1.34.

If as a result of the inspiration of this amount of carbon monoxide the concentration of carboxyhemoglobin rose from $[COHb]_{init}$ to $[COHb]_{final}$, by /87 dividing the amount of hemoglobin bound with CO by this value and multiplying by 100, we will obtain the total amount of hemoglobin in the organism. The final calculation is made according to the formula:

$$\frac{[CO]_{abs} \cdot 100}{1.34 \cdot [COHb]_{final} - [COHb]_{init}}$$

Performance of Test. A gas mixture is prepared which is composed of 2 liters of air, 150 ml carbon monoxide and 20 ml methane. A blood sample is collected from the subject's finger tip for determination of the carboxyhemoglobin content. He is then asked to draw a deep breath, his nose is blocked with a clamp and a mouthpiece is placed in his mouth connecting him to a bag containing a gas mixture. The subject draws as deep a breath as he can from the bag (as much as the gas in the bag will allow) and for 20 seconds (i.e., one blood cycle) breathes in and out of the bag (recycled respiration). Then the subject exhales deeply into the bag, which is sealed with a clamp, and the test is over. Immediately after finishing breathing the gas mixture, the subject must make several deep inspirations and expirations in order to ventilate thoroughly the space in the lungs to get rid of the residual carbon monoxide.

If the rate of movement of the blood is of interest, the blood samples are collected immediately after the end of respiration at intervals of 10 seconds for 1 to 2 minutes and then 3 minutes following the end of respiration. If we are interested only in the total amount of hemoglobin in the organism, the samples are taken only after 3 minutes. For greater accuracy in analysis, several parallel tests are performed.

Preparation of gas mixtures. To prepare gas mixtures 2 liters of air are transferred into a rubber bag from a tank (gasometer), displacing it with water.

As the bag from which the mixture is breathed, one can use an ordinary rubber or plastic football bladder.

To prepare the gas mixtures, 2 liters of air and 150 ml of carbon monoxide as well as 20 ml of methane are added to a bottle of water, which may be drained out of it through a lower tube (gasometer). The carbon monoxide is obtained by heating formic and sulfuric acid and ordinary illuminating gas is used as the methane source. Then this mixture, displaced by water, is transferred to the /88 rubber bag. In order to be sure that there is really been nothing in the bag prior to the time it is filled, it is suggested that it be evacuated with a vacuum pump. It is also convenient to transfer the gas from the gasometer to the bag through the cuvette of the infrared spectrophotometer, passing the gas beforehand through a dryer; in this case the original sample is collected immediately. After the end of the experiment it is recommended that all of the volume of the

gas be passed through the gas cuvette during sampling. In this case we can be sure that the cuvette has been properly scrubbed.

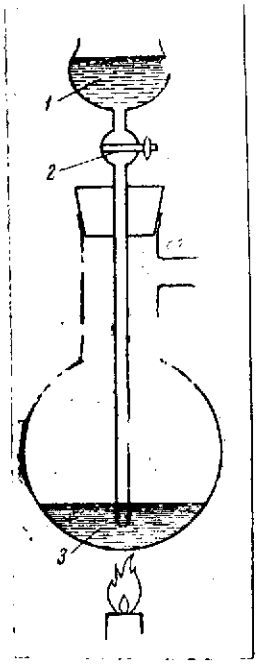

Figure 17. Apparatus for Obtaining Carbon Monoxide. 1, Formic acid; 2, stopcock; 3, sulfuric acid.

The device shown in Figure 17 is /88 used to obtain carbon monoxide. Sulfuric acid is placed in the lower flask and formic acid in the upper one. By opening the stopcock, formic acid is added in small amounts to the sulfuric acid. At the same time, by carefully heating the mixture over the flame of a gas burner, heating is regulated and acid added so that the reaction does not proceed too violently. In order to obtain carbon monoxide without contaminating it with air, which is necessary when plotting the calibration curves, the device is initially evacuated.

PART II

METABOLISM AND KIDNEY FUNCTION DURING SPACEFLIGHT /89

Dehydration As a Cause of Weight Loss

The changes in water-salt metabolism which take the form of a loss of body weight after flight were the first metabolic changes which were seen after the cosmonauts' return to Earth. The fact that this initially attracted attention is not surprising since weighing is a routine medical procedure usually employed to evaluate the health of an individual. The changes in body weight of both the Soviet cosmonauts and the American astronauts are shown in Tables 6 and 7, from which it is apparent that the total decrease was approximately the same for flights of different durations (from 1-24 days), although recovery proceeded differently.[2] Neither was there any apparent relationship between the general condition of the cosmonaut following the flight and the weight he lost. The weight decrease was the same aboard the craft in the "Soyuz" and "Apollo" series, although the dietary conditions, daily programs and gas compositions of the atmospheres in the cabins were different.

The American astronauts were weighed immediately after splashdown, and their weight loss was quite pronounced; however, in many instances they regained their original weight very rapidly. When these data are examined, it is necessary to keep in mind that their flights were terminated by splashdown at sea in a tropical area where the organism can lose water comparatively rapidly, immediately after the end of a flight, as a result of overheating.

The Soviet cosmonauts landed in a steppe region with a dry, temperate climate. Table 6 compares their weights before flight with the results of weighings which were performed on the morning of the day following their landings, on an empty stomach. As we can see from the table, the weight always decreased, regardless of the fact that they had been allowed to eat and drink /91 as much as they liked in the period of time between their landing and the weighing. The slight "non-compensated" weight loss in some of the cosmonauts on flights that lasted less than five days sometimes lasted several days but as a rule all of them had returned to normal by the second or third day after their landing. In the case of the crew of the "Soyuz" who spent 18 days in space, their weights returned to normal much more slowly.

As a rule the weight loss was not accompanied by concentration of the blood — this can be seen from Table 6, which summarizes the published data

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[2]Performing a formal statistical analysis of the published data on weight loss during flight, Davis [186] derived the formula $W = 2.207 + 0.4072 \ln t$, where W is the weight in kilograms, t is the time in days. In fact, this expression assumes that most of the weight loss took place during the first days of the flight.

on the changes in the concentration of the hemoglobin in the blood. In the majority of cases, they were so small that they were within the limits of error of the method. On the basis of these data we can conclude that if the reason for weight loss was dehydration, it was comparatively minor.

In order to ascertain the significance of weight loss for the organism and its pathophysiological mechanisms, it is very important to establish whether or not there was a breakdown of tissues or merely dehydration. This problem can be solved most simply by determining the nitrogen excretion of the organism during the flight. Inasmuch as each kilogram of the human body contains an /92 average of 30 grams of nitrogen, the breakdown of 2 to 3 kilograms of tissue would result in an increase of 60 to 90 grams; this could not go unnoticed on a flight that lasted several days if we take into account that a human being excretes 15 to 20 grams of this element every 24 hours. In those cases when the excretion of this amount of nitrogen was extended over 15 days or more, it would be possible to detect the additional excretion of 4 to 6 grams a day only under the conditions of balanced experiments where the intake of nitrogen with the diet and its excretion from the organism were strictly monitored.

Such studies were performed during the flight of the "Gemini VII" spacecraft which was in space for two weeks. Although Lutwak et al. [237] observed a negative nitrogen balance in this experiment, i.e., symptoms of tissue breakdown, its intensity was not so great that one could explain the weight loss by it (the results of these experiments are shown in Tables 12 and 22). In addition, it is risky to transfer results obtained in this fashion to flights lasting 3 and 5 days. In order to establish the reason for the weight loss during brief flights, it is necessary to use results obtained by an indirect method.

The nitrogen formed from amino acids in the liver during breakdown of tissues is converted into urea. The content of this substance in the entire organism amounts to several tens of grams and the possibility of its excretion through the kidneys is limited, so that the physiological system for excretion of nitrogenous products of tissue catabolism from the organism is quite persistent. Due to this persistence it is possible to study the composition of the urine after the flight to get some idea of the degree of breakdown of the tissues during a stay in space.

After all spaceflights, during the first 48 hours nitrogen excretion was practically the same as under normal conditions. This could be followed easily on the basis of the results of postflight examinations of the crew of the "Vostok" spacecraft, three of whose members remained in space for several days. Corresponding data on control and experimental periods are shown in Table 8 and in Figure 18. Since the excretion of nitrogen at night was always less than in the day, the results are shown separately for day and night. As we can see, there was no pronounced increase in the content of nitrogenous products in the urine, while the weight loss was 1.9 kilograms for the commander, 2.9 for the flight engineer and 3.0 for the physician. If this amount of tissue had been broken down, an additional 50-90 grams of nitrogen would have been excreted with the urine; this, of course, would not have remained unnoticed. Tables 14 and 15 contain data on the composition of the urine which was collected from the

cosmonauts after landing in the spacecraft in the "Soyuz" series; as we can see, the picture is similar to that which was contained for the crew of the "Voskhod".

TABLE 6. INFLUENCE OF SPACEFLIGHT ON BODY WEIGHT AND BLOOD HEMOGLOBIN CONCENTRATION IN SOVIET COSMONAUTS

Cosmonaut	Craft	Launch date	Length of flight (approximate), days	Change in weight in comparison with pre-flight weight, kilogram: Immediately afterwards	After 24 hours	After 48 hours	Change in hemoglobin concentration
G. S. T.	"Vostok-2"	—	1	−1,8	—	—	−1,4
A. G. N.	"Vostok-3"	—	4	−1,6	—	—	−0,2
P. R. P.	"Vostok-4"	—	3	−0,8	—	—	−0,2
V. I. T.	"Vostok-5"	—	3	−1,9	—	—	−0,4
V. F. B.	"Vostok-6"	—	5	−2,4	—	—	+0,2
		1964 г.					
V. M. K.	"Voskhod"	12 Oct.	1	−1,9	—	—	+1,1
K. P. F.	Ditto	12 Oct.	1	−2,9	—	—	+2,0
B. B. Ye.	Ditto	12 Oct.	1	−3,0	—	—	+0,4
G. T. B.	"Soyuz-3"	26 Oct. 1968	3	−4,0	—	—	+0,2
		1969 г.					
V. A. Sh.	"Soyuz-4"	14 Jan.	3	−3,9	−0,2	−0,7	—
Ye. V. Kh.	Ditto	14 Jan.	2	−1,9	−0,7	−0,2	—
A. S. Ye.	Ditto	14 Jan.	2	−2,0	−2,0	−1,5	—
G. S. Sh.	"Soyuz-6"	11 Oct.	5	−2,5	−1,5	−1,4	—
V. N. K.	Ditto	11 Oct.	5	−2,1	−0,9	−0,7	—
V. V. G.	"Soyuz-7"	12 Oct.	5	−2,0	−0,8	−0,8	—
A. V. F.	Ditto	12 Oct.	5	−3,9	−2,1	−1,8	—
V. N. V.	Ditto	12 Oct.	5	−2,4	−0,6	−1,3	—
V. A. Sh.	"Soyuz-8"	13 Oct.	5	−2,2	+0,2	+0,2	—
A. S. Ye.	Ditto	13 Oct.	5	−3,6	−1,6	−1,3	—
Average values for 5-day flights, M±σ				−2,7± ±0,6	−1,07± ±0,7	−1,01± ±0,7	—
		1970 г.					
A. G. N.	"Soyuz-9"	1 June	18	−2,7	−1,3	−1,3	+0,9
V. I. S.	Ditto	1 June	18	−4,0	−2,3	−2,7	+3,5
		1971 г.					
G. T. D.	"Salyut"	6 June	24	−2,9	—	—	—
V. N. V.	Ditto	6 June	24	−1,1		—	—
V. I. P.	Ditto	6 June	24	−2,4		—	—

Commas indicate decimal points.

TABLE 7. CHANGE IN BODY WEIGHT OF AMERICAN ASTRONAUTS WHO FLEW ABOARD APOLLO SPACECRAFT, AS COMPARED TO THE ACTUAL CALORIC CONSUMPTION (ACCORDING TO DATA FROM C. BERRY [163])

Astronaut	Craft	Lanch date	Duration of flight (approximate), days	Change in weight, kg: Immediate	Change in weight, kg: After 24 hours	Average actual caloric content of diet
		1968 г.				
Schirra	Apollo 7	11. Oct.	11	−2,7	−1,35	2000
Eisele		11. Oct.		−4,5	−2,7	2150
Cunningham		11. Oct.		−3,6	−0,9	1800
Borman	Apollo 8	21. Dec.	5	−3,6	−2,7	1500
Lovell		21. Dec.		−3,6	−3,2	1700
Anders		21. Dec.		−1,8	−1,35	1350
		1969 г.				
MacDivitt	Apollo 9	3. March	10	−2,2	−1,35	1900
Scott		3. March		−2,2	+1,35	1700
Schweikert		3. March		−2,7	−0,9	1650
Stafford	Apollo 10	18. March	8	−0,9	0	1400
Young		18. March		−2,2	−1,8	1500
Cernan		18. March		−4,5	−3,6	1700
Armstrong	Apollo 11	16. July	8	−3,6	−0,9	2050
Collins		16. July		−3,2	−3,2	1650
Aldrin		16. July		−0,45	+1,4	2300

Commas indicate decimal points.

Of all the tissues in the organism, the least quantity of nitrogen (0.6%) /93 is contained in the fatty tissue, so that theoretically, the breakdown of fat is not necessarily accompanied by pronounced azoturia. In connection with spaceflight conditions, the breakdown of several kilograms of fat in 1-5 days does not appear likely, since each kilogram of fatty tissue is equivalent to /94 approximately 7,000 kcal (assuming that its fat content is 80%). This amount of energy is equivalent to all of the energy expenditures of a cosmonaut for 3 to 4 days, and a great deal of oxygen would be required to oxidize it. According to the data of A. M. Genin et al. and L. G. Voronin [37], oxygen consumption during the flight amounts to 20 liters per hour, which is equivalent to 2,400 kcal a day. These results were obtained by means of an analysis of the remaining supplies of oxygen in the life-support system of the craft and they correspond approximately to the caloric content of the diet which was consumed and completely exclude the possibility of oxidation of a large quantity of fat in the organism.

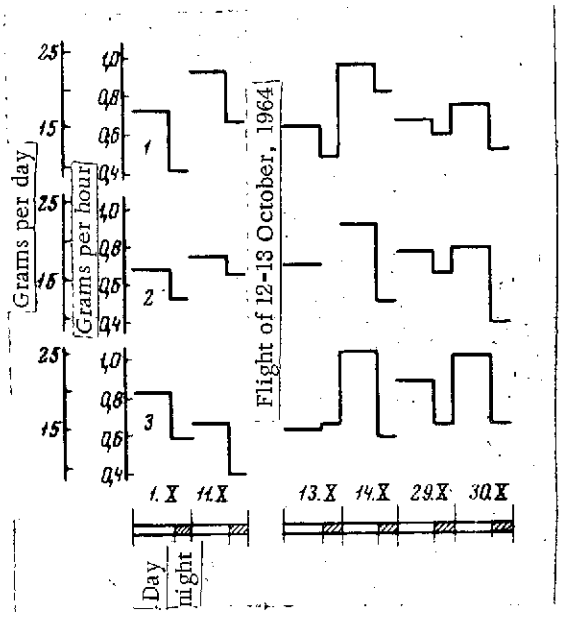

Figure 18. Excretion of Total Nitrogen in the Urine for the Crew Members of the "Voskhod" Spacecraft Before and After the Flight. 1, V.M.K.; 2, K.P.F.; 3, B.B.Ye.; a, grams per day; b, grams per hour; c, flight of 12-13 October, 1964; d, day; e, night.

The weights of both Soviet and American spacemen returned to normal two to three days after brief flights; this is also indicative of dehydration. To illustrate this view, Figure 19 presents data on observations of two young persons who spent three days under high temperature conditions with insufficient utilization of water. As a result of this exposure, each of them lost several kilograms which were replaced 24 hours after the end of the experiment. During this time, regardless of the fact that the subjects were given unlimited amounts to eat and drink and could take in as much salt as they wished, the excretion of sodium and chloride was lower than normal. Similar changes occurred in the cosmonauts of the "Soyuz-6", "Soyuz-7" and "Soyuz-8" spacecraft, as we can see from Figure 20. These data confirm the viewpoint that weight losses were the result of a decrease in water content in the organism.

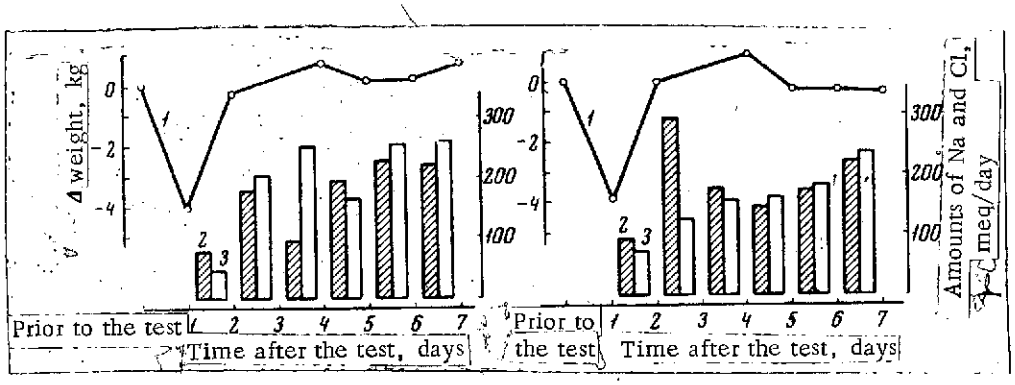

Figure 19. Dynamics of the Recovery of Body Weight and Excretion of Electrolytes with the Urine Following Dehydration Caused by a Stay in a Thermostatic Chamber by 2 Subjects. 1, Change in weight; 2, excretion of sodium; 3, excretion of chlorine. a, Δ weight, kilograms; b, prior to the test; c, time after the test, days; d, amounts of Na and Cl, meq/day.

TABLE. EXCRETION OF NITROGEN WITH THE URINE IN THE CREW MEMBERS OF THE "VOSKHOD" SPACECRAFT IN GRAMS PER HOUR).

Time of the investigation, 1964	V. M. K.	K. P. F.	B. B. Ye.
Before the flight			
Day, 1 October	0,72	0,67	0,82
Night of 1-2 October	0,40	0,53	0,58
day, 11 October	0,93	0,75	0,66
Night, 11-12 October	0,65	0,65	0,39
After the flight			
Day, 13 October	0,63	0,69	0,62
Day, 13-14 October	0,47	—	0,63
Day, 14 October	0,97	0,90	1,05
Night, 14-15 October	0,82	0,50	0,58
Day, 28 October	0,65	0,76	0,87
Night, 29-30 October	0,58	0,64	0,64
Day, 30 October	0,73	0,77	1,0
Night, 30-31 October	0,49	0,39	0,64

Commas indicate decimal points.

The total amounts of water and salt in the organism are closely related, inasmuch as the osmotic concentration of both the extracellular and intracellular fluids is one of the most important biological constants. In dehydration (or on the other hand retention) of water, there is always a corresponding loss or /95 accumulation of salts. In individual cases, the source of salt may be the internal reserves, particularly the bones of the skeleton. Viewing dehydration as a possible cause of weight loss during spaceflight, it cannot be imagined without an adequate excretion of salt.

G. I. Kozyrevskaya and A. I. Grigor'yev have shown that the changes in the osmotic concentration of blood serum following landing in comparison with preflight data are small and essentially within the limits of error of the method. Thus, in cosmonauts who flew aboard the spacecraft in the "Soyuz" series for five days, this increase, amounted on the average to only milliosmols while those who flew for three days lost six milliosmols (normal values are 290-310 milliosmols).

The pathophysiological mechanisms which resulted in this excretion of fluid cannot be considered to be completely understood. The decrease in the water and salt content in the organism, generally speaking, may be the result of either a decreased input of these substances to the organism or increased excretion.

During the flight, obtaining the exact information on the amount of water and salt taken in by the body on each day of the flight, as well as the composition and quantity of urine, was very complicated for a number of reasons. The greatest difficulties arose in collecting and returning the urine to earth for analysis. This is because it is not simple to drop the collected material to the ground. In order to facilitate this problem, during the flight of the American spacecraft "Gemini VII" the following procedure was used [237]. During the flight, the astronauts collected urine in plastic bags which were sealed with special valves that allowed the fluid to pass in only one direction, thus precluding accidental loss of the contents of the bag in the atmosphere of the spacecraft. Such care is very important since under conditions of weightlessness it is much more complicated to deal with fluids than it is on Earth. Before the flight, each bag was loaded with a small quantity of water which contained a radioactive isotope of hydrogen, tritium. After each diuresis, the astronaut would shake the contents of the bag, take out a small sample for analysis, and throw the remaining quantity overboard. The use of this system made it possible /96 to collect one sample from each batch of urine all during the 14-day flight. After returning to Earth, the radioactivity of each sample and its chemical composition were studied. Knowing the original radioactivity of the water that was in the bag before the flight, it was an easy matter to calculate the volume of each batch. In fact, the use of this methodological approach did not yield absolutely reliable results, since daily diuresis was reckoned as the sum of a number of individual fractions, some of which were lost and others apparently were insufficiently well mixed with the labeled water. Therefore, Lutwak, Whedon, Lachence, Reid and Lipscomb [237] considered it more reliable to calculate the excretion of all substances on the basis of the daily amount of creatinine, and the data on diuresis were not presented in the article.

Direct studies of urine composition during several days of flight were also performed onboard the Soviet spacecraft "Soyuz-9" (18-day flight around the Earth). This work was carried out by G. I. Kozyrevskaya and A. I. Grigor'yev. Each cosmonaut had available three rubberized bags so that he could collect all of the urine that was excreted on the first, second and eighteenth days of the flight and return all of it to Earth. There was no possibility of collecting diuresis during the entire flight, but only on certain days. However, this excluded the possibility of the error which disturbed the measurement of the amount of urine excreted during the flight of the "Gemini-7".

During the flight of the "Soyuz-9", collection of urine on the first day did not begin at the moment of takeoff but on the "morning" of the next day. Experience has shown that in the daytime, for various reasons, excretion of both soils and water is not uniform and these differences are compensated by nocturnal diuresis. This was the basis of the well-known Zimnitskiy test, in which urine is collected every four hours. If the functional capacities of the kidneys are limited, for example by cardiac decompensation, and the urine excretion during the day is low, the nocturnal diuresis will increase. In an

individual with healthy kidneys and heart, urine excretion during the day is always more than at night. If we begin to count the time of day not from the morning but from some other hour, for random reasons, the excretion of urine on one day may be more than on another. This random diversity of diurnal diuresis is compensated by appropriate changes in nocturnal excretion, so that if collection of urine is begun in the morning when the subject arises, the diurnal excretion of water and all substances will be more constant.

The quantity and composition of the urine collected from the crew of the "Soyuz-9" spacecraft during the flight, shown in Table 13, clearly shows that during the first two days the excretion of urine by both cosmonauts was somewhat less than usual for them under terrestrial conditions. The excretion of sodium and chlorine in A.G.N. was approximately the same level as before the flight while in V.I.S. it was somewhat less. At first glance these data indicate that /97 dehydration was caused rather by an insufficient intake of water and salt into the organism than by an increase in their excretion. However, this conclusion is premature, since during the very first hours after injection into orbit no urine was collected. The observations that were made on these two cosmonauts are inadequate for any conclusive judgements to be made.

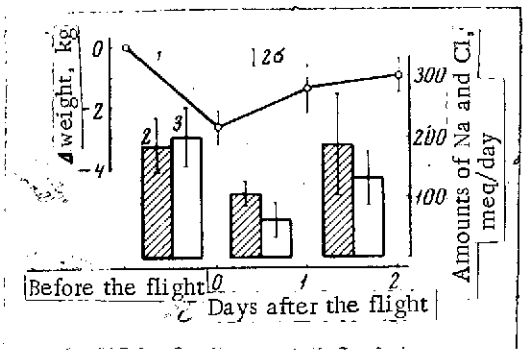

Figure 20. Dynamics of the Recovery of Body Weight (1) and Excretion of Sodium (2) and Chlorine (3) with the Urine in Cosmonauts Following a Five Day Flight Aboard the Spacecraft "Soyuz-6". "Soyuz-7" and "Soyuz-8" (Average Values).

In contrast to many animals, man has no physiological mechanisms that he can depend upon to keep the water content in the organism at a constant level. According to Denton [188], this ability is possessed by dogs, young rabbits, sheep, camels and goats. If animals of one of these species are kept without drinking for some time, so that their weight loss is 5-8% of the body weight, and then allowed to drink, they will regain their original weight in exactly ten minutes. With large water losses, some species lose this ability. At the same time, man, the rat, the adult rabbit, and the guinea pig, after losing even only a small part of their weight as a result of dehydration, cannot make it up by a single intake of fluid.

The reasons for these differences are completely unknown. It is possible that they have to do with the fact that some species retain salts by losing water, so that the osmotic pressure increases. The animal drinks until the concentration of salts reaches the required level. In man, a considerable amount of water is lost with the perspiration, and salts are lost too, so that the increase in osmotic pressure of the blood is not proportional to the water loss. It is difficult to support this conclusion experimentally since there are no sufficiently accurate methods

of determining the osmotic pressure of blood plasma. Of the other methods, the most accurate one is the cryoscopic method [132], but even that does not allow this theory to be verified. Therefore, we cannot reject the explanation provided by Denton [188] that the ability to compensate rapidly for water loss was developed in the course of evolution, since the threat of attack by predators is great while drinking water.

The ability of man to make up his water losses has been thoroughly studied by military hygienists of many countries and these problems are dealt with in detail in the book by E. Adolf, entitled, "Man under Desert Conditions" [2]. There an example is provided in which soldiers, performing heavy physical and emotionally stressful work under desert conditions, suffered significant dehydration, although they had adequate supplies of water in their canteens. Their attention was directed to other problems. If we express this idea by using terms that are employed in physiology, we can say that the system of reflexes that supports the constancy of the water content in the organisms of man was inadequate. This is supported by the observations made by military hygienists that water losses by soldiers during hard work in the heat are not made up immediately but only after several meals.

According to the observations of G. I. Kozyrevskaya, almost all of the cosmonauts noticed a significant onset of a feeling of thirst during the flights and initially drank only in the evening on the day of the launch, during their dinner. However, some of them (G.S.Sh., V.N.K., V.V.G.) felt that there had not been any change in their subjective sensation of thirst. This subjective observation was not supposed by the analysis of the functioning of the life support system which showed that they used 700 to 800 milliliters of fluid each per day, while under terrestrial conditions the water use was 1,200 to 1,400 milliliters. During the space flight, the food and the form in which the water was supplied unavoidably differed from those that were usual for terrestrial drinking conditions. It was impossible to eat soup out of bowls or drink coffee or tea and so on out of glasses. Water, juice and beverages were not so much drunk as sucked up through straws. The lack of normal external stimuli which accompany eating and drinking under terrestrial conditions may have meant that the cosmonauts simply forgot to drink. Such a mechanism could possibly explain weight loss during flights lasting one or two days, but it is difficult to imagine that the human organism could not adjust to the new conditions in three to five days and begin to use water in the necessary amounts. /98

It is also impossible to explain weight loss by inadequate intake of salts into the organism, since their content is sufficient in both the American and Soviet diets. According to V. P. Bychkov and P. P. Ivanov [29] the food ration of the Soviet cosmonauts contains 5.91 grams (225 meq) sodium, 1.89 grams (47 meq) potassium and 4.29 grams (122 meq) chlorine. The solid ration of the "Gemini VII" astronauts, according to Lutwak et al. [237] included 145 meq of solid sodium and 36 meq potassium. These amounts do not differ significantly from those which are usually found in the diet and are much greater than the minimum requirements of man. On various flights by craft in the Vostok and Soyuz series, as in the case of the Apollos, the composition of the ration changed for various reasons, but should not have had an important effect on the

total content of salts, since the makeup of the basic products remained the same. One might expect a sharp decrease in the content of salts only in the event that primarily lyophilized products were used aboard one of the craft, but their use makes sense only for flights of long duration, when regenerated water is used for drinking. On the flights of this duration which have taken place thus far, such products were not employed.

The influence of salt-free diets on the state of the human organism was checked in our experiments; subjects spent 10 days at an ambient temperature of 37 degrees so that the thermoregulation was maintained exclusively by evaporation of sweat, and several grams of salt were lost each day. The young persons who took part in these experiments received a diet that contained approximately 40 meq of sodium, i.e., the minimum possible amount of salt and water, without limitation. Even under such maximally stressful conditions, by the end of the 10th day of the period the subjects had practically not changed their original weight. /99

The explanation of weight loss by the cosmonauts proposed by O. Gauer has received wide attention; it follows directly from a series of his works written jointly with D. Henry and other authors [40, 204-207], dealing with the regulation of blood volume in the organism. According to the concepts which they developed, under conditions of weightlessness the blood flows from those portions in which it is usually stored as a result of the action of the force of gravity and the vessels of the chest become overfilled, thus creating a false sensation of an increase of blood volume in the organism. The distention of the portions of the vena cava located beneath the pericardium and the portions of the auricle which are adjacent to it leads to the development of a reflex whose action results in an increase in excretion of water and salts in the organism. The afferent portion of this reflex is formed by the fibers of the vagus nerve and the efferent portion by the sympathetic innervation of the kidneys. The experimental proof of the correctness of this hypothesis is provided by experiments in which it is possible to produce an increase in water and salt diuresis, changing the pressure in the vicinity of the mouth of the vena cava in dogs, as well as by administering various solutions into the blood stream. By using electrophysiological methods it was also possible to show that the vagus nerve contains fibers which react by discharges in response to distention of the openings of the vena cava by rubber balloons.. Cooling or sectioning the vagus nerve eliminates these effects.

At papers delivered at the second and fourth international symposia entitled "Man in Space", which were held in Paris in 1965 and in Yerevan in 1971 [40, 205], O. Gauer presented the results of numerous observations which confirmed the existence of a similar reflex in man. In these observations, the subjects were subjected to influences that resulted in a redistribution of the blood, similar to that which can be anticipated in a state of weightlessness (or, on the other hand, the reverse of it).

The simplest model of such blood redistribution is the stay of a human being in a horizontal position in bed (clinostatic hypodynamia) or in water, (water immersion). When a human being is in water, the weight of the blood is

the balanced weight of the displaced fluid so that the blood does not collect in the lower parts of the body under the influence of gravity.

When a person is in a horizontal position in bed, the orthostatic gradient is eliminated and the blood does not collect in the lower extremities, but some amount may be deposited in the lower soft tissues, for example, when lying on one's back, in the skin and muscles of the back. Therefore, this model is considered not so much a strict model of weightlessness as water immersion. Table 9 summarizes some of the data on the change in blood volume and blood plasma under these influences. As we can see, in the overwhelming majority of /101 cases, particularly in relatively short experiments involving water immersion, the plasma volume decreased. Under these same influences, during the first hours and days of the experiment, increased excretion of water and electrolytes with the urine was observed (Table 9).

Another influence which causes a decrease in the mass of blood which is deposited in the extremities is respiration under negative pressure. In this case, pressure lower than atmospheric is produced inside the chest cavity (by 10-30 cm water column) as a result of which the blood is "sucked" into the lungs. In the majority of subjects, increased diuresis results [170].

The opposite effect, accumulation of blood in the lower part of the body, occurs when a negative pressure is imposed upon it measuring tens of millimeters of mercury. The lower part of the body of the subject is placed in a container which is sealed at the level of the waist or stomach. This effect is called NMLB for short and it leads to a decrease in diuresis and the development of ADH in the blood [257].

In a number of observations it has been shown that all of the factors which lead to the accumulation of blood in the lower part of the body also produce an increase in the antidiuretic activity of the blood and the content of aldesterone in it — factors which retain water in the organism. On the other hand, when there is a redistribution of blood from the extremities to the chest, the activity of the latter begins to decrease and even human plasma acquires the ability to increase diuresis in anesthetized rats [40].

The ability and the necessity of systematically changing from a horizontal /102 to a vertical position is very important in distinguishing the regulation of blood circulation in man from its regulation in many (but not all) species of animals. Modern man, standing erect, spends part of the time lying down, when the difference in blood pressure between the vessels in the brain and the internal organs depends solely on the vascular tone and the laws of motion of the blood through them. Man spends another part of his time standing so that in /103 addition to the blood pressure produced by the work of the heart its own weight is added — about 75 mm of mercury per meter of a column of blood. Therefore, it is no surprise that each transition to a vertical position causes a regular storm of physiological reactions, the first indication of which is a speeding up of the pulse. Later, humoral changes occur — increased secretion of aldesterone, renin and angiotensin — substances which also have a profound influence on the water-salt exchange. Some papers dealing with this problem have been summarized in Table 10. The humoral reaction to orthostasis is so regular that

in describing new methods of determining aldesterone, it is necessary to check how completely they are manifested.

TABLE 9. INFLUENCE OF HYPODYNAMIA ON BLOOD VOLUME AND BODY COMPOSITION (M OR M ± σ)

Parameters and Methods of study	Duration of experiment, days	Number of subjects	Results	Bibliographic Reference
Volume of plasma, albumens, labeled with I^{131}	6	10	Decrease from 38.8 ± 2.2 to 36.6 ± 1.4 ml per kilogram in one day and up to 34.9 ± ± 1.6 ml per kilogram in 6 days	[288]
	6	10		
	6	4	Decrease by 506 ml	[274]
	10	4	Decrease by 287 ml	[167]
	10	11	Decrease from 3387 to 3055 ml on the 4th day and up to 2963 ml on the 10th day	[280]
	14	4	Decrease in 3 days by approximately 250 ml, further decrease statistically unreliable	[281]
	14	10	Decrease in 5 days by 0.6% of the body weight, then unchanged	[223]
Mass of erythrocytes Cr^{51}	6	10	Unchanged (prior to the experiment, 27.9 ± 1.4; after 24 hours, 27.9 ± 1.4; on the 6th day, 27.9 ± 1.4)	[288]
	Various	11	Decrease by 8.6%, studies performed on orthopedic patients subjected to bed rest for a long period of time)	[250]
Total metabolizable water in the organism H_2^3O	11	10	Decrease from 45.8 to 45.1 liters on the 4th day and to 45.1 liters on the 10th day	[280]
Extracellular fluid	6	10	Unchanged (217 ± 10 prior to the experiment, after 1 day, 216 ± 9; on the 6th day 213 ± ± 10 ml per kilogram)	[288]
	11	10	Decrease from 16.3 to 15.2 liters on the 4th day and to 14.1 liters on the 10th day	[280]

Parameters and Methods of study	Duration of experiment, days	Number of subjects	Results	Bibliographic reference
Metabolizable potassium	10	10	Insignificant decrease in the control from 172 to 161 grams and in the group with physical stress from 164 to 159 grams	[203]
Fat component of the body (weighing under water)	10	10	Increase in the control group from 12.85 to 13.25%; in the group with physical stress from 13.93 to 15.25%	[203]
	Excretion during bed rest			
Water balance	20 days	10	Decrease by 250 ml per day on the average	[223]
	6	4	During the first days, negative	[274]
			During the 1st days, decrease	[61]
Volume of urine	6 days	10	Increased by 18% during the first days	[203]
	up to 6 months	22	Increase in diuresis by 200--700 ml during the 1st few days	[48]
Sodium balance	20 days	10	Decrease by 16 meq per day on the average	[223]
	6 days	10	On the 1st day, negative, 109 meq/day	[203]
Excretion of sodium	6 days	4	Increase during 1st days	[274]
	Excretion during water immersion			
Volume of urine	6 hours	5	Increase from 1.25 ml per minute in the control to 3.47 ml per minute in the experimentals	[185]
	6 hours	16	Increase from 304 ml in 6 hours in the control to 1203 ml in 6 hours in the experimentals	[211]

TABLE 10. CHANGE IN THE CONTENT OF ALDESTERONE, RENIN AND ANGIOTENSIN IN THE PLASMA WITH A SHIFT FROM A HORIZONTAL TO A VERTICAL POSITION

Substance, Method and Units of measurement	Conditions and Time of Experiment	Results	Source
	Aldesterone		
Double isotope label, concentration in the plasma, ng% (M ± σ)	6 men, 2 hrs. lying down	2.62 ± 0.92	
	Ditto, 2 hrs. standing	25.3 ± 6.11	[158]
	6 men, 2 hrs. lying down	1.12 ± 0.59	
	Ditto, 2 hrs. standing	6.88 ± 2.14	[158]
Gas chromatography, concentration in the plasma, ng% (M ± σ)	7 men, 4 hrs. lying down	2.6 ± 0.7	
	27 men, ambulatory	7.7 ± 3.1	[249]
Concentration in the plasma, ng%	Lying down 1 hr.	5.2 ± 0.4	[222]
	Seated	9.2 ± 0.9	
Infusion of radioactive aldesterone, rate of secretion, ng/minutes (M ± σ)	6 men, 2 hrs. lying down	43.6 ± 17	[158]
	Ditto, 2 hrs. standing	330.4 ± 126	
	6 men, 2 hrs. lying down	22.1 ± 12	[158]
	Ditto, 2 hrs. seated	113.9 ± 46	
	Renin		
Biological method, activity of angiotensin in plasma, ng/ml per hr. (M ± m)	9 women, lying down, usual diet	0.7 ± 0.1	[216]
	Ditto, standing, 4 hrs	1.0 ± 0.2	
	9 women, lying down, salt--free diet	2.1 ± 0.3	[216]
	Ditto, standing, 4 hrs.	3.5 ± 0.2	
Radioimmunological method, activity of angiotensin in the plasma equal to 1, ng/ml per hr. (M ± σ)	Lying down, usual diet	1.02 ± 0.21	[216]
	Ditto, standing 4 hrs.	1.75 ± 0.43	
	Lying down, salt-free diet	2.16 ± 0.45	[216]
	Ditto, standing	5.92 ± 0.4	
	Angiotensin		
Radioimmunological method, concentration in the plasma, ng/ml (M ± σ)	Lying down, normal diet	26.3 ± 6.7	[216]
	Ditto, standing	37.4 ± 4.5	
	Lying down, salt-free diet	47.4 ± 11	[216]
	Ditto, standing	66.7 ± 12	

The ability to maintain blood pressure during a transition from a horizontal position to a vertical one is closely linked to the adrenal cortex. One of the symptoms of Addison's disease (the clinical form of insufficiency of the adrenal cortex) is a tendency toward orthostatic collapse. Under conditions of weightlessness, a human being immediately loses all of the group of stimuli that are associated with a change in body position in a gravitational field and it makes no sense to think that this will have no effect on him.

The entire body of all the data presented above leaves no doubt that man actually has a system of reflexes which, when the central volume of blood increases, the organism escapes a "shortage" of fluid. Another problem is the nature of the role played by these reflexes in the mechanism of weight loss by cosmonauts during flight. To be sure, thus far there have been no reliable experimental data which could show that cosmonauts, immediately after finding themselves in a state of weightlessness, undergo an increase in diuresis. Even when such studies can be carried out, it will not be a simple matter to process the results obtained, since the transition to weightlessness causes disappearance of thermoconvection and there is a change in the thermoconductivity of the gas medium. Therefore, the temperature regime of the human body will be disrupted and perspiration excretion as well. This applies to experiments with water immersion to a still greater degree, as was pointed out by G. P. Conradi.

In a paper by O. Gower [40] as well as one by Boyland and Antnowiak [170], it was shown that increased excretion of water and salt under submersion in water and breathing under negative pressure may be eliminated by administering very small doses of vasopressin. It is also known that it is by no means always certain when an individual shifts from a vertical to a horizontal position that diuresis increases. Thus, in healthy human beings diurnal diuresis is always greater than nocturnal, although we spend more time in a vertical position in the daytime and in the horizontal at night. This indicates that the Gower--Henry reflex is less powerful in comparison with the diurnal rhythm. Could it be that all of the extreme flight factors with which the astronaut comes in /104
contact particularly during the time the rocket lifts off, constitute a weaker stimulus then the administration of small doses of ADH or simple alternation of day and night? Can we expect that the reflex in which we are interested will function during a spaceflight in the same fashion as it does during laboratory experimental conditions?

For this reason, the reflex mechanism in question must be considered as only one of the possible hypotheses: probable, but still not proved as far as spaceflight conditions are concerned. Another, alternative explanation is that because of the lack of applied stimuli, the abrupt change in the situation and the overload of work, the cosmonauts simply forget to drink at the beginning of the flight.

It can also be assumed that excretion of water is affected by reflexes from other parts of the vascular tree, particularly the vessels of the brain; thus, many cosmonauts have complained of unpleasant sensations of a rushing of the blood to the head during the first few minutes of the flight. As they put it, the sensation is created that they are upside down [35, 36]. Obviously this

sensation arises because the difference in hydrostatic pressure between the vessels of the internal organs of the head has disappeared and the flow of blood from the brain has been disrupted. We cannot exclude the possibility that this zone may be the source of a reflex directed at the kidneys, although in older studies Cathcart and Williams were unable to find a relationship between the position of a patient's head and the composition of the urine [176].

In dealing with the problems of water-salt balance under spaceflight conditions, several investigators expressed still other viewpoints in 1967 concerning the mechanisms of weight loss during spaceflights. Yu. V. Natochin, essentially sharing the view of O. Gauer, was more inclined to think that the effector mechanism of the reflex is not the sympathetic nervous sytem but the secretion of the antidiuretic hormone. Ya. V. Bagrov held to the opinion that it is not so much the redistribution of the blood and the reflexes from the portions of the vascular tree with low pressure that is important as it is the redistribution of pressure and the secretion of angiotensin that occur. In an article written as far back as 1956, i.e., before the beginning of the spaceflight era [17], we also devoted more attention to the redistribution of the blood pressure than we did to the mass of the blood. This view is supported by the observations of I. D. Pestov [117] and published in an article by Berry [25, 163], indicating that when a human being changes from a horizontal position to a vertical one (orthostasis), the change in the pulse rate is not correlated with the amount of blood which is deposited in the lower extremities. Thus, with poor tolerance of the test, the pulse increases in the course of ten to twenty minutes in a standing /105 position, while the volume of the legs (or their perimeter) increases immediately to a certain level and then remains constant. The degree of this increase shows no correlation with the tolerance of the test.

In drawing conclusions from the available data on the state of water metabolism during flights and in model tests, we can conclude that the direct cause of weight loss during brief flights is the loss of water. The explanation of the reasons for this dehydration, proposed by O. Gauer, shows rather good agreement of the majority of results of model experiments, but fails to take into account the influence of other spaceflight factors which may be combined with the action of weightlessness.

Excretion of Salts

The supplies of all mineral substances in the organism are so great that they cannot be significantly exhausted in the course of three to four to five days of spaceflight, even with complete suspension of intake of salts with the food (Table 11). The investigation of their excretion under these conditions is interesting primarily because it makes it possible to obtain information on how those mechanisms function in weightlessness which are responsible for salt homeostasis in the organism and primarily to evaluate the state of the most important of these mechanisms — the renal system.

The dynamics of the excretion of salts has been studied directly during flight on only two spacecraft, so that in order to evaluate the ability of the organism to maintain a state of water-salt balance, it is necessary to use

basically those data which were collected in the studies following landing. However, during this time it is not always possible to differentiate simply between those changes which developed during the stay in space and those which are a reaction following the transition from weightlessness to terrestrial gravitational conditions.

American researchers who studied mineral exchange in the crew members of the "Gemini-VII" during flight observed pronounced negative sodium, potassium, phosphorus and sulfur balance [237] (Table 12). The excretion figures given in this paper for the loss of potassium and sodium with the urine are within the limits which are normal for healthy human beings (men) while they are much higher than all of the losses of these elements with the feces and sweat. It is possible that the negative balance is the result of random variations and the retention of a stereotype of excretion of these substances with a restriction of the intake with the diet. It is not known why sodium losses are accompanied by aldesteronism. In general, a similar picture was found in the crew members of the "Soyuz-9" spacecraft, in whom the potassium excretion was relatively low. One is truck by the high level of excretion of sodium and chlorine during the first days in A.G.N. (Table 13). On the whole, the available material on the /106 flight is inadequate for us to draw any final conclusions.

The data on the condition of electrolyte metabolism in the postflight period are much more extensive, since it was possible to examine 17 cosmonauts who had made flights lasting from one to 18 days aboard spacecraft in the /107 "Vostok" and "Soyuz" series. The parameters of water-salt metabolism were studied with particular care in the crew members in the "Voskhod" spacecraft which flew for 1 day and the "Soyuz-6, 7 and 8" craft which were in space for five days each. The data on the excretion of the various electrolytes during the first two days following landing are shown in Tables 14 and 15 and in Figure 21 [15]. The duration of urine collection during the first day following the end of the flight was not always equal to 24 hours, since the landings took place at different hours and it was not always a simple matter to determine precisely what period should be considered as the first 24 hours. For this reason, some of the portions of the urine from G.S.Sh. and V.N.K. were included in both the first and second days. This method of analyzing the data involves a certain degree of inaccuracy, but it makes it possible correctly to understand the dynamics of the process. Any other method of counting the days, as we have already pointed out, involves more errors due to the irregularity of excretion of various substances in the course of the day.

In examining the Soviet cosmonauts during the postflight period, only the excretion of salts with the urine was taken into account; their excretion with the perspiration and the feces were not taken into account; since the intake with the food was not measured, i.e., balance studies were not carried out. This approach is governed by the fact that it makes sense to study the balance only in the case when the individual spends a sufficiently long period of time under the same conditions, since it is necessary to have at least three to four days during which equilibrium can be established. On flights lasting only a few days it is merely necessary to study transitory processes — initially, accomodation to flight conditions; later, readaptation to terrestrial conditions. Excretion of substances with the feces, especially calcium, is an inertial process; since /109

the fecal masses are formed in the intestine in the course of several days, their composition is a summary function of a number of long-term processes.

TABLE 11. COMPOSITION OF THE BODY OF AN ADULT MAN*

Substance or tissue studied	Calculated on the basis of		Method used and Source in the Literature
	1 kg	70 kg	
Water	--	41.4 kg	--
Fat	--	12.6 kg	--
Muscle	--	30.0 kg	--
Nitrogen	30 g	2.1 kg	--
Total sodium	--	2,750 meq (63 g)	Chemical analysis [275]
Total potassium	--	4,250 meq (150 g)	
Total calcium	--	58,000 meq (1.16 kg)	
Total magnesium	--	1,700 meq (20.7 g)	
Total phosphorus	--	670 g	
Total sulfur	--	120 g	
Total amount of water in the organism	548 ± 24 ml/kg	--	H_2^3O [228]
Extracellular fluid	217 ± 10 ml/kg	--	S^{35} [228]
Plasma volume	38 ± 2.2 ml/kg	--	Albumin, labeled with I^{131} [228]
Volume of erythrocytes	27.9 ± 1.4 ml/kg	--	Cr^{51} [228]
Mass of hemoglobin	9.8 1.4 g/kg	--	CO, our data, see page 83
Metabolizable sodium	42.4 meq	2,950 meq	Na^{24} [123]
Ditto	40.5-41.4 meq	2,850-2,900 meq	Survey of the literature [121]
Metabolizable potassium	41.4 ± 1.22 meq	--	K^{42} [124]
(85-97% of total potassium)	45-48 meq	--	Survey of the literature [121]
Ditto	49 ± 0.75 meq	--	K^{40} [25]
Potassium, metabolizable in 2 days	234 ± 1 meq	16.4 meq (328 g)	Ca^{47} [276]

*Whereever possible, data were used that were obtained on groups with physical conditions similar to those of the cosmonauts.

TABLE 11. CONTINUED.

Substance or tissue studied	Calculated on the basis of		Method used and source in the literature
	1 kg	70 kg	
Amount of metabolizable potassium/kg of muscle	0.071-0.108 meq	--	The muscle mass was determined on the basis of distribution of radioactive creatinine; the metabolizable potassium was determined on the basis of K^{42} [233].

TABLE 12. AVERAGE VALUES OF BALANCE (DIFFERENCE BETWEEN INTAKE AND EXCRETION) OF SEVERAL ELEMENTS DURING THE FLIGHT OF "GEMINI VII" AS CALCULATED OVER SEVERAL DAYS (ON THE BASIS OF THE WORK OF LUTWAK ET AL. [263]).

Time of Study	Nitrogen g/day	Sulfur g/day	Phosphorus g/day	Sodium meq/day	Potassium meq/day	Calcium meq/day	Magnesium meq/day
Commander of the Craft							
Before the flight (10 days)	−0,02	+1,207	+0,668	−48,4	+11,7	+ 4,7	+ 3,6
During the flight (14 days)	−3,43	−0,51	−0,69	−72,1	−64,6	− 0,3	− 4,3
After the flight (4 days)	−3,99	+0,838	+0,358	+ 8,6	+12,0	+ 0,2	+ 9,6
Copilot							
Before the flight (10 days)	+0,38	+1, 39	+0,707	−50,2	+20,1	+24,7	+ 7,0
During the flight (14 days)	−1,34	−0,243	−0,504	−49,9	−22,9	+ 4,9	− 1,2
After the flight (4 days)	+0,84	+0,953	+0,791	+30,7	+35,6	− 5,4	+11,6

Commas indicate decimal points.

As we can see from the table, in the majority of Soviet cosmonauts the excretion of sodium, potassium and chloride during the first day following the end of the flight was considerably below the average levels of the preflight period. Evidently this was due to a somewhat lower content of these substances in the diet aboard the craft (more precisely, in that part which the cosmonauts actually ate) and also the fact that during this time there was a recovery of the weight that had decreased during the flight and the salts were being retained in the organism together with the water. In the American "Gemini-VII" astronauts, judging by the average data for the four days of the postflight examination, there was no such retention of salts in the organism. However, according to the

data which appear in the article by Berry [263] on the results of medical examinations aboard the spacecraft in the "Apollo" series, the crew members, after landing, showed an excretion of potassium, sodium and chlorine that was less than that for the Soviet cosmonauts.

TABLE 13. EXCRETION OF VARIOUS SUBSTANCES WITH THE URINE IN THE CREW MEMBERS OF "SOYUZ-9" SPACECRAFT AS COMPARED WITH THE DATA OBTAINED ABOARD THE AMERICAN SPACECRAFT "GEMINI-VII"

Duration of the flight. cosmonaut	Time of study, days	Diuresis, ml	Total nitrogen g/day	Creatinine g/day	Phosphorus g/day	Chlorine meq/day	Sodium meq/day	Potassium meq/day	Calcium meq/day	Magnesium meq/day
				"Soyuz-9"						
18 days A. G. N.	1	830	10,0	1,7	0,47	225	300	35	13	—
	2	840	13,8	2,1	0,84	183	188	38	10	—
	18	1028	19,1	2,6	1,27	175	188	48	20	—
V.I. S.	1	800	9,2	0,64	4,6	132	205	27	12	—
	2	750	13,2	2,1	4,3	123	145	37	12	—
	18	830	15,0	2,1	4,8	137	188	42	15	—
				"Gemini-VII"						
14 days Commander of the craft	During the entire flight M ± σ		17,9±2,3	1,6±0,3	1,74±0,4	66±42	196±41	93±42	12±1,6	10±2,8
Copilot			16,2±1,9	1,7±0,3	1,57±0,15	146±22	181±23	51±6	8,1±0,95	8,0±1,3

Commas indicate decimal points.

Special attention should be paid to the high levels of excretion of certain salts during the postflight period, which were most clearly pronounced in V.N.K. following a five-day flight and occurred to a lesser degree than in other cosmonauts. /110 In the case of V.N.K., during the first days following the landing, the excretion of all salts with the exception of calcium was lower than the values which were normal for him, but during the second day the content of chlorine, sodium and calcium was 1-1/2 to 2 times higher than usual. On the second day following the landing, there was also a slight increase in the excretion of potassium in some of the other cosmonauts as well. The differences between the excretion of salts during the first and second days following the landing cannot be the result of random errors in the collection of urine or in the determination of the time periods which were to be considered as the first /111 day, inasmuch as the excretion of creatinine was within normal limits.

One gets the impression that during the flight and immediately afterward the mechanisms of regulation of salt excretion are under the influence of two opposite stimuli — on the one hand, a relative deficit of salts leads to their retention, while on the other hand there is an influence of some powerful factor under whose influence the salts are powerfully expelled from the organism. It is evident that in the majority of cases these tendencies balance each other, but in some persons, under the influence of factors which we do not understand, the

compensation is less accurate or even shows a tendency toward a clear predominance of salt excretion.

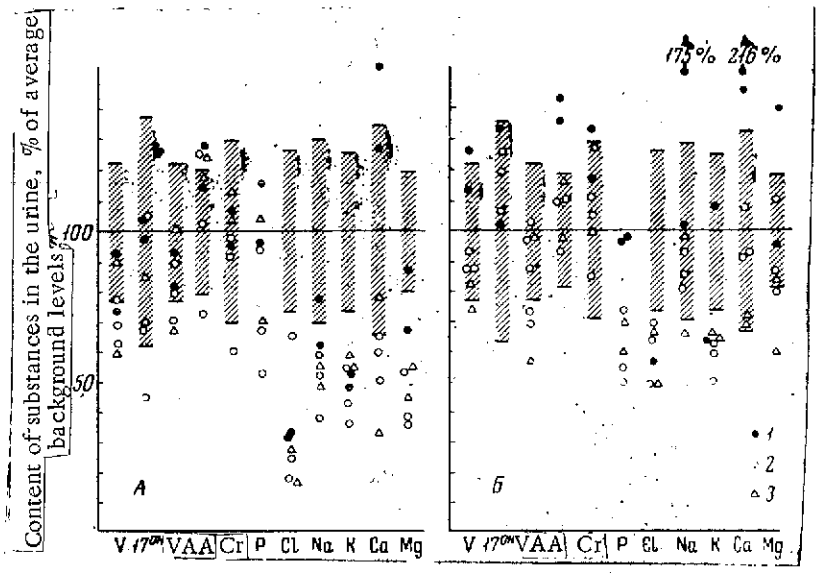

Figure 21. Excretion of Various Substances with the Urine in the Cosmonauts During the First (A) and Second (B) Days Following the 5-Day Flights Aboard the "Soyuz-6" (1), "Soyuz-7" (2) and "Soyuz-8" (3) Spacecraft. The shaded areas correspond to the two mean square deviations of normal values; v = diuresis; 17OH = 17-oxycorticosteroids; VAA = = vanilyl-amygdalic acid, N = total nitrogen; Cr = creatinine.

Test with Water Load

/112

In order to understand the functional state of the mechanisms of regulation of excretion of water and salts following flights, considerable importance attaches to the results that were obtained following the performance of tests involving a water load. This test, which was called the Robinson test or the Pauler and Tepler test [8] was originally proposed for determining very diverse states, including hypocorticism [156]. The test consists in the following: the subject is allowed to drink on an empty stomach an amount of water which corresponds roughly to 2% of the body weight and the excretion of urine is measured for the next two or four hours. A healthy person, in a normal state of hydration, usually excretes in the course of two hours 60% or more of the water drunk, and in four hours more may be excreted than was drunk. This phenomenon was referred to by the old clinicists on some occasions as "water shock" and was used as a distinct diuretic substance. The test is very sensitive and makes it possible to determine even comparatively moderate degrees of dehydration. In 1964, we first employed this test in cosmonauts [104] for the purpose of determining possible disruptions involving the function of the kidneys, since there was reason to believe that g-forces during launching or landing could affect the function of

this organ. The results of the study were somewhat unexpected. While there were no signs of any damage to the kidneys, both of the cosmonauts who were studied, especially the commander of the craft, clearly showed themselves to be incapable of rapidly excreting the water they had drunk. Inasmuch as the ratio of potassium to sodium in the urine and the daily excretion of 17-oxycorticosteroids did not undergo any important changes, the pronounced disturbances in the function of the adrenals may be excluded. Similar changes in the water-excretion function were found in practically all cosmonauts and the results of these studies are shown in Table 16 and Figures 22 and 23. One is truck by the fact that on the 18-day flight there was no water retention. Since this observation refers to only two cosmonauts, it is difficult to say whether or not it has a regular nature of whether it is a result of individual characteristics.

TABLE 14. EXCRETION OF VARIOUS SUBSTANCES WITH THE URINE IN COSMONAUTS ON THE FIRST DAY FOLLOWING LANDING

Craft, cosmonaut	Duration of the urine collection in hours	Urine volume, ml	Total nitrogen, grams	Creatinine, grams	Phosphorus grams	Chlorine meq	Sodium meq	Potassium meq	Calcium meq	Magnesium meq
"Soyuz-3"										
GTB	24	690	14,8	1,53	1,12	—	52	80	—	—
"Soyuz-4"										
VASh	24	—			0,67	64	117	35	9,0	—
"Soyuz-5"										
BVV	24	1 100	18,2	1,86	0,97	164	200	62	10,6	—
Ye. V. Kh.	24	1 200	21,8	2,1	1,05	160	206	58	10,2	—
A. S. Ye.	24	1 110	20,4	2,26	1,09	96	165	51	11,6	—
"Soyuz-6"										
G. S. Sh.	24	880	16,0	1,66	0,83	61	144	37	21,9	6,2
VNK	23	705	14,5	1,47	1,0	67	116	35	17,8	8,0
"Soyuz-7"										
AVF	22	655	12,8	1,43	0,46	129	109	39	9,3	3,5
VNB	21,7	590	9,1	0,94	0,58	38	71	26	7,1	3,3
AVG	22,5	730	15,7	1,5	0,82	50	97	31	8,6	5,0
"Soyuz-8"										
VASh	21	580	14,6	1,75	0,60	54	88	39	4,6	5,1
ASYe.	21	875	15,6	1,62	0,90	34	102	42	11,1	4,1
"Soyuz-9"										
AGN	24	690	15,0	2,1	0,8	92	104	25	11,5	—
VIS	24	665	13,1	1,5	0,76	65	125	30	11	—
Average values for the preflight period	M	952	12,6	1,55	0,865	188	185	72	14,2	9,2
	σ	216	2,4	0,47	—	54	55	19	4,7	1,8

Commas indicate decimal points.

TABLE 15. EXCRETION OF VARIOUS SUBSTANCES WITH THE URINE IN COSMONAUTS ON THE SECOND DAY FOLLOWING LANDING

Craft, cosmonaut	Duration of the urine collection in hours	Urine volume ml	Total nitrogen, grams	Creatinine grams	Phosphorus grams	Chlorine meq	Sodium meq	Potassium meq	Calcium meq	Magnesium meq
"Soyuz-3"										
GTB	24	600	13,2	1,43	0,54	—	32	33	—	—
"Soyuz-6"										
GSSh	23	1080	17,3	1,83	0,85	110	190	44	21,1	8,7
VNK	23	1220	18,3	2,07	0,83	229	325	78	32,1	13,0
"Soyuz-7"										
AVF	23	885	14,0	1,73	0,63	94	158	44	13,0	7,2
VNB	22,7	830	11,7	1,30	0,42	125	171	35	15,2	10,1
AVG	22,5	830	13,9	1,64	0,46	135	149	42	13,2	7,9
"Soyuz-8"										
VASh	23	780	14,7	2,0	0,59	128	184	45	9,7	7,6
ASYe	23	690	12,2	1,54	0,51	94	121	44	9,9	5,2
"Soyuz-9"										
AGN	24	2000	15,6	2,0	1,15	96	91	15,0	18	—
VIS	24	1430	16,9	2,1	1,0	135	224	42	15	—
Average values for the preflight period	M	952	12,6	1,55	0,865	188	185	72	14,2	9,2
	σ	216	2,4	0,47	—	54	55	19	4,7	1,8

Note: AGN and VIS performed a test with a water-load on the second day.

Commas indicate decimal points.

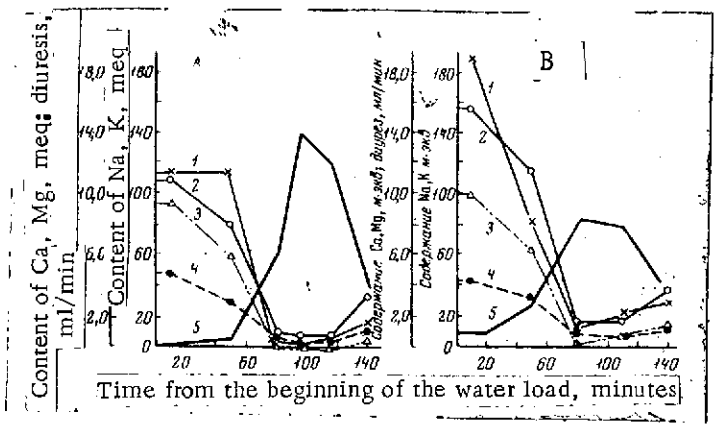

Figure 22. Excretion of Water and Concentration of Electrolytes in the Urine Following Water/Loads in G.S.Sh. Before (A) and After (B) Flight (Meq/Liter). 1, Ca; 2, Na; 3, Mg; 4, K; 5, diuresis.

TABLE 16. SEVERAL INDICATORS OF KIDNEY FUNCTION DURING A TEST INVOLVING A WATER STRESS IN COSMONAUTS FOLLOWING FLIGHTS

Craft and cosmonauts		Excretion of water, %		Maximum minute diuresis, ml/min kg	Concentration of Na at peak diuresis, meq/liter	Excretion of Na at peak diuresis, meq/minute	17-OH, mg	Excretion in 2 hours, Electrolytes, mg/eq			
		in 2 hours	in 4 hours					Na	K	Ca	Mg
"Voskhod" (1 day flight)											
VMK		21	—	2,7	25	67	—	17	14	—	—
BBYe		42	—	12,2	13	158	—	15	8	—	—
"Soyuz-6", "Soyuz-7" and "Soyuz-8" (5 day flight)											
GSSh		44	64	8,3	15	125	1,3	21	6,7	1,7	1,0
VNK		63	71	16,6	18	299	1,3	32	6,9	8,3	1,5
VNB		48	55	9,5	13	123	1,5	25	7,0	1,4	—
AVF		30	46	6,2	48	296	1,6	40	8,5	2,2	1,1
VVG		37	50	9,5	9	86	1,4	7	3,0	0,6	0,5
VASh		64	92	16,0	8	128	1,3	18	9,3	0,7	—
ASYe		65	91	14,1	15	211	0,8	27	10,8	1,4	0,7
"Soyuz-9" (18 day flight)											
AGN		84	—	11,3	8	96	1,2	14	3,8	2,4	1,2
VIS		60	—	10,2	33	336	2,2	39	13,7	2,5	0,9
Normal values	M	60	85	15,0	7,6	106	0,64	11	10,5	0,67	0,66
	σ	12	12	3,8	3,8	48	0,4	6	3,9	0,21	0,3

Commas indicate decimal points.

When the test was performed two weeks following the end of the flight, no deviations from the norm were observed. In subjects who spent a month or more in a horizontal position in bed (clinostatic hypokinesia), the test with the water load developed rather complex dynamics. The results were not always clear or well perceived. According to the data of G. I. Kozyrevskaya and A. I. Grigor'yev, on the eighth day of the experiment the water was retained in the organism. According to our studies, performed on the 14th-15th and 57th-58th days of the test, when the subjects were in a horizontal position, retention of drinking water did not show up (Table 17). However, if the studies were conducted on the second day following the end of a 30 day experiment, i.e., under conditions similar to those which exist following the end of a spaceflight, the /113 water was retained somewhat. Two weeks later after that, the test gives the usual results for healthy individuals.

The functional tests which were performed revealed significant changes not only in the water but also the salt excretory systems. In six out of seven cosmonauts who had completed five-day flights aboard spacecraft in the "Soyuz" series, the minute excretion of sodium at the peak of diuresis was greater than the average values of the controls, and in two instances exceeded it by nearly three times. Excretion of calcium in the majority of cases also increased, but excretion of potassium and magnesium did not change. Therefore, the changes in /114

the magnitude of osmotic clearance were not as great as the changes in the minute excretion of sodium at the peak of diuresis (Table 18).

TABLE 17. INDICES OF FUNCTIONAL STATE OF THE KIDNEYS UNDER CONDITIONS INVOLVING PROLONGED CLINOSTATIC HYPOKINESIA (AVERAGE DATA)

Experimental conditions	Number of subjects	Excretion of drinking water in two hours, %	Maximum minutes diuresis, ml/minute	Concentration of Na at peak diuresis, meq/l	Excretion of Na at peak diuresis, meq/minute	Excretion with urine in two hours, meq		
						Na	K	Ca
14-15 days of hypokinesia in bed	6	67	16	6.8	98	13	7.1	-
Same experiment, on the 57-58th day	6	68	18	4.1	80	6.9	7.2	-
On the day following the 30-day stay in bed	9	56	12.4	3.9	50	5.8	5.6	1.1
Normal values of M	-	60	15.0	7.6	106	11	10.5	0.66
" σ	-	12	3.8	3.8	48	6	3.9	0.3

The excretion of sodium and calcium was particularly high in V.N.K., in whom, as we have already pointed out, excretion of salts was much higher than normal on the second day following the landing. Judging by the results obtained in these studies, there is no relationship between salt excretion and water retention. Thus, in V.N.K. salt excretion was maximum but on the basis of the quantity of water excreted during the test is concerned he fell in the middle of the group. In the case of A.V.F., the excretion of electrolytes was quite pronounced, while in V.V.G. on the other hand it was below normal; yet they excreted practically the same amount of water. One gets the impression that as the duration of the flight increases the ability to excrete drinking water is restored but the incapacity to retain electrolytes at the level of the water load is progressive. Thus, the most pronounced water retention was in V.M.K. who was in flight for only one day, while the maximum sodium excretion at peak diuresis was in V.I.S. who flew for 18 days. /115

In model experiments involving prolonged bed rest, excretion of electrolytes against a water-load background showed no increase (see Table 17) either during

those periods when the water that was drunk was retained for a short time or when it was excreted normally. Thus, inability to retain electrolytes during the performance of this test was characteristic only of the state of the organism after the flight. Inasmuch as there was no relationship between salt and water excretion, their change must be viewed as a result of damage to various mechanisms. This is in agreement with the data of Yu. V. Natochin and G. P. Gusev [49] who showed in experiments on dogs that under conditions of various functional stresses there is no relationship between the excretion of water and ions by the kidneys.

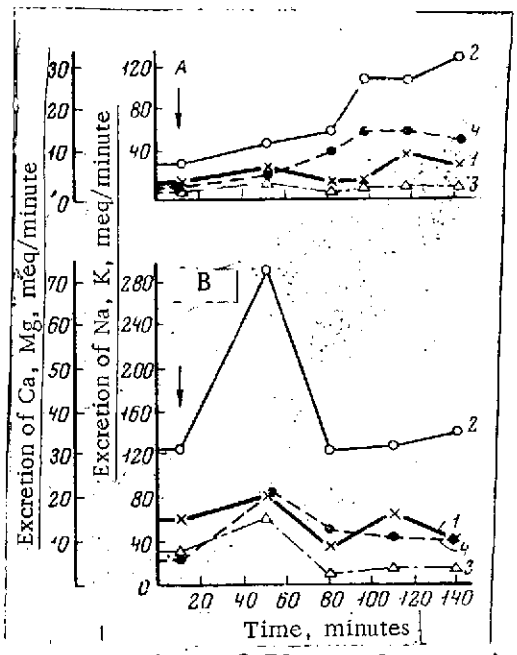

Figure 23. Excretion of Electrolytes with the Urine Following Water Load in G.S.Sh. Before (A) and After (B) Flight. Symbols same as in Figure 22.

Analyzing the results of these experiments, we must keep in mind that the test with the water load, generally speaking, was very sensitive and the retention of water may develop as the result of the action of the most diverse causes which are not always possible to find, for example, the result of a slight limitation of the drinking schedule, previous physical stress, and so on. Therefore, the test is always performed in the morning on an empty stomach.

The inability of the kidneys to excrete drinking water rapidly on the general level characterizes tonization of systems aimed at water retention in the organism. Let us try to analyze the possible mechanism for this phenomenon. It is considered that the water which is drunk reduces the osmotic concentration of the extracellular fluid, so that the secretion of the antidiuretic hormone is suppressed and the excretion of urine is increased. Retention of urine may be the consequences of several causes: /116

- The drinking water does not lead to a decrease in osmotic concentration of extracellular fluid to such an extent that it suppresses the secretion of ADH;

- the receptor mechanisms are disturbed (including the central branch) as a result of which the decrease in osmotic pressure suppresses the secretion of ADH; /117

- a quantity of ADH is circulating in the blood such that although the water load does suppress its secretion, the concentration of hormone remains high;

- the kidney becomes uncontrollable, losing its ability to handle high diuresis because its intimate mechanisms have been damaged.

TABLE 18. MAGNITUDE OF WATER AND OSMOTIC CLEARANCE AT PEAK DIURESIS DURING PERFORMANCE OF TEST WITH A WATER LOAD (ACCORDING TO THE DATA OF A. N. GRIGOR'YEV AND G. I. KOZYREVSKAYA [48])

Craft and cosmonauts	Excretion of water %		Maximum minute diuresis ml	V_{osm}	C_{osm}	C_{H_2O}
	in 2 hours	in 4 hours				
"Voskhod"						
VMK	21	—	2,7	0,269	2,5	0,19
BBBYe	42	—	12,2	0,078	3,2	9,0
"Soyuz-4"						
VASh.	—	36	7,0	0,181	4,06	2,94
"Soyuz-5"						
BVV	—	65	7,8	0,132	3,33	4,45
YeVKH	—	61	10,7	0,080	2,89	7,81
ASYe	—	40	6,2	0,190	3,91	2,29
"Soyuz-6"						
GSSh	44	64	8,3	0,118	3,32	4,98
VNK	63	71	16,6	0,080	4,41	12,29
"Soyuz-7"						
AVF	30	46	6,2	0,212	4,40	1,8
VNV	48	55	9,5	0,109	3,53	5,97
VVG	37	50	9,5	0,129	4,08	5,42
"Soyuz-8"						
VASh	64	92	16,0	0,066	3,52	12,48
ASYe	65	91	14,1	0,087	4,12	10,08
"Soyuz-9"						
AGN	84	—	11,3	0,074	2,77	8,53
VIS	60	—	10,2	0,073	2,45	7,73
Normal	60	85	15,0	0,064	3,22	11,8

Note: V_{osm} is the osmotic concentration of the urine at peak diuresis (in osmols); C_{osm} is the ostmotic clearance at peak diuresis; C_{H_2O} is the clearance of osmotically free water at peak diuresis.

Commas indicate decimal points.

At the present time we do not have any data to allow adoption of any one of these proposals even as a working hypothesis, but each of these possibilities may be reinforced by certain arguments. The water load may not lead to a decrease in the osmotic concentration of the extracellular fluid if the water was not absorbed by the gastrointestinal tract, or, having been absorbed, crossed over immediately into the cells. The first of these possibilities is not very likely, inasmuch as there was no damage of any kind to the functions of the gastrointestinal tract. In examining astronauts following flight aboard Apollo XIV, a slight increase in intracellular space was found at the expense of extracellular space (Table 19), i.e., there was a tendency toward intracellular edema [25]. This is an indirect argument in favor of the fact that after landing, while carrying out the test with the water load, the water might also have passed into the cells. Evidently, in Addison's disease the transfer of water into the intracellular space is also the cause of retention of drinking water. /118

TABLE 19. CHANGE (IN %) IN VARIOUS COMPONENTS OF THE BODIES OF AMERICAN ASTRONAUTS DURING THE FLIGHT OF APOLLO 14 [25].

Investigated parameters	Method of measurement	Astronauts			Control subjects		
		CDR	CMP*	LMP	1	2	3
Mass of erythrocytes	Cr^{51}	−1,7	−9,1	−4,0	+4,4	−3,5	+0,3
Plasma volume	Albumin I^{25}	+1,2	−9,7	+0,1	16,0	+6,1	+10,7
Volume of extracellular fluid	$S^{35}O_4$	−0,5	0,0	−0,3	+4,2	−2,8	+3,3
Total content of water in the organism	H_2^3O	−1,9	−17,7	−1,8	+1,6	−2,1	+3,0
Intracellular water Difference between space	and H_2^3O $S^{35}O_4$	−2,7	−27,0	−2,6	0,0	−1,6	−2,9

Note: The CMP (Command module pilot), unlike the other 2 members of the crew, did not have any EVA on the lunar surface. His water losses were much greater.

Commas indicate decimal points.

The concepts of O. Gauer correspond most closely to the second of these possibilities, to wit, as a result of the change to a vertical position under conditions of action of the force of gravity there is a decrease in the mass of blood which is located in the chest cavity, which is the reason for the antidiuretic reflex which is quite pronounced, strong enough so that it stops the diuresis caused by the drop in osmotic concentration of tissue fluids. If such inhibition actually takes place, it must be accomplished in the central branch of both these reflexes.

The third suggestion consists in the fact that in the organism of cosmonauts following flight, so much antidiuretic hormone is circulating that the water load, although it does lead to a slight suppression of its synthesis, does not

actually have an effect on the concentration. There are two possibilities here. First of all, the rate of excretion of this hormone from the organism may be so slow that the change in its production has no real effect on the concentration. This appears to be not very probable, since the rate of semiexcretion of protein hormones from the pituitary changes by the minute. The second possibility consists in the fact that the mechanism of ADH production in the pituitary following the flight is stimulated so strongly that the osmoregulatory reflex cannot be inhibited. This possibility is based on the fact that many powerful stimulants including pain, physical stress, and so on cause excretion of ADH, aside from dependence upon the actual degree of hydration of the organism.

We know that the ability of the kidneys to excrete urine with a low osmotic concentration is evident only at a certain stage of ontogenesis. Thus, according to the data of E. Adol'f [1], the ability of added water to stimulate diuresis develops completely in puppies only on the 21st day after birth, while in rats it occurs on the 10th day. In this case, we are speaking about the inability of the kidneys to excrete water, inasmuch as the central mechanisms for the regulation of water excretion are not yet developed and the concentration of ADH is either very low or cannot be detected at all in the blood. According to view of A. G. Ginetsinskiy [1], absorption of water in the kidney tubules takes place because the hyaluoronic acid is destroyed by the influence of the antidiuretic hormone and this acid makes the intercellular space impermeable to water. In order to obtain urine with a low osmotic concentration, the hyaluoronic acid must be synthesized anew. Hence, excretion of water from the organism is an active process which takes place only at a certain stage of ontogenesis and is possible only under the condition of synthesis of hyaluoronic acid. In the event that a general weakening of the organ takes place (this cannot be completely excluded at the end of a long spaceflight), the retention of drinking water may be associated with a decrease in the functional capacity of the kidneys, for example, the /119 inability to synthesize hyaluoronic acid rapidly. Inasmuch as an inability of the kidneys to hold electrolytes develops after flights, it would be very handy to be able to explain both of these factors by the same mechanism, in other words, a functional disturbance of the kidneys themselves.

No such explanation is found in the previously noted lack of agreement between the disruption of the salt and water excretory functions in the cosmonauts during the performance of tests with water stress after flights. If the basis for these disruptions had been the same mechanism, one would have expected that the cosmonauts in whom the greatest water retention was observed would also have excreted salt to the greatest extent. However, there was in fact no such relationship. Retention of water was not pronounced during the brief flights when the general status of the cosmonauts was good and there was no basis for fearing any nonspecific damage to the tissues. One of the sufficiently well studied pathological states in which pronounced retention of water in organism occurs is edema due to hunger, including edema which develops in kwashiorkor, a disease which attacks children who are fed primarily on corn. According to the studies of Skirantia and Mohanram [268], in this disease the ADH concentration in the blood serum and urine is increased, although it would seem that with insufficient nutrition the synthesis of hormones would be reduced. Hence, even with a clear disruption of nutrition and protein synthesis the retention of water in the

organism does not occur because the kidneys lose their ability to synthesize hyaluoronic acid.

Explaining the mechanism of increased salt excretion against a background of water load is still more complicated than in the case of the mechanism of water retention. Water diuresis itself does not lead to an increase in salt excretion, as can be seen from the results of our model experiments with prolonged hypodynamia when, regardless of the change in water excretion, the salt concentration at peak diuresis was always low (see Table 17), as well as from the experiments of G. P. Gusev and Yu. P. Natochin [49] which showed that in dogs, even with a water load that reached 5% of body weight, water diuresis was accompanied by salt excretion only in those cases when there was a simultaneous increase in the concentration of the corresponding ions in the blood due to intravenous administration of salt solutions. In those cases, there was an increase in the excretion of only that ion which was administered into the circulatory system. Metzger et al., who applied a water load of 20 ml/kg to healthy and hypertonic individuals, also failed to observe any increase in sodium excretion under these conditions [243].

Under certain conditions, the antidiuretic hormone is capable of increasing salt excretion as well as water retention [67, 210, 234]. It would be very interesting indeed if it were precisely this that explained the changes which /120 occur. However, this apsect of its action can be realized only under these very special conditions, aside from the fact that, as we have already mentioned, there is no relationship between water and salt excretion with water loads.

One is struck by a certain degree of parallelism between the excretion of salts when water loads are given and their excretion during the first two days following landing.

Other Possible Mechanisms for the Change in Water-Salt Exchange During Spaceflights

In the previous section, we discussed two attempts to explain the changes in water-salt metabolism during spaceflights — a reflex influence from large vessels in the chest, caused by redistribution of the blood, and the disruption of biochemical processes in the kidneys as the result of a general weakening of the organism. Neither of these theories can totally explain all of the known facts, and it is therefore interesting to study some of the other possibilities.

Following the reading of the paper by Neuman [248] at the COSPAR meeting in Warsaw in 1962, as well as the work of P. A. Korzhuyev [75], researchers began to devote more attention to the possible processes of demineralization of the bone tissue during flight. It was interesting to try to connect the processes of excretion of calcium from the organism with other disturbances in water-salt metabolism under the same conditions. The simplest diagram of such a syndrome looks something like this.

As was demonstrated in the works of Giordano et al. [209], Walsev [283], G. Gusev and Yu. Natochin [49] as well as a number of other researchers, there is a definite relationship between the excretion of sodium and calcium by the kidneys:

in those instances when the excretion of one of these substances increases, the excretion of the other increases as well. If there really is a demineralization of the bones during flight and an increase in the excretion of calcium, then we can expect on the basis of this physiological relationship that there will be an increase in the excretion of sodium and water as well. A check of the correctness of this assumption may be made in two ways: first of all, making sure whether or not there actually is a loss of calcium from the bone tissue during the flight and secondly, how strictly, practically speaking, the relationship between the excretion of sodium and calcium by the kidneys is maintained.

In order to check the relationship between the excretion of calcium and sodium under various physiological conditions, special experiments were performed in which the subjects, healthy young people, were exposed for 10 days to maximum possible desalinization [14]. For this purpose they remained continuously in a thermochamber at an ambient air temperature of approximately 38-40°. Under such /121 conditions, practically the entire heat from the organism is given off through the liberation and evaporation of prespiration, and a considerable quantity of salt is lost together with the prespiration. Some of the subjects were on a milk-vegetable diet with a caloric content of 2,500 kcal, containing a significant quantity of proteins and other components of the diet with the exception of sodium chloride, while others had the usual food ration with a high content of sodium chloride, or the same milk-vegetable diet but with the addition of various amounts of salt. In all cases, water was given *ad libitum*. The details of the composition of the diet and the conditions of the experiment are shown in Table 20.

TABLE 20. EXCRETION OF ELECTROLYTES WITH THE URINE WHILE ON DIETS WITH VARIOUS SALT CONTENTS DURING A TEN-DAY STAY AT A TEMPERATURE OF 38-40° [14]

Subject	Conditions of diet and regime	Content of sodium in the daily ration	Excretion of electrolytes with the urine, meq/day				Diuresis, ml/day	Note
			Na	K	Ca	Mg		
L-s	Usual diet	130	56	42	6,2	10,3	650	Usual caloric diet 2700 kcal/day, potassium content 77 meq, calcium - 41, magnesium - 32 meq.
A-v			53	50	3,7	10,5	780	
L-ch			53	46	15,0	10,5	780	
Kh-p	Usual diet + + 10 grams NaCl daily	300	136	46	12,0	10,6	770	
L-f	Usual diet + + 10 grams NaCl daily and bed rest	300	137	76	13,5	6,3	670	Normal caloric diet 2500 kcal, potassium 33, calcium 60 meq.
O-k			142	60	5,9	11,1	840	
R-y	Milk-vegetable diet	40	16	61	3,3	13,0	800	
N-v			31	61	7,3	10,0	700	
P-v			10	68	4,3	9,1	680	
Sh-v	Milk-vegetable diet and bed rest	40	34	59	5,5	10,0	680	
B-n			33	69	19,0	8,2	740	

Commas indicate decimal points.

Under the conditions of such experiments, the relationship between the excretion of sodium and calcium with the urine is disrupted (Figure 24). There was also no strict relationship between the excretion of these substances following the end of a spaceflight. All of this indicates that calciuria, even if it in fact exists during flight, cannot be the reason for the loss of water and salts.

Hence, none of the examined hypotheses can explain all of the changes which have been observed. It still remains possible that those changes in water-salt metabolism which occur following spaceflights are characteristic of precisely those conditions and occur rather as the result of a restructuring of regulatory processes in the organism as a whole and in the kidney tissue alone. The kidneys in this case are an effector organ which is either most sensitive to this change /122 or the changes in its function are simply more accessible to observation.

The direct cause for increased excretion of salt at the level of water diuresis and immediately following the flight may be hormonal changes. In experiments on dogs it has been shown that progesterone has the ability to cause salt diuresis, i.e., increased excretion of water and salts from the organism. According to the clinical observations of Bachev [22], premedication with prednizolon of patients with hyperaldesteronism increases the diuretic action of the diuretics, which obviously has to do with its antagonistic effect with respect to aldesterone. There is sufficient basis for believing that during spaceflight the production of aldesterone is increased (this problem is dealt with in greater detail in the section on hormonal changes), and it is possible that in response there is also an increase in the production of other hormonal substances which are intended to compensate for its action, and the change in salt excretion following the flight is a result of a complex mosiac of dynamic relationships among these substances.

From the practical standpoint it is very important to separate those changes which develop during flights from those which are the reaction of the organism to a change from a weightless condition to conditions of life under terrestrial gravitation, i.e., to the natural fact of the end of the flight. Judging by the fact that in model experiments the retention of drinking water occurred immediately after the end of the experiment, we can conclude that it is the result of its termination after the flight as well, i.e., the return from weightlessness to gravitation. On the other hand, increased salt excretion at the peak of diuresis following the end of flights is logically related to the prolonged action of weightlessness, since it cannot be observed in model experiments. At the present time we have no direct data to indicate that the changes described in water-salt metabolism have an effect on the ability of the organism of a cosmonaut to withstand the action of harmful environmental factors. Some of the changes in the volume of the circulating plasma, which the Americans have observed following some flights (Table 21), in most cases were less than 10%, /123 i.e., they did not go beyond the limits of error of the method. Instead, the reduced orthostatic stability which was observed after the flights, like the changes in water-salt metabolism, was the consequence of some general cause having to do with the effect of weightlessness, but whose pathogenic mechanism has not been discovered. Therefore, excretion of salts or retention of water must be viewed not as a cause but as a symptom of some "space syndrome".

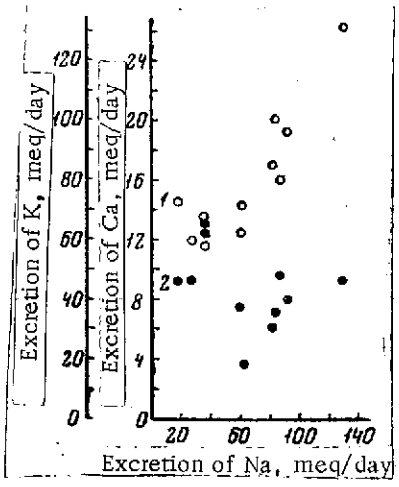

Figure 24. Relationship Between Excretion of Calcium and Sodium in Controlled Experiments. 1, Ca; 2, K.

State of Nitrogen Metabolism During Flights and in Simulation Experiments

As we have already pointed out in the section on the change in body weight, during flights lasting up to 5 days there was no symptom of an increase in tissue catabolism and a related increase in the excretion of nitrogenous substances with the urine. The calculations performed there of the amount of nitrogen which would have to be excreted from the organism if all of the weight loss were to take place solely as the result of a breakdown of tissues apply to short time intervals. During longer flights, even small differences in the intake and excretion of nitrogenous products, accumulated from day to day, may lead to total values which have definite physiological significance.

TABLE 21. CHANGE IN BLOOD PLASMA VOLUME AND MASS OF ERYTHROCYTES IN AMERICAN ASTRONAUTS (ACCORDING TO THE DATA IN [25, 198])

Craft	Duration of flight days	Change in plasma volume in % of original level for each member of the crew			Change in mass of erythrocytes in % of original level for each member of crew		
		1	2	3	1	2	3
Gemini IV	4	— 4	—13	—	—12	—13	—
Gemini V	8	— 9	—15	—	—20	—22	—
Gemini VII	14	+18	+ 4	—	—19	— 8	—
Apollo 7	11	— 5	— 4	+ 1	— 1	— 9	0
Apollo 8	7	—16	—15	— 8	+ 2	— 2	— 4
Apollo 9	11	— 4	— 7	—13	— 4	— 7	—10
Apollo 14	11	+ 1,2	— 9,7	+ 0,1	— 1,7	— 9,1	— 4,0

Commas indicate decimal points.

Similar studies performed by Lutwak, Whedon, Lachence, Reid and Lipscomb [237] aboard the Gemini VII showed that the average values of negative nitrogen balance in one of the astronauts were 1.34 and for another 3.43 grams/day. According to these data, and also according to other results of research conducted in [10, 237], Table 22 was compiled and a calculation made of the amount /124 of tissue which would have to be broken down in order to serve as the source of the substances found in the urine. As we can see, the actual weight loss was considerably in excess of the possible values of tissue breakdown as calculated according to the data from biochemical studies. This indicates that even on a

14-day flight dehydration continues to remain as one of the causes of weight loss and the breakdown of tissues still does not take on such dimensions that it would be worthwhile to fear its influence on the physiological functions of the organism.

The data obtained during the 18-day flight of Soyuz-9 on total nitrogen excretion with the urine during the first, second and 18th days of the flight, although they do not go beyond the limits of the values which are usual under normal conditions, do approach the upper limit of normal, as for example in A.G.N. at the end of the flight (see Table 13, page 99; the standards given for this contingent of men are presented in Table 15 on page 102). The recovery of weight following this flight, especially in V.I.S., took place more slowly than on the 5-day flights (see Table 6). This is an indirect argument in favor of the development of a negative nitrogen balance in him.

In order to get some idea of the state of protein metabolism, it is also possible to present data on the change in mass of the erythrocytes and volume of plasma proteins during flights, obtained by American researchers. Since we know that the lifetime of an erythrocyte is about 100 days, each day about 1/100th of the total amount of hemoglobin in the organism is renewed and all of this pro- /125 tein is synthesized anew from amino acids. The plasma proteins are renewed even more rapidly, so that a decrease in their numbers can serve as an indirect symptom of a retardation of protein synthesis, although these processes depend on a number of other factors as well. American researchers, in examining the crews of the "Gemini" spacecraft, found a decrease in the erythrocyte mass in some astronauts but this observation was not confirmed [27] during the flights in the "Apollo" series (Table 21).

TABLE 22. COMPARISON OF THE RESULTS OF BALANCE STUDIES WITH THE CHANGE IN THE WEIGHT OF ASTRONAUTS DURING THE FLIGHTS OF THE GEMINI VII SPACECRAFT (ON THE BASIS OF DATA OF LUTWAK ET AL. AND BERRY [10, 237])

Indices	F. B.		J. L.	
	In 1 day	For the whole flight	In 1 day	For the whole flight
Nitrogen loss, g	3,43	48	1,34	19
Equivalent to a mass of tissue, g	—	1600	—	630
Potassium loss, meq	64,6	870	2,29	320
Potassium loss, g	2,58	36	0,9	13
Ratio K/N	—	0,75	—	0,67
Loss of phosphorus, g	0,69	9,5	0,504	7,0
Ratio P/N, g	—	0,2	—	0,37
Loss of sulfur, g	0,51	7,1	0,24	3,3
Ratio S/N, g	—	0,15	—	0,17
Weight loss during the flight, kg	—	3,4	—	3,0

Commas indicate decimal points.

Those tissue metabolism values which developed in cosmonauts who were in space for 14 and 18 days do not in themselves pose a danger to health and are worthy of attention only as a starting point for extrapolation to longer periods. Disruption of nitrogen metabolism during long flights constitutes a threat to the health of cosmonauts for several reasons. The first and probably the most important one at this stage is the breakdown of muscle tissue and the corresponding weakening of the muscles of the support-motor apparatus, concerning which scientists and science fiction writers wrote long before the start of the space age. In fact, it has been firmly established that in the course of all experiments involving limitation of mobility the functional possibilities of the muscles of the lower extremities and the muscles which maintain the posture of the trunk are considerably shortened and the forces of the grasping muscles of the hands remains practically unchanged [55, 114]. The extent to which this has to do with losses of muscle mass or whether we are talking here only about a disruption of these functional characteristics cannot be determined without carrying out special studies.

This problem is partially answered by the work of N. P. Mikhaleva, I. I. Ivanov, I. V. Fedorov and E. M. Amdiy [101], in which it was shown that with limitation of mobility of rats the changes in protein composition of the muscles are reminiscent of those which occur when their function is disrupted by other factors — there is a relative decrease in proteins of the actomyosin complex [143, 58]. Similar results were obtained on rabbits by M. S. Gayevskaya et al. [39]. I. V. Fedorov, Yu. I. Milov, L. A. Grishanina and Ye. Ye. Simonov [141, 142] have shown that in the case of immobilization of rats the rate of inclusion of radioactive amino acids in the muscle tissue is retarded. On the basis of the results of these experiments performed in animals, drawing conclusions about the nature of metabolic processes in human beings must be done with great care, inasmuch as the rat model used was much more strict, i.e., for all of the animals the effect turned out to be much more serious than that to which cosmonauts are exposed during flights or subjects are exposed in bed rest. This conclusion is made because some of the rats died during the experiments involving prolonged hypodynamia.

Other factors that support devotion of particular attention to the synthesis of proteins are not so widely known during flight but are also worthy of serious evaluation. There is a close relationship between them and the adaptational /126 capacities of the organism, which was pointed out by F. C. Meyerson and V. P. Malkin et al. [98, 99, 100]. In their studies, performed in rats, it was shown that adaptation to oxygen insufficiency does not occur if protein synthesis is suspended in the animal by administration of an antibiotic which blocks its synthesis. This is in good agreement with the role which is played by the increase in the mass of cardiac muscle and the number of erythrocytes in the accomodation to hypoxia. Inasmuch as the protein in all tissues, including the myocardium, is constantly being renewed, disruption of synthetic processes must unavoidably lead to an overcoming of the catabolic processes and a weakening of the capacities of the organ. The above mentioned experiments of I. V. Fedorov, et al., on rats [141, 149], with the animals kept for various periods of time under conditions of limited mobility, shows that there is a disruption of protein synthesis not only in the skeletal muscles but also in the myocardium and other internal organs. If such characteristics were observed in human beings,

any catabolism of protein would lead to disruption of the function of the cardiovascular system. Under spaceflight conditions this is of critical significance since in the final analysis it is precisely the condition of hemodynamics that determines the ability of the human organism to counteract the harmful action of extreme factors.

Maintenance of the level of protein synthesis at a sufficiently high level is also of importance for systems of immunological protection of the organism and the function of its central nervous system. Practically all of the authors who have been involved in the study of the condition of antiinfectious immunity both during flights and during experiments on stands have called attention to the threat of a decrease in the resistance of the organism of the cosmonaut to various infectious agents during flight. The close relationship of the mechanisms of immunity to those of protein synthesis is obvious: the origin of all protective processes is the synthesis of protein.

The processes in the nervous system also cannot take place without a constant and intensive renewal of their proteins. This follows from an experiment in which the influence of blockage of protein synthesis on behavior and learning of animals was studied as well as the rate of renewal of proteins during various functional states of the nervous system [50]. Mainly we are speaking here only of the determination of additional details regarding that which is known to every physician — any weakening of the organism, whether it is linked with illness or hunger, has its effect on the ability of man to carry out mental work.

The total study of the condition of nitrogen balance, like potassium and phosphorus balance and the balance of several other substances, has been studied in detail by the first founders of metabolic studies during prolonged bed rest — Cuthbertson [184], Dietrick, Whedon and Shore [187] as well as Schoncheyder et al. /127
[261-262]. These authors succeeded in finding symptoms of negative nitrogen balance which were more pronounced at the start of the studies and later decreased. The values which they observed were not very great in themselves, depended on the concrete conditions of the experiment, and could be reduced by various preventative measures — correct diet, physical exercise and so on. Therefore, it was interesting to evaluate the influence of various factors and the conditions of the experiment on the degree of tissue metabolism. It was very difficult to carry out this work under the conditions of strictly balanced studies for a number of reasons. Even if we do not take into account the organizational problems (considerable expenditure of labor in making chemical analyses of the food), the methodological difficulties associated with purely physiological aspects of the experiment in many cases are insurmountable. First of all, each balance is composed of the results of several determinations, each of which introduces its own errors; in addition, it is necessary to study the composition of the feces and perspiration. Collection of perspiration under conditions of water immersion is impossible, and it is not a simple matter during bed rest, inasmuch as some of it is soaked up by the bed clothes, which are very difficult to analyze. In fact, in a number of cases it is possible to determine the tissue metabolism conditions not by carrying out balance studies but by comparing the characteristic values for nitrogen excretion for a given subject in the preexperimental period with the values during his stay under conditions that to some extent simulate a period of weightlessness. Under these conditions, the appetite

usually decreases, the nitrogen taken in with the diet decreases accordingly and is less than in the control period, and any increase in nitrogen excreted with the urine must be treated as a sign of protein breakdown.

Yu. K. Syzrantsev and Yu. F. Udalov studied the influence of various versions of the experiments on the excretion of nitrogen with the urine. The results of their studies are shown in Table 23 and in Figure 25, from which it is clear that bed rest or water immersion produces a definite increase in nitrogen excretion, approximately by 4.5 to 5 grams/day in 9-12 day experiments, and in the majority of cases the maximum occurs on the fifth or sixth day of hypodynamia. In these experiments, a measure directed at training the peripheral vascular system (binding the soft tissues with bandages) had no influence on nitrogen metabolism. Thus, the physical stress was very effective: the higher it was, the smaller the difference between the excretion of nitrogen in the control and experimental periods.

By limiting the intake of protein with the diet and increasing its nutrient properties and vitamin saturation, we also succeeded in reducing the differences between the excretion of nitrogen in the experimental and control periods. From the experiments of Yu. K. Syzrantsev it follows from that the state of nitrogen metabolism is not linked directly (in any case for the given duration) with the state of the vascular system. Thus, immersion in water as a rule causes more /129 pronounced hemodynamic changes than ordinary bed rest, but it has no more profound effects on nitrogen excretion than does ordinary bed rest.

Similar results were obtained in our experiments, carried out under the general direction of A. D. Voskresenskiy and B. S. Katkovskiy [11, 38], in which nine subjects remained for 30 days under conditions of bed rest (Table 24). Three of them (group 4) carried out physical exercise with an energy value of about 500 kcal/day on a special stand which made it possible to stress different groups of muscles in the horizontal position. One subject from this group of three (B), also received 5 mg daily of an anabolic steroid for 15 days (dianabol). This group was periodically subjected to negative pressure on the lower part of the body (NPLB). In the second group of three (group 5) control subjects were used who were not subjected to the same kind of training which was intended to make them better able to tolerate conditions of hypodynamia, but were merely examined periodically including a sample with NPLB. Finally, in the third group of three (group 6), electrical stimulation of the muscles was performed daily. From the data in Table 24 it is clear that in the group with physical exercise the nitrogen excretion during the experiment was somewhat lower than before, while in the two other groups it was increased instead. On the whole, the differences are not great if we exclude group 3 whose data was studied in more detail on page 117; each subject during the entire 30 days of the experiment lost 390-450 grams, i.e., the maximum difference was about 60 grams. Since each kg of muscle contains about 30 grams of nitrogen, these differences are equivalent to a breakdown of 2 kg of tissue per month, a value which is not very /131 large on the whole. In other words, if we take into account that the diet was the same in all of the groups and in the group with physical training the nitrogen excretion from the organism was equal to its intake with the food, the additional amount of nitrogen which was excreted by the subjects in groups

5 and 6 may have been formed as the result of the breakdown of two kg of muscle tissue. This value is not large in comparison with the total amount of muscles in the body, which is 25 to 30 kg.

TABLE 23. INFLUENCE OF VARIOUS CONDITIONS ON THE EXCRETION OF TOTAL NITROGEN WITH THE URINE IN CLINOSTATIC HYPOKINESIA (ACCORDING TO THE DATA OF Yu. K. SYZRANTSEV AND Yu. F. UDALOV)

Experimental Conditions	Number of Subjects	Control period		Experimental period			t and P	Series of the experiment	Increase in excretion (g/day)
		n*	Excretion of nitrogen, g/day (M ± m)	Duration, days	n*	Excretion of nitrogen, g/day (M ± m)	2		
Bed rest, food ration containing 90-100 g of protein	8	14	12±0,4	12	44	16,6±0,5	6,4 0,001	1	4,6
Bed rest, food ration containing 90-100 g of protein, binding of the legs	5	8	10,05±0,5	9	44	15,02±0,3	5,1 0,001	2	5,0
Water immersion	3	6	11,8±0,7	12	24	16,5±0,5	5,1 0,01	1	4,7
Bed rest, physical exercise (up to 150 kcal/day)	3	11	14,0±0,9	8	28	17,8±0,7	3,18 0,01	3	3,8
Bed rest, physical exercise (up to 400 kcal/day)	3	35	11,6±0,6	8	23	12,3±0,4	0,45 0,5	4	0,7
Bed rest, food ration containing 70 g of proteins, 1950 kcal and vitamin complex	4	14	12,9±0,4	8	29	14,2±0,4	2,04 0,1	5	1,3

n* Total number of man-days of investigation.
Commas indicate decimal points.

As a matter of fact, in these experiments, in all of the groups, the subjects ate the same diet composed of dry (cakes, bread and pastry and so on) or preserved products; the differences could be due only to incomplete eating of the diet, but the leftovers were small.

It is also necessary to take into account that the subjects who were exposed to physical stress during training lost a certain amount of nitrogen with perspiration, so that in fact the differences between nitrogen excretion were less in different groups.

Subject Sh., in whom nitrogen excretion was much higher than in the other two members of the same group, was more developed physically. In carrying out physical exercise, he tried to achieve the best result, so that his energy consumption was high. Obviously, the caloric content of the diet was low for him and the inadequate amount of energy was compensated for by the breakdown of tissues, so that his nitrogen excretion was higher. This is supported by the results of the determination of his blood cholesterol level (see page 144).

Hence, the physical exercise turned out to be capable of reducing the amount of nitrogen excreted with the urine only in the event that the total caloric content of the diet was adequate. /132

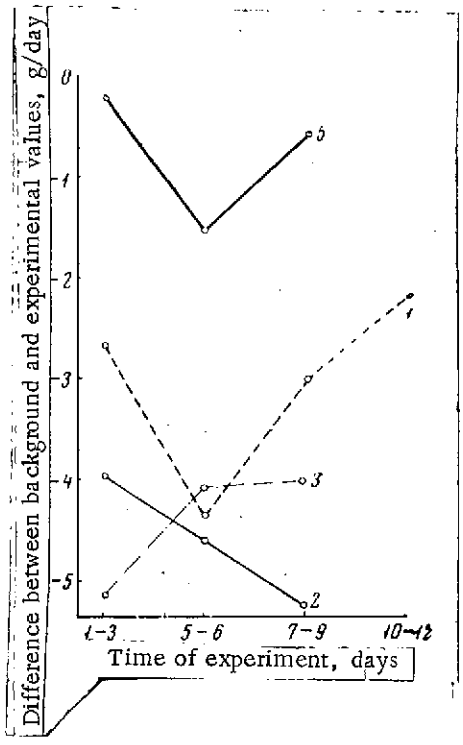

Figure 25. Dynamics of the Excretion of Total Nitrogen with the Urine (Average Values) in Various Versions of the Experiments with Clinostatic Hypokinesia (According to the Data of Yu. K. Syzrantsev and Yu. F. Udalov). 1, 2, 3, 5, Series of experiments according to Table 23.

The nitrogen balance was studied more carefully by us jointly with I. V. Fedorov and Yu. K. Syzrantsev during a 100-day experiment involving bed rest [42, 133]. In this series, we took into account nitrogen excretion with the urine and feces and its intake with the diet. The results of the studies that were carried out on three young persons who had not been subjected to any kind of preventive measures (group 8) are shown on page 26 [of the foreign text]. From them we can see that negative nitrogen balance was found in all three subjects; its dynamics were subhect to considerable individual variations (the studies were performed during individual periods whose duration corresponds to the base of the cone in the figure), from which one can get a partial idea on the basis of the data presented. In this experiment, we did not take into account the excretion of nitrogen with the sweat, and for this reason the real values of the nitrogen imbalance were slightly higher.

Interpolating the values for the losses of nitrogen which occur during the entire experiment, we can calculate the total amount of tissue which is broken down (assuming a nitrogen content of 3%). Corresponding calculations are shown in Table 25. The actual weight loss turned out to be considerably less. If we exclude the possibility of nitrogen intake from the air (which is not very likely although there are no convincing proofs that man can not take in atmospheric nitrogen, even in small quantities), we must suppose that the breakdown of nitrogenous tissues is balanced either by retention of water in the organism or by storage of fat, which contains only 0.6% nitrogen. In the section on water-salt exchange, we dealt with the fact that all existing data indicate dehydration of the organism during such experiments rather than retention of water; it therefore sounds more convincing that there is a partial replacement of water tissue by fat during prolonged hypodynamia and therefore the actual loss of muscle is greater than one would conclude from the weight loss. This is in agreement with the results of the studies of A. G. Zhdanova, who found an increase in fat content in the organism under similar conditions, measuring the specific weight of the subjects [59]. The accumulation of fat in

the organism must be accompanied by an appropriate intake of energy whose only source can be food. In the same Table 25 we have presented the results of a study of basal metabolism which was carried out by M. I. Mikhasev, V. I. Sokolkov and M. A. Tikhonov [102]. It is clear from these calculations that the difference between the real energy expenditures and the intake of energy from food roughly corresponds to the amount of stored fat.

Summing up the data available on the state of protein balance with limited mobility, we must point out that all of the authors who have studied this problem under the most diverse conditions of experimentation and diet found a predominance of nitrogen excretion over its intake with food. The maximum of these differences, as a rule, occurred during the second week of the experiments, with the degree of negative balance being dependent on many factors; the rational organization of the diet and physical stress decreased it. /133

TABLE 24. EXCRETION OF TOTAL NITROGEN WITH THE URINE IN SUBJECTS KEPT FOR 30 DAYS IN BED REST (AVERAGE DATA, g/day)

Group and conditions of experiment	Subject	Before the test	Days of experiment					Immediately after experiment	Average values for 1 day	Total for 30 days
			1—6	7—12	13—18	19—24	25—30			
4 Physical stress	B	13,2	14,5	12,6	12,6	13,5	13,0	12,6	13,2	397,4
	Nk	14,7	13,4	14,6	10,3	14,0	13,1	11,5	13,05	392,6
	Sh	18,3	15,2	17,6	16,6	16,5	18,8	17,3	16,9	508,2
Average		16,1	13,4	14,0	13,2	14,7	15,0	13,8	14,35	—
5 Control	Nf	10,1	15,5	14,7	13,2	10,9	15,0	9,4	13,9	416,8
	K	11,5	13,8	15,2	12,2	15,1	13,3	9,8	13,9	418,3
	S	13,8	10,6	15,1	13,0	16,0	14,2	10,0	13,8	413,4
Average		11,7	13,9	15,0	12,8	14,0	14,1	9,7	13,9	—
6 Electrical stimulation	M	13,1	13,9	15,2	14,3	15,3	17,0	14,5	15,15	454,1
	O	11,9	14,1	15,9	14,2	15,1	16,2	11,5	15,1	453,0
	P	12,8	13,8	15,5	16,8	14,7	14,8	13,2	15,15	454,3
Average		12,6	13,9	15,5	15,1	15,0	16,0	13,1	15,1	—

Commas indicate decimal points.

Metabolic studies, so to speak, only make it possible to see the whole picture — does the intake of nitrogen into the organism predominate or does its excretion predominate? However, this method does not reveal the reason for the disturbances that are observed: is the synthesis of tissue proteins retarded, or is their breakdown intensified? To answer this question, we can use the method of determination of the rate of excretion of nitrogen N^{15} from the organism. As was pointed out in the initial work of Rittenberg et al. [290], only a portion of the nitrogen, amino acids, ingested with the food is used directly for synthesis of tissue proteins, while another part is converted into urea and, we might say, is expelled "in transit" from the organism with the /134

urine. At the same time, tissue proteins are synthesized not only from those amino acids which have been taken in with the food but also those which were formed as a result of the breakdown of proteins.

TABLE 25. COMPONENTS OF THE LEVEL OF ENERGY CONSUMPTION, CALORIC CONTENT OF THE DIET AND DYNAMICS OF BODY WEIGHT IN A 100-DAY EXPERIMENT INVOLVING BED REST

Parameters	Eighth series			Ninth series		
	Without physical stress			Physical stress, 450 kcal/day		
	Ne.	Na.	K.	L.	M.	Yu.
Required basal metabolism, kcal/day	1560	1600	1810	1740	1490	1660
25th day of the experiment						
Caloric content of natural diet, kcal/day	2430	2460	2470	2720	2540	2570
Actual energy consumption under conditions of basal metabolism, kcal/day	1570	1400	2000	1850	1650	1550
Difference, kcal/day	860	1060	470	4420	440	570
89th day of the experiment						
Caloric content of the actual diet, kcal/day	2200	1840	2180	2630	2670	2580
Actual energy consumption under conditions of basal metabolism, kcal/day	1480	1350	1570	1830	1520	1500
Difference, kcal/day	720	500	610	390	700	630
Difference between caloric content of the diet and the average expenditures, extrapolated for the 89 days of the experiment, 1000's of kcal	70	82	48	36	52	54
Nitrogen balance for 89 days, grams	2215	-258	-317	-194	+40	-35
Change in weight, calculated on the basis of the data of nitrogen balance on the basis of a nitrogen content of 3%, kg	-7.1	-8.5	-10.5	-6.5	+1.3	-1.1
Actual change in weight after 89 days of the experiment, kg	-1.5	-1.5	-0.9	-1.2	+0.3	+1.1
Difference between actual and calculated change in weight, kg	-6.6	-7.0	-9.6	-5.3	+1.0	-2.2
Quantity of fatty tissue equivalent to the difference between the caloric content of the diet and the energy expenditure, kg	7.7	9.1	5.3	4.0	5.8	6.0

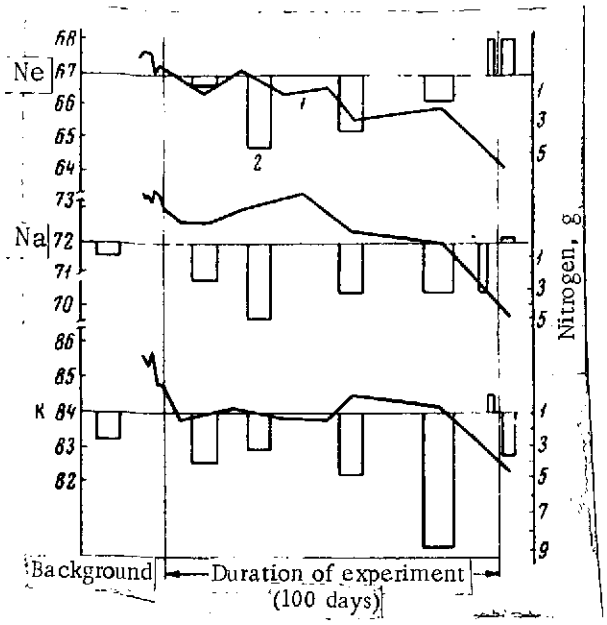

Figure 26. Dynamics of Weight (1) and Level of Nitrogen Balance (2) in Subjects Ne., Na. and K. (8 Groups of Experiments), Kept 100 Days in Bed Rest.

The rate of synthesis of tissue proteins during hypodynamia was first changed by means of heavy nitrogen by Heilskov, Schonheyder and Olssen [219] in 1955. They found that if a man spends 10 days in bed and his extremities are fastened down, the negative balance is caused by a decrease in the rate of protein synthesis and not by an increase in the breakdown rate. A similar study was conducted by us in the already quoted experiment with 100 days of hypodynamia. The results are shown in Table 26, from which it is evident that although the rate of synthesis decreased on the whole, this decrease was comparatively slight and did not go beyond the limits of scatter of the values in normal individuals. Various results obtained by us and by Swedish researchers are not surprising: our subjects were under less harsh conditions, their extremities were not fastened down, and the goal of these experiments was not to /135 produce pronounced physiological changes but (rather) to check possible methods of protecting the organism against the harmful action of prolonged hypodynamia. Therefore, we used the most rational choice of nutrition product, which was a prototype of the diet for feeding cosmonauts during flight.

Most of the nitrogen contained in the human organism goes to make up the muscle tissue; therefore, concluding that nitrogen metabolism is not disruptive on the whole, we thereby suggest that it is harmless in the muscles as well. However, other approaches may be used to test them during hypodynamia, including the results of a study of creatinine and creatine metabolism. Creatine in the muscles is not synthesized but is their most important component: creatinophosphoric acid acts as a reserve source of energy. It is excreted from the organism in the form of a cyclic anhydride — creatinine; in an adult man the creatine content in the urine is very low, 1/10th or even less of the creatinine content. According to L. L. Ferdman [143], creatinuria in the norm is found only in children or young animals and its appearance in an adult man is a symptom of muscular dystrophy. By using more sensitive analytical methods, this substance can be detected in an adult man, but only in small amounts. Regardless of their points of view concerning the normal level of creatinuria, all authors agree that an increase is a symptom of muscular pathology [58]. Schonheyder and Christensen [261] studied the rate of inclusion of N^{15} glycine in the composition of creatine and creatinine in the urine in one healthy man. The studies were performed on the 11th to 14th days of the test, when the subject showed a negative nitrogen balance. It was found that the magnitude of the excess isotope in the creatine

three hours after administration of the isotope was about 10 times higher than in the creatinine, from which it was concluded that the rate of inclusion of creatine in the muscles had been reduced.

TABLE 26. STATE OF NITROGEN METABOLISM AND CERTAIN PARAMETERS OF PROCESSES IN MUSCLES DURING 100 DAYS OF BED REST FOR 3 SUBJECTS (AVERAGE DATA)

Parameters	Eighth series			Ninth series		
	Without physical training			With physical training		
	Before the experiment	Third week	Ninth week	Before the experiment	Third week	Ninth week
Excretion of total nitrogen with the urine, g/day	17.4	16.4	16.0	16.0	13.9	17.2
Nitrogen balance, g/day	-0.12	-1.7	-3.2	0	-0.1	-1.2
Rate of protein synthesis, g/kg/day	0.36	0.41	0.31	0.51	0.44	0.41
Excretion of creatinine with the urine, g/day	1.8	2.2	1.8	2.0	2.1	1.8
Excretion of creatine with the urine, g/day	0.39	0.52	0.52	0.6	0.58	0.41
Uptake of administered creatinine, %	89	85	85	97	97	71

Note: Physical exercises were performed in the horizontal trainer and their energetic value was 450 kcal. The daily food ration on the average contained 2620 kcal, 130 grams of protein, 91 grams of fat and 320 grams of carbohydrates.

Certain changes in the state of creatinine metabolism in the organism can also be obtained by giving the substance orally (so-called creatinine stress). If its uptake by the muscles is disturbed, the substance which has been administered is excreted from the organism with the urine. It may be that this method is more sensitive and ensures fewer disruptions in the muscles than a simple study of creatinuria. The results of such studies performed by us jointly with I. V. Fedorov during the above mentioned experiment involving prolonged hypodynamia are shown in Table 26. As we can see from this table, the excretion of creatinine was not increased in comparison with the original levels, nor was

there any disruption of the handling of the creatinine stress that we could see.

In this work, the determination of the creatine content in the urine was performed according to the Tauski method, which consists in the creatine being converted to creatinine by heating with picric acid at the temperature of a boiling water bath. This method is always used for determining creatine in the urine of healthy adults. /137

In order to evaluate the biochemical processes in muscle tissue during hypodynamia, it was interesting to study the parameters which usually characterize the diseases of muscle: disruption of amino acid composition of the blood and urine, increase in alkaline phosphotase activity, isoenzymes of lactate dehydrogenase and creatinekinase in blood serum [10, 58, 78, 143, 213]. Studies performed by I. I. Ivanov, B. F. Korovkin and N. D. Mikhaleva [59] on individuals who spent long periods of time under conditions of bed rest failed to show any such changes in aspartate and alanine aminotransferase activity or aldolase and creatinekinase which would have had diagnostic significance (Table 27). In some series of experiments, the alkaline phosphotase activity was increased, while in others it was not increased. The reason for this increase remains unclear: it could have been processes in the bones or muscles, inasmuch as the source of the enzyme in the blood serum could be located either there or in some other tissue.

The experiments described above, like the data in the literature, indicate that with prolonged limitation of mobility under conditions of bed rest (clinostatic hypokinesia) metabolism in muscle tissue on the whole is not disturbed. This conclusion refers to the case when the goal of the experiment is to simulate spaceflight conditions, so that the subjects receive a complete diet, they change their position in bed, preventive measures are taken in a number of cases to increase the working capacity following the end of the experiment: physical exercise, tonic muscle contractions, electrical stimulation and so forth. Under these conditions, regardless of the relatively favorable level of metabolic indices, functional capacities of the muscle system are found to be disturbed. This is expressed in a general weakness, loss of muscle strength, especially in the lower extremities and muscles responsible for maintaining posture, disruption of gait, as well as leg pains following the end of the experiment [55, 66, 114].

This contradiction between the results of functional and biochemical studies may be explained in two ways. First of all, in hypodynamia the nervous branch of the motor apparatus is disturbed; secondly, there is a selective disruption of the function of individual muscle groups which somehow turn out to be *locus minoris resistentia*. If their mass is small with respect to the entire remaining mass of muscle in the body, disruptions of metabolism may occur unnoticed.

During spaceflights, as during various experiments on the test stand, simulating certain flight conditions, the urea content in the blood increases. /138
This increase was first noticed during the flight of the "Voskhod" spacecraft in 1964 [12] as well as during the flight of the "Salyut" station in 1971 (Table 28). On the whole, when comparing the urea content in the blood of 17 cosmonauts

who flew 1-5 days, its content is found to have increased statistically reliably, although very slightly — by 3 mg% in all, which has no clinical significance whatever. After the 18-day flight of the "Soyuz-9" spacecraft, the urea concentration in the blood was practically unchanged. American researchers also failed to notice any changes in the concentration of this substance following flights.

TABLE 27. CHANGE IN ACTIVITY OF CERTAIN BLOOD SERUM ENZYMES IN SUBJECT WHO SPENT 70 DAYS IN BED (ACCORDING TO DATA OF I.I. IVANOV, B. F. KOROVKIN AND N. P. MIKHALEVA [59])

Enzymes, arbitrary units	Series of the experiment	Before the experiment	Hypodynamia, days			Recovery period
			7 to 15	30	60 to 65	
Aspartate-aminotransferase	2nd	6,2±0,39	6,6±0,55	5,3±0,09	3,5±0,07	4,1±0,47
	3rd	6,5±0,23	6,2±0,85	7,2±1,10	4,9±0,66	5,0±0,33
	4th	5,4±0,70	6,2±0,25	3,2±0,18	4,7±0,69	4,5±0,35
	5th	4,5±0,37	4,7±0,38	4,5±0,73	3,2±0,18	4,0±0,70
Alanine-aminotransferase	2nd	2,5±0,28	2,0±0,28	2,0±0,14	2,3±0,41	3,3±0,47
	3rd	3,3±0,09	4,5±0,28	4,8±0,34	4,1±0,54	5,0±0,33
	4th	2,0±0,28	3,2±0,53	4,7±0,18	4,2±0,09	3,0±0,01
	5th	4,2±1,01	2,3±0,19	5,8±0,48	2,5±0,35	2,2±0,18
Aldolase	2nd	12,8±0,52	11,2±1,01	8,1±0,80	9,3±1,59	8,7±0,83
	3rd	8,7±0,75	16,7±0,96	11,5±0,80	9,5±0,38	10,7±0,83
	4th	4,8±0,19	7,0±0,47	5,2±0,18	4,5±0,72	8,5±0,35
	5th	5,0±0,52	7,2±0,09	7,5±0,60	4,7±0,035	7,0±0,70
Alkaline phosphatase	2nd	18,5±1,06	31,3±0,76	16,0±0,33	21,3±0,01	24,7±0,47
	3rd	20,5±0,35	34,7±3,66	27,7±4,21	26,0±2,30	28,7±2,43
	4th	15,7±0,96	25,3±2,16	22,0±2,12	25,3±1,06	35,7±0,70
	5th	17,3±1,93	29,0±1,85	29,0±1,85	32,0±0,38	35,0±0,67
Creatine kinase	2nd	1,0±0,01	1,1±0,07	1,2±0,13	0,9±0,02	1,6±0,07
	3rd	1,0±0,02	2,2±0,19	2,2±0,34	0,8±0,04	1,4±0,11

Commas indicate decimal points.

In various versions of the experiments in which the influence of bed rest was studied or the subjects were placed in small-volume chambers for developing a life-support system, but had the opportunity to move around inside, they sat down or stood during the daytime and the urea concentration increased slightly by the tenth to fifteenth day of the experiment, then fell [111]. A different amount of physical exercise, a change in the composition of the gas mixture, electrical stimulation of the muscles — none of these had any influence on this effect (Table 29). In order to be sure that this increase was not an artifact caused by the entrance of some other substances into the blood, during the /139 experiment mentioned above with 100 days of hypokinesia (simultaneously with analyses carried out using the method adopted in our laboratory with diacetylmonoxime) the urea content and the residual nitrogen were determined by the urease method and after mineralization. All three methods showed the same dynamics — a maximum increase on the 17th day of the experiment; on the 60th day the content of urea and residual nitrogen had decreased.

TABLE 28. UREA CONTENT IN THE BLOOD OF COSMONAUTS DURING SPACEFLIGHTS*

Craft, time of flight	Cosmonaut	background values	Before the flight	Flight, days 1	5	15	22	After the flight
"Voskhod" 1 day	K. P. F.	25	31	47	—	—	—	40
	B. B. Ye.	23	—	51,41	—	—	—	40
average data of 5 day flights	M	—	33	—	—	—	—	36
	σ	—	5,9	—	—	—	—	5,6
	n	—	13	—	—	—	—	17
"Soyuz-11" 18 days	A. G. N.	36	28	—	—	—	—	29
	V. I. S.	25	28	—	—	—	—	24
"Salyut" 24 days	G. T. D.	32	38	—	50	41	43	32**
	V. N. V.	34	32	—	33	—	39	34
	V. I. P.	42	33	—	53	53	50	48

*Average content of urea determined in 190 tests of cosmonauts and cosmonaut-candidates, was 31 mg%, while the mean square deviation was 6.4 mg%.

**The results were obtained in an analysis of blood collected during an autopsy.

Commas indicate decimal points.

The urea content in the blood is determined primarily by the functional capacities of the kidneys and to only a slight extent by the rate of protein breakdown. Since this substance passes freely through the cell walls, the "urea space" is practically equal to the total water volume in the organism, and it is even used to determine the total water content in the organism [148]. Therefore, at a concentration of 30 mg%, assuming its volume is equal to 50 liters, we will obtain a total urea content of 15 grams in the organism, i.e., the supply is approximately equal to half the daily excretion. These results are in good agreement with direct studies performed by means of stable isotopes. This supply (or, as has become conventional in biochemistry) "volume fund", acts as a buffer which levels off the random variations in the rate of its formation. At the same time, the capacity of the kidneys to excrete urea is considerably in excess of the actual requirements, which can be ascertained on the basis of the fact that urea is not excreted uniformly throughout the day and the "peak" values are much higher than average ones. Therefore, the change in urea concentration in the blood can only be explained by a restructuring of the regulation of renal function.

The reason for this restructuring may be found in the loss of electrolytes, since according to the old data of Achard [154], as well as other researchers, significant chlorine losses from the organism cause the kidneys to lose their ability to excrete urea — so-called "chloroprivic" uremia develops. However, those degrees of electrolyte loss which may occur during flights or during stimulation experiments are insufficient to cause such an effect. This follows from our above mentioned experiments in which subjects were kept on a salt-free

diet for ten days in a thermostatically controlled chamber, i.e., under conditions that led to maximum sodium chloride loss from the organism:

		Before the experiment	Days 1	2	3	4	5	6	7	8	9	10
Urea content, mg%	M	18	21	25	25	23	21	22	21	20	20	18
	m	2.2	1.0	2.5	2.3	1.3	1.5	2.1	1.0	1.7	1.8	1.3

However, as we can see, their urea content did not increase.

In order to evaluate the functional capacities of the kidneys during an experiment involving 100-day hypodynamia, a test was performed with a water load whose results are shown in page 115 [of the foreign text] as well as a test with a soda stress. This test consisted in the subject being given 10 grams of sodium bicarbonate, after which the pH of the urine was measured for two hours. According to V. A. Veysman [131], when kidney function is disrupted the urine does not become alkaline, or is alkalized more slowly than normal. Rapid excretion of alkali indicates good functioning of the renal tissue. As we can see from Table 30, no functional disturbances involving the kidneys were found by this method. If the retention of urea in the organism were the consequence of an insufficient intake of water into the organism, excretion of nitrogenous substances would have increased when performing an experiment with a water load, but as we can see from Table 30, no such increase occurred. /140

TABLE 29. CHANGE IN UREA CONTENT IN THE BLOOD IN VARIOUS SERIES OF TERRESTRIAL EXPERIMENTS INVOLVING BED REST OR A STAY IN SMALL-VOLUME (M ± m, mg%)

Duration of experiments, days	Series	Experimental conditions	No. of subjects	Material studied	Urea content, mg%: Before the experiment	Time after start of experiment, days: 2nd-3rd	8th-9th	15th-17th	19th-20th	25th-30th	60th	100th	Time after end of experiment, days: 2nd	7th-8th
		Stay in small volume chambers	Up to 19	Blood	30±1,4 $n=19$	37±2,1 $n=8$	—	38±1,6 $n=8$	—	38±1,8 $n=8$	—	—	—	—
20	First control for series 2	Bed rest	6	Blood	29±1,3	—	34±2,1	—	36±0,9	—	—	—	—	27+1,6
		Bed rest	2	Blood	28	—	32	—	33	—	—	—	—	25
	Two	Bed rest and hypoxia	4	Blood	30±1,9	—	36±2,6	—	38±2,5	—	—	—	—	28±2,2
30	Four, five six	Bed rest, in the 4th series physical stress and NPLB, in the 6th	9	Serum	33±1,6	—	—	—	36±2,3	—	—	—	32±1,7	—
100	Eight and nine	In the 8th series, bed rest, in the 9th series bed rest and physical stress	6	Blood	32±2,2	—	—	35±2,4	—	—	36±1,4	34±2		—

Commas indicate decimal points.

TABLE 30. UREA AND RESIDUAL NITROGEN CONTENT IN THE BLOOD AND ABILITY OF KIDNEYS TO EXCRETE UREA, CREATININE AND ALKALI ON THE 57th-60th DAYS OF BED REST AS COMPARED WITH ORIGINAL VALUES

Series of experiments and conditions	Subject	Urea, mg%				Residual nitrogen, mg%		Excretion during water load, g/hr				pH of urine following administration of load of 10 grams $NaHCO_3$	
		Diacetylmonoxime method		Urease method				Urea		Creatinine			
		Before the test	On the 60th day	Before the test	On the 60th day	Before the test	On the 60th day	Before the test	On the 57th day	Before the test	On the 57th day	Before the test	On the 58th day
Series eight bed rest (control)		31	33	31	40	36	40	0,8	1,4	30	57	8,0	7,6
		42	40	41	41	46	42	1,1	2,0	47	99	8,2	7,7
		30	34	33	34	41	38	2,1	1,8	60	92	8,2	8,0
Series nine bed rest and physical exercise		27	33	31	34	37	35	1,6	3,5	68	68	8,1	7,6
		30	40	33	41	38	40	2,3	2,0	63	82	7,8	8,0
		30	34	38	34	38	36	2,0	1,9	73	78	8,2	7,0
Average		32	36	35	37	39	39	1,7	2,1	56	80	8,1	7,8
		2,2	1,4	1,7	1,1	3,7	3,1	0,25	0,3	7	7	0,07	0,1

Commas indicate decimal points.

With an increase in the urea content in the blood, due to damage to the kidneys, there also is frequently an increase in creatinine content, although it is felt that this substance begins to be stored after more severe damage than that which leads to retention of urea. Results of determinations of creatinine content in the blood, performed on 9 subjects kept in conditions of bed rest for 30 days, are shown in Table 31. It is clear from this table that those /141 persons in whom the urea content in the blood serum increased the creatinine concentration did not increase. An increase in this substance was observed in only three subjects out of nine, so that it cannot be considered regular. In these experiments, the creatinine is determined by a micromodification of the method of Popper et al. [254].

No data are available on changes in creatinine content during spaceflight.

Hence, the very slight but completely regular increase in urea content in the blood, observed in practically all experiments involving prolonged hypodynamia, is not accompanied by a regular increase in creatinine content in the blood serum or statistically reliable functional changes in the kidneys, that could be revealed by stress tests. During spaceflights, this increase in urea content is quite obvious. It is evident that the reason for it is not a general injury to the renal tissue but some specific disruption of the processes responsible for excretion of urea specifically. We know that at night, when man rests in a lying position, the excretion of urea with the urine (or what is practically the same thing, total nitrogen, inasmuch as urea contains 80 to 90% of the total amount of nitrogen in the urine), is less than in the day. This can

be seen, for example, from Figure 18, where corresponding data have been presented for the crew of the "Voskhod-1" spacecraft. One of the stimuli that determined the level of renal glomerular filtration is the reflex with the working muscles. It may be that a weakening of precisely these influences was the cause of urea accumulation in the blood. /144

TABLE 31. DYNAMICS OF THE CHANGE IN UREA CONTENT IN BLOOD SERUM AS COMPARED WITH THE CHANGE IN THE CREATININE CONTENT IN 30-DAY HYPODYNAMIA (IN mg%)

Series of experiments and conditions	Subject	Periods of investigation				
		Before the experiment	Diet, standard	20th day of experiment	After experiment, days	
					2nd	16th
Urea						
Fourth						
Bed rest, physical exercise, NPLB	B	28	34	32	29	25
	Nk	18	24	22	20	23
	Sh	27	31	32	35	24
Average		24	30	29	28	24
Fifth						
Bed rest (control)	Ne	29	36	36	38	27
	K	33	35	45	35	35
	S	25	29	38	33	25
Average		29	33	40	35	29
Sixth						
Bed rest, electrical stimulation	M	19	33	39	33	26
	O	—	30	43	32	26
	D	—	41	39	33	32
Average		—	35	40	33	28
Creatinine						
Fourth						
Bed rest, physical exercise, NPLB	B	1,5	1,10	1,6	1,8	1,2
	Nk	1,0	1,0	1,5	1,6	1,4
	Sh	1,2	1,2	1,6	1,6	1,2
Average		1,2	1,1	1,6	1,7	1,3
Fifth						
Bed rest (control)	Ne	1,0	1,3	0,9	1,6	1,2
	K	1,2	1,4	0,9	1,6	1,1
	S	—	1,2	1,2	1,4	1,3
Average		1,1	1,3	1,0	1,5	1,2
Sixth						
Bed rest, electrical stimulation	M	1,3	1,6	0,6	1,4	1,3
	O	—	1,6	1,1	1,3	1,4
	D	—	1,6	1,0	1,5	1,3
Average		—	1,6	0,9	1,4	1,4

Commas indicate decimal points.

Change in Total Mass of Hemoglobin in the Organism

Hemoglobin is one of several proteins whose total amount in the organism may be measured. The change in its mass during a spaceflight is of interest for two reasons: first of all because it can serve as a model that characterizes the general conditions of protein synthesis, and secondly, because it makes it possible to estimate the mass of circulating blood. Special emphasis must be placed on the fact that in this case we are speaking not of the concentration of hemoglobin in the peripheral blood but the total content of this substance in the entire organism.

TABLE 32. CHANGE IN TOTAL HEMOGLOBIN MASS (IN GRAMS/m^2 OF BODY SURFACE) IN MODEL EXPERIMENTS WITH 30-DAY BED REST (M ± m)

Series of experiments	Conditions	Angle of inclination of the bed*	No. of subjects	Mass of hemoglobin		Recovery period	
				Before the experiment	At end of experiment	4th day	9-11 days
-	Head end of bed lowered	-6°	6	403 ± 13	308 ± 14	-	332 ± 31
5th	Head end of bed lowered	-4°	3	410	315	290	307
-	Head end of bed lowered	-2°	6	417 ± 23	313 ± 8	-	329 ± 9
6th	Head end of bed lowered and electrical stimulation of the muscles	-4°	3	445	330	340	348
-	Head end of bed raised	+6°	6	395 ± 11	353 ± 4	-	377 ± 8
4th	Head end of bed raised, physical exercise	-4°	3	420	416	397	401
-	Control (no influences applied)		6	392 ± 27	-	-	386 ± 28

*The "minus" sign indicates sloping of the bed when the head end of the bed was lower than the foot and the "plus" sign indicates the reverse.

Evidently the first to draw attention to possible disruptions in circulation of the blood during weightlessness was P. A. Korzhuyev [75]. He proceeded on the basis of purely theoretical considerations and concluded that absence of the force of gravity would necessarily lead to involution of the bones of the skeleton which, in addition to its function in the support-motor apparatus, constitutes a reservoir for the bone marrow. Therefore, one should expect disturbances of hemopoiesis. Although on some spaceflights the American researchers found a decrease in the mass of erythrocytes (see Table 21), on the whole the changes were not reliable. Since the partial pressure of oxygen in their craft was much higher than at sea level, the assumption arose that the reason for the breakdown of the erythrocytes was hyperoxia. In our spacecraft, the composition of the atmosphere is the same as on Earth, so that it is possible to study the influence exerted on the blood by weightlessness alone. For this purpose, a method was devised in our laboratory for the direct determination of hemoglobin mass in the organism on the basis of the amount of carbon monoxide absorbed, which was described in detail in the methodological part of this work. Until now, it has not been possible to use it to examine cosmonauts after flights, but in simulation experiments involving hypodynamia such data were collected. R. K. Kiselev examined a total of 33 individuals in various experiments, including controls. /145

The results they obtained are shown in Table 32. It is clear from this table that the mass of hemoglobin decreased in those experiments in which the foot of the bed was elevated slightly with respect to the head. On the other hand, in those groups where the foot was lower than the head, the decrease in hemoglobin mass was slight or completely absent. Electrical stimulation of the muscles had practically no influence on this decrease, but physical exercise with an energy value of about 500 kcal daily, performed on a special stand that allowed "running" and "walking" in a horizontal position, had positive results, on the whole. In evaluating these data, it is necessary to keep in mind that the physical exercise was performed by a total of 3 individuals, two of whom showed a slight decrease in the mass of hemoglobin while the third, in whom the original quantity of hemoglobin in the organism was low (calculated on the basis of 1 m^2 of body surface), it increased during the test. In addition, this subject received 5 mg of dianabol daily for two weeks. It is difficult to say to what precisely the change was linked, the physical stress or the intake of the anabolic steroid, so that the data for the entire group taken together may be viewed only as a rough approximation. It is interesting that in those groups of subjects who were in beds with slightly elevated heads no decrease in hemoglobin mass occurred. This group consisted of 6 individuals, so that the results are considered statistically reliable. /146

In these experiments, there was no reliable change in the blood hemoglobin concentration or the number of erythrocytes, and the bilirubin content in the blood serum likewise did not increase (this part of the study was carried out by T. A. Kislovskaya) (Table 33). In other words, the reduction of the total amount of hemoglobin in the organism occurred in proportion to the change in plasma volume. /147

One can get some idea of the direct cause of the decrease in hemoglobin mass from the number of reticulocytes in the peripheral blood. During hypodynamia

their number did not change, but immediately after the end of the experiment, during readaptation to normal living conditions, it decreased sharply (Figure 27). If the decrease in hemoglobin mass was the consequence of an increased breakdown of erythrocytes, the bilirubin content would have increased during the experiment as well as the quantity of reticulocytes, since hemolysis is usually accompanied by increased regeneration of blood. Since the number of reticulocytes increased only during readaptation, the direct cause of anemia can only be a slowing down of the rate of erythropoiesis and hemoglobin synthesis. However, the nature of the pathophysiological mechanism of this slowdown remains unclear, or why even a slight elevation of the head of the bed prevents it.

Use of Anabolic Steroids to Make Up For Possible Disruptions of Metabolic Processes During Spaceflight

As may be concluded from the previous sections, it is possible to eliminate practically completely any disturbances of nitrogen metabolism during prolonged hypodynamia by using a proper diet, physical exercise and other nonspecific measures. However, it is not clear whether these are sufficient to prevent tissue catabolism on long flights. These dangers are due to the fact that on a real flight the organism of a cosmonaut is not influenced by weightlessness and limited mobility alone. There is considerable emotional stress, caused by a feeling of responsibility for the assigned task and the large volume of scientific studies, as well as unusual conditions for working, resting and eating. It is therefore desirable to study methods of compensation for possible negative nitrogen balance by using pharmacological substances, and to work out methods of using and prescribing them.

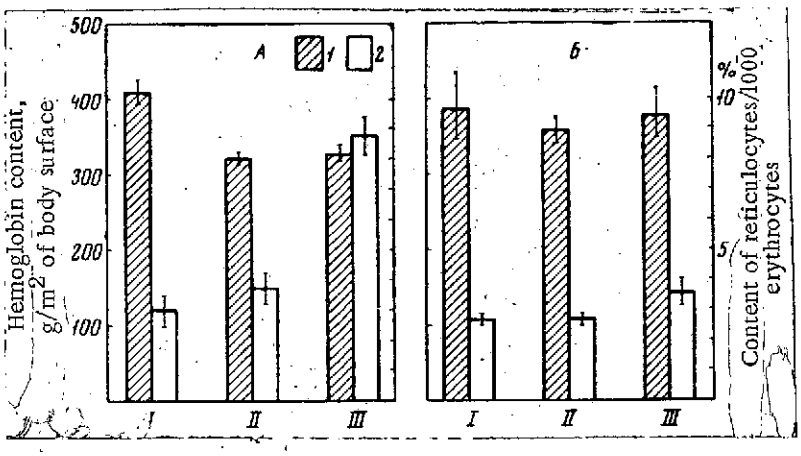

Figure 27. Total Hemoglobin Content in the Organism (1) and the Number of Reticulocytes in the Peripheral Blood (2). A, Subjects who spent a month in a horizontal position with the foot of the bed slightly elevated (-2°); B, ditto, but with the head elevated (+6°). In each group of 6 individuals, the average values are given (M ± m); I, before the test; II, end of experiment; III, recovery period.

TABLE 33. AMOUNT OF ERYTHROCYTES AND HEMOGLOBIN AND BILIRUBIN CONTENT IN THE BLOOD AS COMPARED WITH THE TOTAL MASS OF HEMOGLOBIN IN THE ORGANISM BEFORE AND AT THE END OF A 30-DAY PERIOD OF HYPODYNAMIA

Series of experiments and subjects	Mass of g/m^2		No. of erythrocytes in the peripheral blood millions/1 ml		Concentration of Hb in the peripheral blood, g%		Content of indirect bilirubin in the blood serum, mg%	
	Before the experiment	End of experiment	Before the experiment	End of experiment	Before the experiment	End of experiment	Before the experiment	End of experiment
Four								
B.	380	470	5.2	4.7	14.8	14.8	0.28	0.33
Nk.	410	355	5.2	4.7	15.0	14.6	0.26	0.55
Sh.	470	425	5.1	4.7	14.5	13.8	0.55	0.77
Average	420	416	5.2	4.7	14.8	14.4	0.36	0.55
Five								
Nf.	330	335	4.7	4.6	13.5	14.5	0.26	0.22
K.	465	340	4.8	4.6	14.0	13.8	0.37	0.33
S.	435	280	4.8	4.7	13.6	13.7	0.7	1.1
Average	410	315	4.8	4.6	13.7	14.0	0.42	0.55
Six								
O.M.	434	350	4.4	4.7	14.2	14.2	0.66	0.44
M.	402	275	4.8	4.9	14.9	15.1	0.77	0.44
D.	500	365	4.7	4.8	14.1	15.0	0.77	0.88
Average	475	430	4.6	4.8	14.4	14.8	0.73	0.57

In this respect, we are unavoidably led to consider the anabolic steroids — synthetic medicinal substances which have seen increasingly wide clinical application in recent years for the treatment of various ailments associated with disruption of protein synthesis; they are used for the treatment of radiation sickness, burns, poorly healing fractures, and dietary problems. These substances are the strongest of the known pharmacological agents that stimulate protein synthesis in the living organism. Their use has been proposed for purposes of space medicine by V. V. Parin, T. N. Krupin, G. P. Mikhaylovskiy and A. Ya. Tizul [115] in an article published in 1970, but this publication did not contain the necessary basis or a body of detailed research material. /148

At the present time, several dozen anabolic steroids are known which are distinguished by their biological activity and are used in various medicinal forms. All of these substances are similar to the male sex hormones testosterone and androsterone in their chemical composition. By adding additional groups to various parts of the molecule, they can be practically completely stripped of their virilizing effects (i.e., the ability to lead to the development or intensification of secondary male sexual characteristics — growth of the beard and mustache, deep voice, and so on), but they possess increased anabolic activity, i.e., the ability to increase protein synthesis.

As a model for solving the problem in which we were interested, we selected patients who were being treated in the traumatological clinic of Professor V. P. Okhotskiy. This work was carried out with his direct participation, as well as that of Dr. Ye. I. Doronin, Professor I. V. Fedorov and I. P. Yakovleva [19]. Patients with various fractures were observed who had not previously suffered from any severe pathology, and who by virtue of the conditions of their ailment were compelled to remain in a horizontal position in bed for a long time. As we have already pointed out, healthy persons confined to bed rest may not develop negative nitrogen balance, so that it is difficult to study the prophylactic action of anabolic steroids on them. Under the conditions of the traumatological clinic, there is always a certain contingent of patients who are forced to remain in a horizontal position and whose traumas are so severe that they cause severe tissue breakdown.

A total of 62 men aged 17 to 62 with various injuries was studied: their problems ranged from exposed fractures of the pelvis and compression fractures of the spine to operations for the purpose of contracture of three to four inches of the wrist (Dupuytren's contraction). Patients with contractions were practically healthy persons in all other respects, were not confined to bed rest and constituted the control group. The compression fractures of the spine were accompanied by relatively slight traumas, and proceeded without any disturbances of the function of the pelvic organs and retroperitoneal hematomas. Patients with fractures of the pelvis were subjected to an operation on the eighth to ninth days; ossetosynthesis using a large metal rod was being performed on them. A number of the patients received 20 mg of an anabolic steroid called oranabol daily; it is also known as 17-alpha-methyladrostene-5-diol-4, 17-alpha-an-ZOL).

In patients with pelvic fractures as well as those with fractures of the shin, who did not receive any preparation, the excretion of total nitrogen with the urine during the first 20 days of hospitalization was high, reaching 20-25 /149 g/day, after which it dropped off to the usual levels on the order of 15 to 16 grams.

The average values for excretion of various substances with the urine during the entire period of observation, calculated on the basis of 1 day, are shown in Table 34. Table 35 gives the results that refer only to the fourth-sixth days of hospitalization. No balance studies were conducted in this work, but the influence of trauma and anabolics on metabolic processes was evaluated by comparing the composition of urine from different groups of patients. This approach was justified by the fact that the subjects received the same diet but the number of observations was sufficiently high that random variations could be smoothed out.

TABLE 34. EXCRETION OF PRODUCTS OF NITROGEN METABOLISM AND ELECTROLYTES IN PATIENTS WHO RECEIVED (A) AND DID NOT RECEIVE (B) ANABOLICS (AVERAGE DATA FOR THE ENTIRE PERIOD OF OBSERVATION; IN GRAMS/DAY)

Nature of the trauma	Number of subjects	Total nitrogen	Potassium	Phosphorus	Creatinine	Creatine	Sodium	Muscle tissue
Pelvic fracture								
a	5	12,9	2,20	0,78	1,40	0,40	2,69	5600
b	4	21,1	3,20	1,17	1,80	0,82	3,62	—
Open fracture of the shin								
a	5	15,8	2,40	0,90	1,87	0,46	3,23	1400
b	4	18,3	2,81	0,90	1,72	0,29	3,73	—
Closed fracture of the shin								
a	5	12,9	2,19	0,85	1,09	0,38	4,73	2500
b	5	16,6	2,46	0,94	1,75	0,45	5,30	—
Fracture of the spine								
a	9	12,9	2,18	0,81	1,24	0,24	3,70	515
b	7	13,4	2,17	0,81	1,30	0,44	3,73	—
Dupuytren's contraction								
a	10	11,5	1,96	0,82	1,31	0,11	4,45	—
b	8	11,2	1,77	0,73	1,13	0,15	4,35	—

Commas indicate decimal points.

In patients with fractures of the spine, as well as those with Dupuytren's contraction, the anabolitics had practically no effect as far as reducing nitrogen excretion is concerned. In those groups, it showed insignificant differences and was at about the same level as in the case of fractures of the pelvis treated with steroids. From this it follows that limitation of mobility /150 alone (patients with spinal traumas were confined to strict bed rest!) does not lead to the disruptions of nitrogen metabolism which are indicated by excretion of nitrogen from the organism and require medicinal treatment. The picture is different in the case of patients with fractures of the pelvis or shin. Here in a number of cases the anabolics turned out to be capable of preventing general metabolic reactions to trauma. This is clearly evident from Figure 29, which shows data for nitrogen excretion in patients with pelvic fractures during the period of the operation of ostosynthesis which was carried out on the 8th-9th day of hospitalization. In 4 out of 5 patients, oranobol prevented an increase in nitrogen excretion after the operation and only 1 patient turned out to be resistant.

Anabolic steroids are not completely indifferent pharmacological substances; they must be used with care and only in those cases when they are actually needed. Among the possible complications are disturbances of water metabolism, asthenization, and disruptions of sexual activity. At the Department of Traumatology and Orthopedics of the Second Military-Medical Institute, where

these preparations have been used for a number of years, no such complications were observed, which indicates that the dosage selected was correct and the periods of therapy were proper. However, it is impossible to disregard the /151 possibility of complications. In choosing rational methods for the use of anabolics in space medicine it is also necessary to take into account that they are not always effective. As we can see from Figure 28, in one patient they did not produce the expected effect. In experiments on rats, when the rate of protein synthesis was studied on the basis of the inclusion of labeled amino acids, I. V. Fedorov was unable to select conditions in which anabolic steroids could have increased the synthesis of proteins, suppressed by immobilization of animals in special confining cages.

TABLE 35. EXCRETION OF TOTAL NITROGEN, POTASSIUM AND PHOSPHORUS WITH THE URINE ON THE FOURTH-SIXTH DAYS OF HOSPITALIZATION IN PATIENTS WHO RECEIVED (a) AND DID NOT RECEIVE (b) ORANABOL (AVERAGE VALUES IN GRAMS/DAY AND MEAN SQUARE DEVIATIONS)

Nature of the trauma	No. of Patients	No. of tests	Total nitrogen	Potassium	Phosphorus
Fracture of the pelvis					
a	5	15	13,6±3,4	2,1±0,39	0,59±0,49
b	4	12	19,6±4,1	3,0±0,50	0,97±0,57
Open fracture of the shin					
a	4	10	20,4±4,7	2,1±0,60	0,95±0,35
b	5	11	21,8±4,1	3,5±1,50	1,14±0,54
Closed fracture of the shin					
a	5	12	12,7±3,56	2,30±0,68	0,85±0,15
b	5	15	15,7±3,92	2,30±0,69	0,86±0,54
Fracture of the spine					
a	9	26	15,0±3,9	2,1±0,56	0,82±0,29
b	7	17	16,0±3,8	2,0±0,61	0,81±0,37
Dupuyten's contraction					
a	11	38	12,3±3,08	2,01±0,48	0,80±0,22
b	8	24	11,8±2,9	1,80±0,42	0,89±0,30

Commas indicate decimal points.

Inasmuch as oranabol has been found in the majority of cases to be capable of preventing protein breakdown caused by considerable trauma, it is highly likely that similar substances may be of value on spaceflights in the event of increased tissue metabolism. These substances have pronounced therapeutic effects only a few days after the beginning of their use, and in any case their effect will be completely evident in 10 days. If it does not show up in this time from it must be concluded that the substance was ineffective.

In surgical traumas, when anabolics have not been employed, nitrogen metabolism returns to normal in 20 days. Although it is difficult to draw a parallel between the reaction of the human organism to a bone fracture and to a spaceflight, it may be assumed that if being sick for 20 days involves

breakdown of proteins which begins after surgical trauma, it is highly likely that during this same period of time the organism would cope with tissue catabolism if he were on a spaceflight. Therefore, treatment with anabolics should be given especially during precisely this period. /152

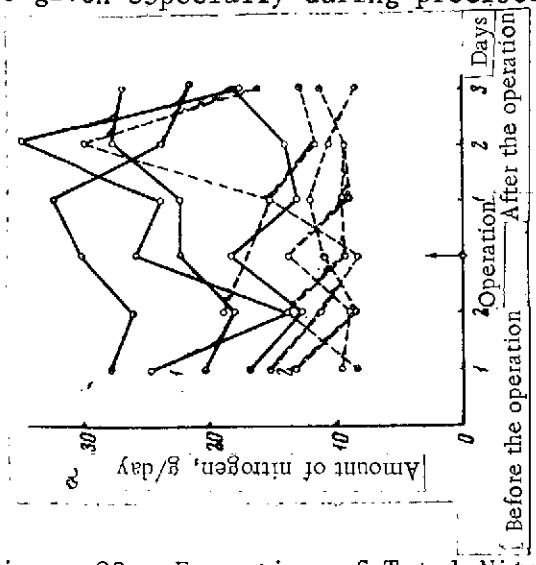

Figure 28. Excretion of Total Nitrogen with the Urine (in Grams/Day) in Patients Who Did Not Receive (1) Anabolics and Those Who Did Receive (2) Daily at the Rate of 20 mg of Oranabol Per Day.

In recommending a certain substance for use aboard a spacecraft, it is necessary to check carefully for negative side effects associated with the specific conditions of the work of the cosmonaut. Such work was carried out in connection with anabolics by A. K. Sgibnev and V. K. Filosofov, who studied the influence of these substances on tolerance to g-forces, performance of special psychological tests and resistance to orthostatis. No harmful effects were found [126].

Blood Sugar[1]

The glucose and nonesterified fatty acids (NEFA) contained in the blood serve as the basic sources of energy for all tissue of the organism including the muscles. Therefore, the ability to perform physical work depends to a large extent on the adequacy of the supply of this substance to the muscle cells, i.e., in the final analysis, how effectively their concentration in the blood is maintained.

The change in blood sugar level during physical work is well known. This question was studied in detail in the monographs of L. G. Leibson [92] and T. Cahn [173], so there is no need to dwell on the question of selecting specific experimental and clinical studies. It has been firmly established that if a trained athlete perform considerable physical work, the blood sugar level will remain about the same. However, if the stress is extreme for this person, as his capacities are exhausted hypoglycemia will develop, corresponding to a sharp decrease in working capacity. At the beginning of work, in the case of inadequately trained athletes, one frequently sees hypoglycemia caused by nervous and emotional excitation and the secretion of adrenaline and noradrenaline.

[1] In the Russian scientific literature, as in the German, blood sugar is always understood to be the amount of glucose, although strictly speaking the term is incorrect since glucose is not the only sugar in the blood. In the literature in the English and French languages, the term "glucose" is usually employed. In the present monograph, the term "blood sugar" will be used as a synonym for the word "glucose" in accordance with tradition.

According to the data presented in the source article by S. Berry [163], the American astronauts, following their flights aboard the spacecraft in the Apollo series (8 to 10 days), showed an increase in the blood glucose level. In the case of Soviet cosmonauts, following brief flights (up to 5 days), there /153 was no such increase (Table 36). In A.G.N. and V.I.S., who spent 18 days in space aboard the spacecraft in the Apollo series (8 to 10 days), showed an increase in the blood glucose level. In the case of Soviet cosmonauts, following brief flights (up to 5 days), there was no such increase (Table 36). In A.G.N. and V.I.S., who spent 18 days in space aboard "Soyuz-9", the sugar content had increased somewhat after the end of the flight in comparison with the preflight values, but did not go beyond the limits of normal variation. In fact, this increase took place not because there was hyperglycemia following the flight but because the additional values were lower than normal.

TABLE 36. BLOOD SUGAR LEVEL IN COSMONAUTS DURING SPACEFLIGHTS (IN mg%)

Craft and time of flight	Cosmonaut	Background values	Before the Flight	Flight, days				After the flight
				1	5	15	22	
"Voskhod", 1 day	K.P.F.	75	100	85	—	—	—	105
	B.B.Ye.	115	115	76; 112	—	—	—	55
Craft of the "Soyuz" type, 5 days	Average values							
	M	—	87	—	—	—	—	90
	σ	—	12	—	—	—	—	16
	n	—	13	—	—	—	—	17
"Soyuz-11", 18 days	A.G.N.	74	61	—	—	—	—	84
	V.I.S.	71	54	—	—	—	—	84
"Salyut", 24 days	G.T.D.	95	80	—	83	80	140	—
	V.N.V.	93	81	—	66	—	110	—
	V.I.P.	92	84	—	75	78	92	—

Note: The average blood sugar level obtained in the course of 185 tests of cosmonauts and cosmonaut-candidates was 85 mg%, with the mean square deviation being 17 mg%. Commas indicate decimal points.

The difference between the results obtained in a study of Soviet and American cosmonauts and astronauts is not surprising, and there are sufficient reasons for this. First of all, there are test conditions: in the case of the American astronauts, the blood was collected for analysis immediately after splashdown, while in the Soviets it took place on the morning of the next day, under standard conditions, when the joyful emotions associated with the successful conclusion of the flight had already partially evaporated. It must also be kept in mind that there were differences in the diet and the duration of the flight.

During the time spent in space by the "Salyut" station, its members collected blood samples for biochemical study. Regardless of the tragic ending of this

flight, all of the material was returned to Earth and analyzed. The studies /154
were carried out by means of the "AMAK" method, which was described in the first part of this book: the blood was dried on pieces of paper and the sugar content was determined by the anthrone method. The results that were obtained are shown in Table 36 in comparison with data from other flights. As we can see from this table, during the 1-day flight of the "Voskhod" craft in 1964, there were no pronounced changes in the blood sugar level. On the 5th and 15th days of the flight of the "Salyut" station there was a slight tendency toward hypoglycemia which developed primarily in V.N.V. On the 22nd day, both cosmonauts showed a sharp increase in sugar concentration. In V.I.P. the content increased in comparison with the 5th and 15th days of the flight but remained at background level.

The direct causes which could have led to such reactions could not be determined. We cannot completely exclude the possibility that the cosmonauts, as the result of some error, collected blood immediately after eating food or some event took place that led to emotional hypoglycemia. However, both of these factors are not very likely, since during the two previous blood sample collections the data were obtained normally and an analysis of the entries in the log book showed no additional factors. We only know that on this day the entire crew of the spacecraft was carrying out more physical work than usual, since they were preparing for the end of the flight.

In order to explain the changes in the blood sugar level during the flight, we can examine the results of some model experiments with prolonged hypodynamia (Table 37). These experiments were performed at different times with various groups of researchers, but the sugar content of the blood in all cases was analyzed in our laboratory by the same anthrone method so that the results are completely compatible.

The majority of the described series of experiments were conducted in order to work out preventive measures that would prevent possible disruptions of the state of health during the flight and mainly during the period of readaptation to terrestrial conditions. For this purpose, hypoxia, physical stress, electrical stimulation of muscles, as well as various methods of training the venous portion of the circulatory system were tested: cutting off the veins at the wrists by means of inflatable cuffs (occlusion training) and applying negative pressure to the lower half of the body (NPLB). For this purpose the lower half of the body was placed in a container that was hermetically sealed at the level of the waist or pelvis and a negative pressure was produced (several tens of mm Hg). It is believed that the accumulation of blood in the lower part of the body as the result of impedimentation of efflux simulates its accumulation there as a result of the action of the orthostatic gradient, thereby training the venous system, which is necessary for preventing orthostatic collapse. Con- /156
siderable attention was always paid to the state of hemodynamics following flights, as well as after prolonged experiments with bed rest, since under these conditions there is an increased tendency toward orthostatic collapse.

In the model experiments, the blood sugar level decreased as a rule. Judging by the results obtained in the 7th, 8th and 9th series, this decrease developed gradually and was clearly evident by the third week of the experiment.

It continued to be evident until the end of the experiment, but immediately before its termination the glycemia level became unstable: in some cases it increased while it fell in others. These changes were most likely caused by the neuro-emotional stimulation which is unavoidable at the end of a long experiment. Judging by the available material, the nature of the reaction was a function of at least two factors: the diet and the level of physical activity. In the 8th and 9th series, in which the sugar level rose prior to the end of the experiment, the subjects received diets that were similar in composition to those aboard the spacecraft. In the 7th series, the usual hospital diet was given and the sugar level fell. Physical training in the 9th series promoted a decrease in the prefinish reaction (in comparison with the 8th series).

TABLE 37. CHANGE IN BLOOD SUGAR LEVEL IN VARIOUS SERIES OF TERRESTRIAL EXPERIMENTS INVOLVING BED REST (AVERAGE VALUES IN mg%)

Duration, days	Series of experiments*	Conditions	No. of Subjects	Substance studied	Before the test	Days after beginning of test					Days after end of test	
						8-9	17-20	29	60	100	2	15-20
20	1	Bed rest (control)	2	Blood	60	66	66	—	—	—	—	
	2	Bed rest and hypoxia	4	Blood	68	72	72	—	—	—	—	73
30	4	Bed rest, physical exercise and NPLB	3	Serum	68	—	60	—	—	—	65	64
30	5	Bed rest (control)	3	Serum	71	—	62	—	—	—	75	67
30	6	Bed rest and electrical stimulation	3		62	—	60	—	—	—	73	64
		Average values for the 4th, 5th and 6th series	9	M±m	67±2,5	—	61±1,3	—	—	—	—	65±1,9
30	7	Bed rest	9	M±m	69±2	66±2,1	62±1,3	45±1,9	—	—	—	—
100	8	Bed rest (control)	3	Blood	91	—	56	—	78	109	—	—
100	9	Bed rest and physical exercise	3	Blood	85	—	60	—	79	89	—	—
100		Average values for the 8th and 9th series	6	M±m	88±4,9	—	58±2,7		78±2,5	99±7,3	—	—

*Number of series same as in Table 29, 30-33, 39.
Commas indicate decimal points.

Immediately following the end of the experiment the sugar level increased in the 5th series, where no preventive measures were taken of any kind. In the 4th and 6th series, which were performed simultaneously with the 5th, this increase was not so pronounced. From this it follows that physical stress and electrical stimulation of muscles are capable of weakening the glycemic reaction at the end of the experiment.

The data presented on the blood sugar level during model experiments is generally in good agreement with the results obtained in the determination of nonesterified fatty acids (see the following section), which serves as an indirect support for the correctness of the results obtained.

On the general levelm the decrease in blood sugar during hypodynamia and its instability at the end of the experiment must be viewed as symptoms of a lack of training which develops as the result of the prolonged effects of hypodynamia. Evidently this is why the glycemia level fell on the 5th and 15th days of the flight. On the 22nd day the additional physical stress caused by preparation for landing acted as a rather strong stimulus to cause a noticeable increase in blood sugar. It is very difficult to evaluate the energy losses during work under weightlessness conditions since the biomechanics of movement differ from the norm. It may be concluded that the work which the cosmonauts performed on the day of the third blood collection was not particularly great from the usual "terrestrial" standpoint but it was sufficient to cause hyperglycemia, since the cosmonauts were already detrained as a result of prolonged /157 flight. This stress caused the same physiological effect in them that would occur in inadequately trained athletes who participated in contests. The only difference is that the threshold to produce such an effect is much higher in athletes.

Nonesterified Fatty Acids (NEFA) in the Blood

The dynamics of the NEFA level in the blood must be viewed together with the dynamics of glycemia, because although these substances differ in chemical nature, in the physiological sense they carry out similar functions, serving as sources of energy. The category of nonesterified fatty acids in the blood contains fatty acids with a number of carbon atoms, at least 16, whose chains may contain double bonds. Most of these substances, as well as the ether-bound fatty acids in the blood, are formed by palmitic, oleic and stearic acid. Existing analytical methods have been inadequately developed in many respects (see more about this in the methodological section), so that we must be very careful when evaluating the data in the literature. The experiments described in this section were performed by means of an original method which differs in many respects from those described previously, so that the results are frequently not compatible. The experiments described in the methodological section indicate that the original titrometric method of determining NEFA following their separation from the serum and appropriate purification gives the most accurate results.

The NEFA are distributed nonuniformly between the blood plasma and the erythrocytes, so that their content in whole blood is lower and less informative in comparison with the serum content. Since it has thus far not been possible to include a determination of the NEFA in complex microchemical analysis of /158 dried blood, the studies were performed only in model experiments with prolonged hypodynamia.

From the data presented in Table 38 it is clear that the NEFA content during prolonged hypodynamia changed slightly all the way to the end of the experiment. On the whole, during hypodynamia there were inverse relationships between the concentrations of NEFA and glucose in the blood (Figure 29). In the 4th, 5th and 6th series, where the subjects received diets that were similar to those aboard the spacecraft, the NEFA content was higher than for the normal hospital diet (7th series) but the inverse relationship to the concentrations of these two substances remained in both instances. This serves as an indirect

indication of the fact that the results of the determination of sugar and NEFA are not an artifact. Following the end of the experiments, at the beginning of readaptation, these relationships were destroyed: in the control group (5th series) which did not undergo training, the NEFA concentration increased on the second day of readaptation simultaneously with an increase in sugar concentration. This can be most simply explained by the fact that in those subjects who were least trained the end of the experiment required considerable mobilization of the adrenaline reserves, so that there was a rise in both sugar and NEFA levels. In those groups with physical training and electrical stimulation, other mechanisms were retained so that the transition from clinostatic hypodynamia to normal activity took place more smoothly.

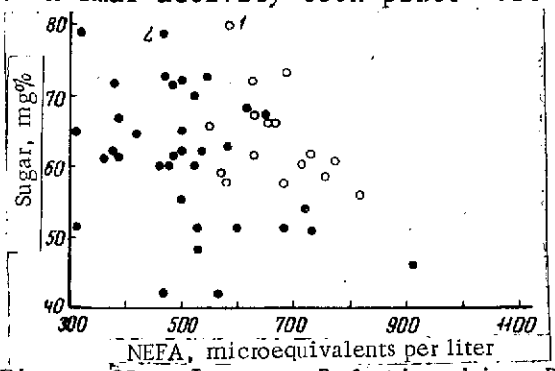

Figure 29. Inverse Relationships Between Sugar Level and NEFA in Blood Plasma During 30-Day Experiments with Hypodynamia. 1, Feeding prototype diet to be used aboard the craft (4th, 5th and 6th series of tests); 2, hospital diet (7th series).

While the physiological mechanisms that provide simultaneous increase of NEFA and sugar appear to be quite obvious at the beginning of readaptation, the intimate reasons for the decrease in the concentration of sugar and the rise in the NEFA during the experiment are not clear. Here we can operate only on the basis of guesses. During hypodynamia, it is possible to conceive of an increase in insulin activity, since the sugar level decreases and the amount of fatty tissue in the subject increases. However, this mechanism is excluded since the administration of insulin not only causes a decrease in blood sugar but also a slight (although less pronounced) decrease in NEFA. /159

These changes can be better explained by the decrease in glucocorticoid activity; as we know, the administration of hydrocortisone increases the sugar content in the blood (steroid diabetes) and decreases the NEFA, while hypoglycemia develops in animals that have had the adrenal cortex removed. This explanation is generally in agreement with the decrease in excretion of 17-oxycorticosteroids with the urine. Inasmuch as no clear relationship has been found between the concentrations of glucose, NEFA and 11-oxycorticosteroids in blood serum, the above is still only a working hypothesis.

This assumption generally agrees with the results of the studies of Lancaster [242] who found that the ability of the muscles to utilize glucose is reduced during hypodynamia. This means that the sugar curves have a hyperglycemic appearance, although the insulin concentration rises sharply following administration of glucose.

Blood Cholesterol

Since atherosclerotic damage to the vessels is the most widespread of human ailments, blood cholesterol has become the object of tremendous amount of

research. The results of this work, although they have expanded our knowledge concerning the role of lipids in the mechanism of the development of atherosclerosis, thus far have not provided any clear answers to certain questions. However, one of the reasons is that there is no reliable way of diagnosing the initial stages of the disease when the pathogenetic role of hypercholesterolemia is most likely, since the results of laboratory analyses cannot be compared with anything. The second reason is that it is very difficult reliably to determine blood cholesterol — it is bound to the proteins in very strong components which we have learned to break down only in recent years. Therefore the results obtained by insufficiently reliable biochemical methods remain doubtful.

As it applies to problems of medical safety of spaceflights, the study of blood cholesterol is important because cosmonauts belong to the age group in which the content of this substance in the blood in men increases rapidly, making the threat of atherosclerosis possible. /160

TABLE 38. CHANGE IN THE CONTENT OF NONESTERIFIED FATTY ACIDS (IN MICROEQUIVALENTS PER LITER) IN BLOOD SERUM UNDER TERRESTRIAL EXPERIMENTAL CONDITIONS WITH HYPODYNAMIA (AVERAGE VALUES FOR EACH SERIES)

Series	Conditions	No. of Subjects	Before the experiment	During the experiment, days			After the experiment, days	
				10th	20th	29th	2nd	16-20th
4th	Bed rest, physical exercise, NPLB	3	600	—	950	—	730	690
5th	Bed rest (control)	3	610	—	700	—	1200	600
6th	Bed rest and electrical stimulation of the muscles	3	660	—	640	—	700	700
3rd-6th	Avg. data for a number of similar experiments, M ± m	3	650±50	—	700±60	—	—	580±40
	Number of observations		16		12			15
7th	Bed rest, M ± m	9	480±90	440±80	450±100	630±130	—	—

Commas indicate decimal points.

Cholesterol and its ethers are insoluble in water, so that they occur in the blood in the form of lipoprotein complexes inhomogeneously distributed between the plasma and erythrocytes. In the red blood cells, these complexes go to make up the stroma, which is more inert physiologically speaking, so that the total cholesterol concentration in the erythrocytes changes more rarely and not so sharply as in the plasma. Therefore, plasma or serum is usually used for investigation, but not whole blood. It is a very complicated affair to collect the liquid part of the blood during a spaceflight, so that all of the analyses were carried out with whole blood treated by a complex microchemical

method specially devised for this purpose. As experience showed, such data, although they are less informative, nevertheless provide interesting results.

Cholesterol is synthesized in at least 3 tissues in the organism — in the liver, the wall of the intestine and the intima of the vessels, in addition to being taken in with the food. Its level in the blood is a function of the intensity of all of these processes as well as the rate of breakdown which (according to a number of researchers) is determined by physical activity [241]. Therefore, the level of cholesterolemia reflects many processes in the organism. According to K. S. Kosyakov [80], hypocholesterolemia shows good agreement with the seriousness of the condition and is a symptom of the syndrome of the general reaction of the organism to nonspecific influences. On the other hand, Leytes [93] considers an increase in cholesterol to be a symptom of stress and describes neurogenic hypercholesterolemia. According to Shidek et al. [264] it is frequently combined with vegetative lability. Although the leading pathogenetic role of blood cholesterol in the development of atherosclerotic damage is not universally recognized, the majority of famous specialists including Professor B. V. Il'inskiy, feel that its increase is of great diagnostic significance. In his program article "Hypertonic Disease and Atherosclerosis"[2] he writes: "...In atherosclerosis, the most prominent early symptoms must be considered to be disruption of metabolism, taking the form of an increase in cholesterol level, beta-lipoproteins, a decrease in the albumin fraction of proteins and the stable colloid solution of cholesterol in the blood plasma, but usually without the noticeable clinical changes that are typical of hypertonic disease involving the nervous system and the level of arterial pressure." Since cholesterolemia is a function of alimentary factors, its study may be useful for evaluating nutrition.

The results of studies aboard the "Salyut" orbital station as well as /161
before and after other flights are shown in Table 39; it is clear that there were no regular changes. During the short brief flights, a slight and statistically unreliable increase occurred; after the 18-day flight of the "Soyuz-9", the cholesterol level in the blood of A.G.N. increased while in V.I.S. it decreased.

During the flight of the "Salyut" station, all 3 cosmonauts showed a decrease in whole-blood cholesterol.

The American astronauts, during their flights aboard Apollo spacecraft, showed a decrease in blood cholesterol [163].

Table 40 sums up several experiments, in most of which (Series 1, 2, 4, 5, 6, 8 and 9) the subjects were young men who were fed various preliminary versions of the food rations eaten aboard the spacecraft. The caloric content was 2700-3000 kcal per day and the diet consisted of concentrated high-nutrient products. In the 3rd series, they received an improved hospital diet while in

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[2]B. V. Il'inskiy: "Hypertonic Disease and Atherosclerosis," Klinicheskaya Meditsina, Vol. 48, No. 8, p. 30, 1970.

the 7th series they received an ordinary hospital diet. The age of the subjects was between 20 and 35 but each group was homogeneous in this regard.

TABLE 39. CHOLESTEROL LEVEL IN THE BLOOD OF COSMONAUTS DURING SPACEFLIGHTS (IN mg%)

Craft and time of flight, days	Cosmonaut	Background values	Before the flight	During the flight, days			After the Flight
				5th	15th	22nd	
"Soyuz" type craft (average data for 5-day flights)	M±m)	—	189±27	—	—	—	199±32
	n	—	10	—	—	—	15
"Soyuz" 9, 18	V.I.S.	171	167	—	—	—	136
	A.G.N.	208	206	—	—	—	225
"Salyut" 24	G.T.D.	190	210	145	140	150	—
	V.N.V.	260	215	190	—	200	—
	V.I.P.	220	225	175	160	165	—

Note: The average cholesterol content obtained for 140 cosmonauts and cosmonaut-candidates studied was 191 mg% with the mean square deviation being 28 mg%.

Since the level of cholesterolemia increases with age, the results of each series had to be compared with the original data.

It is clear from the table that against a background of eating the diet to be consumed aboard the spacecraft, hypodynamia produced hypercholesterolemia. Only 2 effects could have prevented it — hypoxia (Series 2) and electrical stimulation of the muscles (Series 6). The increase in cholesterol was not found in Series 7, when the subjects ate a normal hospital diet. Since 9 persons were studied simultaneously in this group, the results are considered to be completely reliable. All of the analyses were carried out using the original method described in the first part of this paper. In Series 4, 5 and 6 they were duplicated by the Il'k method [the work was carried out by T. A. Kislovskaya, who found the same sort of trend but the numbers were lower, of course (see Figure 10)]. /163

The role of the alimentary factor in these experiments is clear. It can also be reinforced by the following observation. In all of the subjects in Series 4 and 5 the cholesterol level in the blood serum rose during the experiment. The smallest increase was in subject Sh. (Series 4) in whom the original level was 200 mg% and 215 mg% on the 2nd day after the end of the subject on this same diet was an average of 16.9 g per day while for the others it was 13 to 14 g. Subject Sh. was more strongly developed physically and his

energy consumption in performing exercise was highest, so that we are forced to conclude that the caloric content of the diet was insufficient for him and tissue catabolism took place.

TABLE 40. BLOOD CHOLESTEROL IN VARIOUS SERIES OF TERRESTRIAL EXPERIMENTS INVOLVING BED REST (AVERAGE VALUES IN mg%)

Duration of experiments, days	Series	Conditions	Number of subjects	Substance investigated	Before the experiment	Days from the start of the experiment							After the experiment, days	
						2 to 3	8 to 9	12 to 17	20	26 to 29	60	100	2	15 to 20
20	1st	Bed rest (control)	2	Blood	167	—	196	—	187	—	—	—	—	157
	2nd	Bed rest and hypoxia	4	Blood	172	—	167	—	171	—	—	—	—	143
	3rd	Bed rest, NPLB	3	Plasma	185	—	—	—	—	—	—	—	259	249
				Serum	209	—	—	—	231	—	—	—	257	238
	4th	Bed rest, physical exercise, NPLB	3	Blood	—	163	—	153	—	173	—	—	—	—
				Serum	256	—	—	—	293	—	—	—	328	284
	5th	Bed rest (control)	3	Blood	—	162	—	207	—	203	—	—	—	—
		Avg. values for series 3, 4 and 5	9	Plasma M ± m	221±14	—	—	—	262±17	—	—	—	281±14	257±13
30	6th	Bed rest and electrical stimulation of muscles	3	Serum	269	—	—	—	253	—	—	—	251	228
				Blood	—	163	—	183	—	171	—	—	—	—
100	7th	Bed rest	9	Serum M ± m	—	280±14	260±63	—	270±34	270±17	—	—	—	—
	8th	Bed rest	3	Blood	170	—	—	217	—	—	213	231	—	—
	9th	Bed rest and physical exercise	3	Blood	169	—	—	230	—	—	236	227	—	—
		Avg. values for series 8 and 9	6	Blood, M ± m	169±5	—	—	224±7	—	—	224±11	229±13	—	—

The theory of the relative excess of the diet in experiments with hyperdynamia is supported also by the results of a direct study of energy consumption and body composition which is presented in Table 25.

By no means all hypodynamia (any more than all excess nutrition) will lead to hypercholesterolemia. These problems have been very well studied in the clinic. Thusm in the treatment of myocardial infarct patients are kept in strict bed rest for at least 3 weeks and there is a reliable increase in the activity of their sympathetic-adrenal system and a rise in the concentration of nonesterified fatty acids in the blood serum, but the cholesterol level remains constant [109]. These observations, among others, make it possible to exclude "stress", i.e., a certain nonspecific reaction to external stimulation, as the direct cause of the increase in the blood cholesterol during hypodynamia. If in fact such a universal state exists, it would necessarily develop in the case of myocardial infarct!

The excessive nature of the diet, of itself, also is not a sufficient cause for explaining the increased blood cholesterol. This problem has been studied on many occasions and some authors have found a relationship between the nature

of the diet, the blood cholesterol and the development of atherosclerosis, while others failed to find such a relationship; it is obvious that the result is a function of the concrete conditions under which the investigated contingents found themselves. /164

It might seem that hypercholesterolemia developed in our model experiments as the result of an unfavorable combination of two factors — limitation of mobility and relative excess of nutrition. During the flights, there was no such combination; the food frequently was insufficient to compensate for all of the energy expenditures. Thus, in the case of the crew of the "Gemini VII" [237], a negative nitrogen balance was produced and the caloric content of the food that was actually eaten during the flights of the craft in the Apollo series was at the 1500-2000 kcal level [163]. However, in the case of A.G.N. who flew aboard the "Soyuz-9" spacecraft and whose weight loss after the flight was minimal, the cholesterol content in the blood increased slightly.

If these ideas concerning the mechanism of the development of hypercholesterolemia in model experiments are correct, the problem takes on great seriousness. In fact, the shortage of nutrition due to effects on the nitrogen balance and the rate of protein synthesis in the organism leads to a weakening of its reserve capacities, which is extremely undesirable under the extreme conditions of spaceflight, when any unforeseen situation may require maximum exertion on the part of the organism. At the same time, the excess amount of food, by causing hypercholesterolemia, will increase the threat of development of atherosclerosis, a disease which is very dangerous since spaceflight imposes considerable and still incompletely understood demands on the cardiovascular system.

It is difficult exactly to balance the energy consumption and the caloric content of the diet — "terrestrial" standards can be used only with great care. During his flight the cosmonaut has work thrust on him up to the limit, causing great neuro-emotional stress; he must also navigate the craft and carry out scientific research program as well as monitor the operation of numerous assemblies. The food does not have the usual external appearance and size, and there is no weight, which means that the evacuatory function of the gastrointestinal tract is affected. All of this means that there is a change in the accustomed stimuli which influence the feelings of hunger and appetite, thus disrupting the very fine balance between energy consumption and food intake. Therefore the possible pathogenetic mechanisms of the development of hypercholesterolemia in model experiments and the factors which are capable of overcoming it deserve careful analysis.

It is possible that the increase in blood cholesterol in the model experiments was promoted by a slight decrease in the activity of the thyroid gland, which can be concluded from the results of a study of basal metabolism [102]. Since the function of this gland, as far as we know, has not been studied in experiments with hypodynamia by direct methods, the problem can be solved only after carrying out additional tests.

Does an excess of food during hypodynamia always lead to hypercholesterolemia? Judging by the results of our experiments, this is not always the case; /165

at least in two series where we used hypoxia and electrical stimulation of the muscles, there was no such increase. Just why hypoxia has the ability to prevent this increase is difficult to say. It is possible that it is a general intensification of the function of blood circulation and respiration under these conditions that was making itself known, but it is difficult to draw any final conclusions or even to put together any kind of well founded assumptions on the basis of this data, since the number of observations is not great. These experiments were performed when it seemed that hypoxia might be used as a prophylactic measure to prevent possible damage during flights. Later, this possibility was dropped, since orthostatic instability following landing is most significant and cannot be prevented by this kind of training. In addition, periodically producing a rarefied gas medium in the cabin of the spacecraft was very complicated from the technical standpoint. The use of electrical stimulation of the muscles during flight appeared more promising. This method does not require any complicated devices, and training consists simply in applying electrodes to the skin at certain points through which electrical current pulses are delivered periodically to cause contraction of the muscles. Since the position of the parts of the body does not change, contraction occurs without any change in the length of the muscles, i.e., in an isometric fashion. As we know, physiologists differentiate between two types of contraction — isometric and isotonic. In the first case, the length of the muscles does not decrease, so that they do not perform any work against external forces. In isotonic contraction the load remains constant but the length of the muscle decreases, and the work is performed, usually the lifting of a load. According to the laws of mechanics, the amount of work performed is equal to the derivative of the change in the muscle length times the force (i.e., the weight of the burden which was lifted).

As was shown by Fenn, then studied in detail by Hill [43], isometric contraction of an isolated sartorius muscle of a frog involves less energy liberation in comparison with isotonic. Similar results were obtained in our experiments with O. A. Virovets when the oxygen consumption of a similar isolated sartorius muscle of a frog was recorded. It is clear from Figure 31 that the respiration increased in only those cases when the load was lifted. If it was so great that the muscle could not lift it, i.e., the contractions were isometric, oxygen consumption did not rise.

When an individual works, almost all of the muscle contractions have a mixed nature — both a reduction of length and a change in force occur. Most of the energy is expended in performing work against external forces. Obviously /166 electrical stimulation produces a distinctive "false" muscle contraction, and several aspects of metabolism in them are intensified without the total energy consumption being increased significantly. According to the data of Malinov, Perley and McLaughlin [241], the breakdown of cholesterol in the organism is associated with muscular work, so that limitation of mobility would necessarily lead to a slowing down of cholesterol breakdown. Obviously, against a background of excessive caloric intake this is sufficient to cause hypercholesterolemia. It might seem that the isometric muscle contractions which occur during electrical stimulation would intensify the breakdown of cholesterol. So the problem is as follows: are complete, isotonic contractions required for accelerating the breakdown of cholesterol or are more economic isometric contractions adequate with regard to energy?

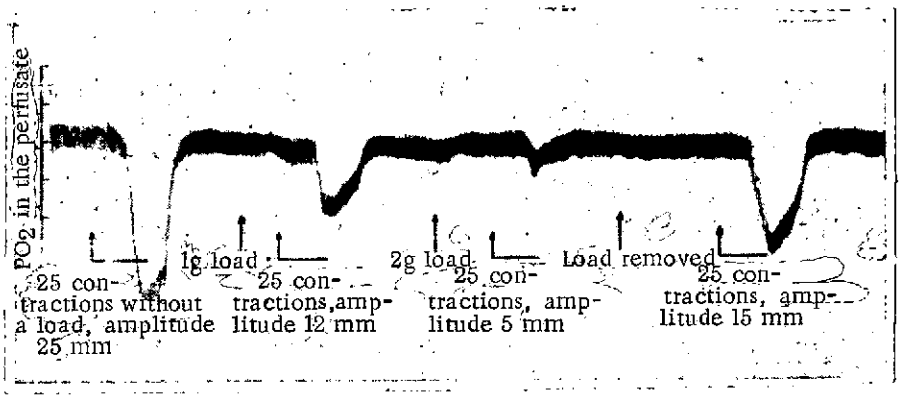

Figure 30. Utilization of Oxygen by Isolated Sartorius Muscle of a Frog During Isometric and Isotonic Contractions.

At the present time there is no final answer to this question but several views might be mentioned. Basically, what is the reason for the increase in the intensity of metabolism in the muscles when they contract — is it the exhaustion of materials which are the sources of energy or does the contraction itself act as a signal which activates mechanisms that result in an increase in everything including catabolism of cholesterol? This is a matter of deep significance since insufficiency of physical activity is an unavoidable aspect of spaceflight. Is it necessary to compensate by means of physical exercises, which are valuable from the energetic standpoint, or is it adequate to have "stimulation" of muscle activity by means of isometric contractions, for example, those produced by electrical stimulation?

Recently, the regulation of metabolism in muscle tissue has normally been studied in disintegrated preparations — mitochondria or cell homogenates. Under these conditions, as was very clearly pointed out first by Lardi, and then in the numerous works of Chance and his associates, the principle of respiratory /167 control is accomplished which consists in the fact that the stimulus for the increased respiration of the tissue preparation is the accumulation of ADP, a substance which appears as a result of the breakdown of the principal accumulator of energy in the living cell — ATP. If this principle is universal, i.e., under all conditions the stimulus for an increase in the intensity of metabolism can only be the expenditure of previously stored energy resources, electrical stimulation as a preventive means has no future in spaceflights. In fact, its advantage consists in the fact that secondary effects caused by muscle activity are realized without performing energetically useful work associated with the consumption of a large quantity of oxygen and food and expenditure of time.

However, there are no indications that muscle metabolism is intensified only after the breakdown of a significant portion of the ATP. In fact, the principle of respiratory control is realized only in disintegrated preparations of muscle fibers. In a series of works by Sheradijan [263] it was shown that the individual contractions of an isolated frog muscle do not lead to a

significant increase in the ADP content in it, although it is well known that oxygen consumption and heat production increase under these conditions. It is possible that the considerations presented are insufficient for a final conclusion to be drawn as to which of the two points of view in question is the correct one, but if it is shown that the concept (which may be provisionally called respiratory control) does not apply to working muscles of an intact organism, i.e., metabolism in them increases not because the ATP has broken down but as the result of more complex regulatory influences, the "molecular" basis of electrostimulation will have been established.

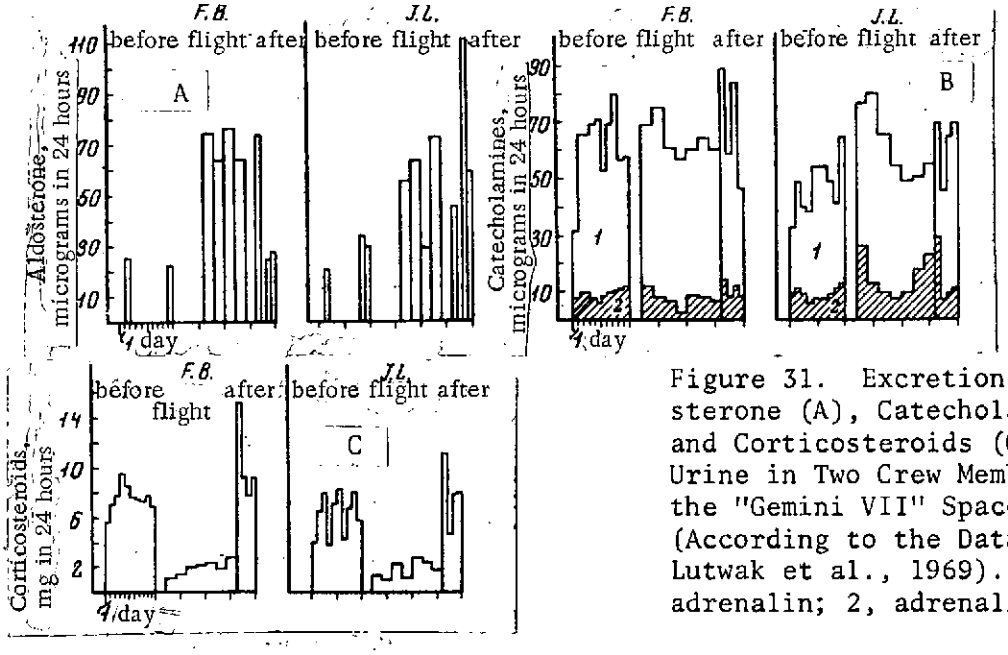

Figure 31. Excretion of Aldosterone (A), Catecholamines (B) and Corticosteroids (C) in the Urine in Two Crew Members of the "Gemini VII" Spacecraft (According to the Data from Lutwak et al., 1969). 1, Noradrenalin; 2, adrenalin.

Change in Functions of the Endocrine System

Knowing the functional state of the endocrine glands may explain many features of metabolism during spaceflight, but it is not an easy matter to obtain the necessary data, due to the methodological difficulties: hormonal substances are unstable and their analysis requires considerable material. Direct determination of the hormones themselves and their direct metabolites in biological substrates does not always provide a complete and correct idea of the condition of the endocrine glands, since the results of the analysis are a function not only of the rate at which the substance in question is formed but also of the pathways it uses and at the rate at which it is excreted from the organism. These difficulties are best seen using the example of the study of the adrenalin system: daily excretion of adrenalin and noradrenalin with the urine varies within considerable limits, but it amounts to several tens of micrograms, i.e., approximately 1% of the amounts synthesized [260].

This is explained by the fact that the overwhelming majority of catecholamines undergo chemical conversion (they are catalized) and in the final analysis excreted with the urine in the form of vanilyl-amygdalic acid. Excretion of the hormones themselves is very sensitive to various influences, and

even minor changes in the rate of its breakdown have a powerful effect on excretion. On the other hand, the excretion of vanilyl-amygdalic acid is quite stable, so small changes are reliable, but this indicator is sluggish and not very informative. It simultaneously provides information on the total rate of formation of several catecholamines in the organism.

Hormonal studies performed by the Americans during the flight of "Gemini VII" [237] yielded results which at first glance appear paradoxical (Figure 31). It was found that during the stay in space the excretion of 17-oxycorticosteroids (substances which characterize glucocorticoid function of the adrenal cortex) was reduced, but immediately after landing there was a significant increase so that the ordinary values were exceeded markedly. Excretion of aldosterone during the flight was very high, 40-80 micrograms per day, and it lasted even after the flight. Excretion of adrenaline and noradrenaline showed practically no change, but the total level from the standpoint of our norms was rather high. Summing up the results of endocrine studies conducted by the Americans in /169 monitoring the astronauts immediately after the spaceflights, Berry [24, 163] noticed an increase in the activity of the antidiuretic hormone, aldosterone, renin, and catecholamines, but these data were not interpreted by him.

The crew of the "Soyuz-9" spacecraft showed a tendency toward a decrease in the content of 17-oxycorticosteroids in the urine during their flight (Table 41). After the flights the excretion of these substances, as revealed by an examination of 13 cosmonauts (Table 42), was normal on the whole, although on certain days and at certain times the values were slightly above normal (Figure 32). Excretion of acid-labile conjugate of aldosterone was studied for only 3 days after the end of the "Soyuz-9" flight and was found to be within ordinary limits (see Table 41). The excretion of vanilyl-amygdalic acid, the final product of the metabolism of adrenaline and noradrenaline, studied after a number of flights, showed no deviation from normal values (see Table 42).

TABLE 41. EXCRETION OF 17-OXYCORTICOSTEROIDS (17-OH) AND ACID-LABILE CONJUGATE OF ALDOSTERONE (AS) IN THE CREW MEMBERS OF THE "SOYUZ-9" SPACECRAFT

Conditions	A.G.N.		V.I.S.	
	17-OH, mg per day	AS, micrograms per day	17-OH mg per day	AS, micrograms per day
One month before flight	2,8	—	6,6	—
On eve of launching	3,8	9	5,9	11
During flight, first day	1,8	—	2,6	—
During flight, second day	2,5	—	1,1	—
During flight, 18th day	3,8	—	5,1	—
After flight, second day	4,3	—	4,5	—
After flight, third day	—	10	—	11

Commas indicate decimal points.

TABLE 42. EXCRETION OF 17-OXYCORTICOSTEROIDS (A) AND VANILYL-AMYGDALIC ACID (B) IN THE URINE FOLLOWING THE END OF SPACEFLIGHTS (IN mg PER DAY)

Craft and cosmonaut	Duration of flight, days	A		B	
		One day	Two days	One day	Two days
"Vostok"					
V.M.K.	1	8,5	3,7	—	—
K.P.F.		5,8	3,6	—	—
B.B.Ye.		7,4	2,5	—	—
"Soyuz-3"					
G.T.B.	3	2,5	2,5	4,1	4,6
"Soyuz-5"					
B.A.V.	3	3,3	—	—	—
Ye.V.Kh.		2,6	—	—	—
A.S.Ye.		4,1	—	—	—
"Soyuz-6"					
G.S.Sh.	5	3,9	4,2	4,8	4,6
V.N.K.		4,2	5,4	4,2	5,1
"Soyuz-7"					
A.V.F.	5	4,2	4,8	4,1	5,4
V.N.V.		1,8	4,3	3,6	3,6
A.V.G.		2,8	5,1	4,3	3,8
"Soyuz-8"					
V.A.Sh.	5	2,8	2,9	3,5	5,1
A.S.Ye.		3,4	2,5	5,2	2,9
"Soyuz-9"					
A.G.N.	18	3,3	4,3	—	—
V.I.S.		3,7	5,4	—	—
Normal values for M		4,0		5,2	
σ		1,5		1,2	

Commas indicate decimal points.

The table shows the results of a urinalysis which was performed during the autopsy. The material obtained provides some idea concerning the excretion of several substances by the kidneys during the last hours of the orbital flight. The results shown in Table 43 do not show any striking changes. According to the calculations, these portions of urine must have been excreted in 3 hours. We are not convinced that the last evacuation of the urinary bladder was complete, so that the calculated values of the hourly excretion must be viewed with a certain degree of caution. But even if we assume a possible error in the calculations, the results shown in the table make it possible to exclude the /170 possibility of a pronounced increase in the activity of the cortical and medullary layers of the adrenal gland.

As we can see, the data we have at our disposal on the functional state of the endocrine system during the flight is small, and the most complete data we

have applies to the glucocorticoid function of the adrenal gland. According to the data of both the American and Soviet investigators, excretion of 17-oxycorticosteroids was reduced. Only one factor leads us to doubt the veracity of /171 these results: oxycorticosteroids are very labile compounds chemically speaking and the development of microbial flora in the urine leads to their destruction; we cannot exclude a partial loss of hormones.

In performing model experiments with hypodynamia various authors have obtained different results. In the majority of cases, it was possible to detect unreliable changes from the original levels [53, 140, 185, 211].

According to our experiments, the adrenal cortex in man reacts much more sharply to submersion in water than it does to immobilization in bed. Water immersion (Figure 33) leads to a pronounced decrease in the excretion of 17-oxycorticosteroids during the first days of the experiment, but later there may be abrupt jumps. During prolonged bed rest (Table 44), it was sometimes possible to observe a decrease in the excretion of these substances but on the whole the levels remained within normal limits. The concentration of 11-oxycorticosteroids in the blood during hypodynamia in bed showed practically no differences with respect to normal values.

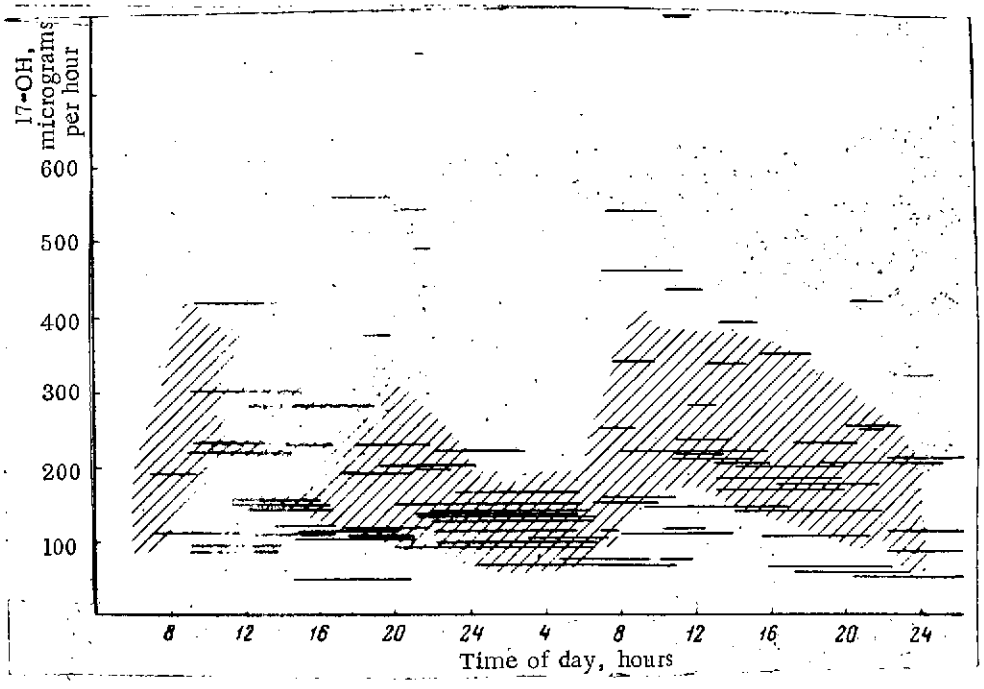

Figure 32. Values for the Hourly Excretion of 17-Oxycorticosteroids with the Urine in 13 Cosmonauts Who Flew Aboard "Soyuz" Type Spacecraft. A, Days when the landing took place; B, days following. Shaded area; normal values (M ± Δ).

In performing the experiment in small-volume chambers or in model spacecraft /172 cabins, where the subjects could move about freely, sitting or standing, there were no reliable changes in the excretion of 17-oxycorticosteroids.

Analyzing the data from the model experiments, we must keep in mind that the last days of preparation for a long experiment, as well as its conclusion, are very stressful. This is because the participants in an experiment lasting 1 or more months are isolated from normal life, and must undergo complicated and sometimes fatiguing medical examinations. It is therefore no surprise that immediately after the experiment the function of the adrenal cortex was slightly increased as the result of a nonspecific hormonal reaction to external stimuli. Such a reaction, which was first noticed by H. Selye in rats [128], was later described on many occasions in man and various species of animals. During the experiment (provided of course that it is not complicated by any special conditions, for example plaster casts on the extremities), the subjects will be in the correct regime, under conditions of relative rest, which leads to a normalization of a number of functions, including the glucocorticoid function, so the excretion of 17-oxycorticosteroids is reduced. The end of the experiment in another stimulus that is strong enough to cause a new rise in the activity of the adrenal cortex. All of this was possibly the reason why various authors observed different reactions with various degrees of severity. /173

TABLE 43. CONTENT OF HORMONAL SUBSTANCES AND CREATININE IN THE URINE OF THE COSMONAUTS ABOARD THE "SALYUT" STATION

Parameters	G.T.D.	V.N.V.	V.I.P.
Volume of urine, ml	150	205	235
Creatinine concentration, mg per ml	1,75	2,15	1,35
Concentration of 17-oxycorticosteroids, mg per ml	5,2	6,0	7,8
Adrenaline concentration, micrograms/liter	4,6	6,3	4,7
Noradrenaline " " "	20,4	26,2	11,2
Coefficient of 17-oxycorticosteroids/ /creatinine, micrograms per mg	3	2,8	5,8
Coefficient adrenaline/creatinine, ng/mg	2,6	2,9	3,5
Coefficient noradrenaline/creatinine, ng/mg	11,6	12,2	8,6
Approximate excretion in 1 hour:			
Creatinine, mg	86	150	110
17-oxycorticosteroids, micrograms	260	420	630
Adrenaline, ng	230	440	380
Noradrenaline, ng	1020	1820	900

Commas indicate decimal points.

Spaceflights and the model using water immersion which is most similar to them in the hemodynamic sense are something else again. Here, even by making a correction to possible methodological errors, we can speak of a decrease in the function of the adrenal cortex. Even such strong stimuli as injection into orbit or return to Earth, which are accomplished not only by complicated and responsible work with g-forces and emotions, do not cause the anticipated strong increase in the excretion of 17-oxycorticosteroids. Evidently the functional capacity of the gland is reduced. /174

In examining experiments involving water immersion, we must take into account a number of additional complications which this model creates for the

human organism: even very minor changes in temperature in the environment have a profound effect on the thermal regulation; the water stimulates the skin, especially in sensitive subjects, sometimes causing generalized dermatoses. It is therefore not surprising that against a background of a general decrease in the content of 17-oxycorticosteroids in the urine, their amounts jump up sharply on individual days.

TABLE 44. EXCRETION OF 17-OXYCORTICOSTEROIDS WITH THE URINE (IN mg PER DAY) DURING A 30-DAY SIMULATION EXPERIMENT WITH BED REST (M ± m)

Series	No. of subjects	Before test	During the test, decade			After the experiment, days	
			1	2	3	2nd	6th
4th	3	4,8±0,47	3,6±0,47	3,3±0,37 P′<0,05	3,2±0,52	7,0±1,5 P″<0,05	5,7±1,1
5th	3	6,5±0,61	4,0±0,46	3,8±0,43 P′<0,01	4,3±0,37	8,5±0,7 P″<0,001	5,4±0,06
6th	3	5,4±0,63	4,2±0,4	3,9±0,49 P′<0,05	3,9±0,49	8,9±1,9 P″<0,05	5,6±0,97

Commas indicate decimal points.

The increase in aldosterone excretion which was observed by the Americans during the flight of "Gemini VII" cannot be understood from the standpoint of the previously established concepts regarding the physiology of this mineralocorticoid. It has repeatedly been demonstrated (see Table 10) that when an individual is lying down the production, excretion from the organism and blood alderosterone are less than when he is standing or sitting. Postural aldosteronic reaction is so characteristic that it is employed for physiological evaluation when working out a new method of analysis. Why it is that under conditions of spaceflight, when weightlessness (according to the generally accepted theory) acts on the hemodynamics in the same way as bed rest, that the production of this hormone increases?

In addition to the change in the position of the body, one reason for the increase in aldesterone production may be a deficiency of sodium in the diet. During the spaceflight of "Gemini VII" the sodium content in the daily diet varied from 88-199 meq, i.e., it was no different from normal and was much higher than the 10 meq per day which is usually used to stimulate aldesteronism.

The examination of A.G.N. and V.I.S. (see Table 41) on the day following the end of the 18-day flight failed to produce any increase in the content of acid-labile conjugate of aldosterone in the urine. However, these results do not disprove the American data since the studies were performed on the 3rd day after the landing. It is possible that the Americans used an improved method of double isotope labeling.

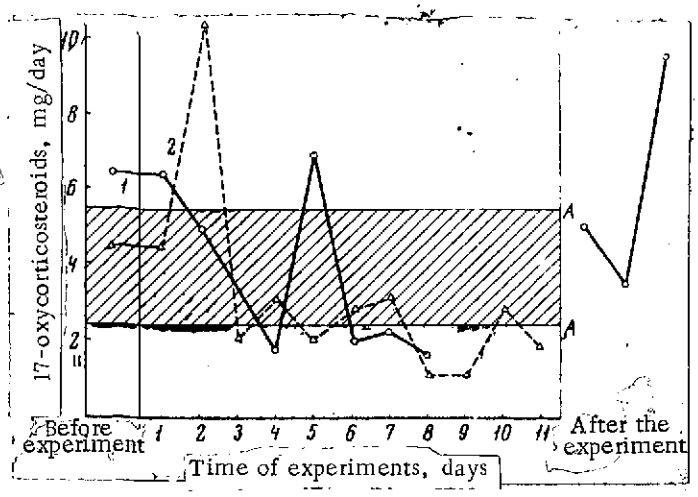

Figure 33. Excretion of 17-Oxycorticosteroids with the Urine in an Experiment Involving Water Immersion. Two observations (1 and 2). A, Ā - normal values (M ± Δ).

In simulation experiments with hypodynamia in bed (Table 45), 5 out of 6 subjects had developed an increase in aldosterone excretion by the end of the experiment. These studies, like the analyses of urine samples for the "Soyuz-9" spacecraft, were carried out by L. A. Vinogradov [53].

In clinical studies, aldosteronism is considered to be a symptom of cardiac decompensation. According to observations published by Karochkin, Matveyeva and Karoleva [79], in healthy human beings the excretion of aldosterone with the urine varies within limits of 6 to 12 micrograms per day with insufficiency of blood circulation to the IIA degree. On the average, it is 12 (variations from 8 to 18) micrograms per day, and with IIB degree insufficiency it is 21.5 (variations from 12 to 32) micrograms per day; with IIB degree insufficiency /175 it is 22 (variations from 16 to 30) micrograms per day. Similar results were obtained by all the other investigators who have dealt with the study of this problem. From the standpoint of such criteria, the participants in our experiments were seriously ill persons, but in fact a very careful clinical examination failed to reveal any kind of pathology in them! Evidently the content of the acid-labile conjugate of aldosterone in the urine is a function not only of the development of stagnation phenomena in the venous system and the need to economize sodium, but some other factors as well. Nevertheless, the tendency toward the development of aldosteronism, which appeared during the spaceflights and in the course of a long simulation experiment, constitutes a threat. It may have some relationship to the development of orthostatic disturbances following flight or prolonged strict bed rest.

An indirect indication of the activity of the aldosterone system in the organism may be the change in the content of electrolytes in the blood serum and urine. When its production increases, the potassium concentration in the serum/ /sodium ratio of the urine increases. Following several flights, minor changes actually did develop in the ionogram, but they were not regular. Therefore, on the whole the study of electrolytes does not support the theory that weightlessness causes activation of the mineralocorticoid function of the adrenals.

The increase in the excretion of catecholamines which the Americans detected /176 after flights was viewed by Berry as an indicator of the "stress reaction". In our observations, as after the flight of the "Gemini VII", there were no regular or reliable symptoms of an increase in the activity of the sympathetic-adrenalin

system. Excretion of vanilyl-amygdalic acid (see Table 42), the final product of catecholamine metabolism, did not increase after the flights.

According to our data of Goodall, McCall and Grivelin [211], submersion in warm water leads to a slight increase in excretion of adrenaline with the urine (from 0.65 ± 0.06 to 0.95 ± 0.13 micrograms per hour) and a decrease in noradrenaline (from 2.50 ± 0.13 to 1.13 ± 0.29 micrograms per hour). Although in this work the differences in noradrenaline were found to be statistically reliable, it is not clear whether they have any physiological meaning due to the considerable lability of this parameter in the norm. In the work by Daniell and Torfi [185] no regular differences were found between the excretion of these substances in an immersion experiment and in the controls.

Our knowledge of the nature of hormonal reactions during s stay in space by man could be divided into 2 parts. One of them would contain all that has been said concerning the influence of weightlessness itself, while the other would contain the nonspecific hormonal reactions of the type described by H. Selye as "stress." On the practical level, we are interested only in those facts that apply to the first group, inasmuch as they are required for determining the mechanisms that may possibly be responsible for the disruption of physiological functions during flight and immediately after its conclusion, during the readaptation period. Nonspecific hormonal reactions are less interesting in this regard; they attract attention rather as obstacles, from which they must be separated if possible.

If we view the facts presented above from this viewpoint, i.e., those concerning the functional status of the adrenal gland, its adrenalin, glucocorticoid and mineralocorticoid formations, we can exclude the nonspecific components in the reactions of the cortical substance — both glucocorticoids and mineralocorticoids. In fact, for all of the nonspecific reactions described thus far, there is a characteristic increase in the function of the glucocorticoids while during weightlessness or its simulation by submersion in water a decrease in the secretion of 17-oxycorticosteroids takes place. Generally speaking, such a decrease may be the result of exhaustion of the gland following prolonged stress. Such a possibility is excluded since no stage of increase in the function of the gland has not been found. Numerous studies performed in cosmonauts during preparations for flights, as well as directly during the launch, failed to turn up anything surprising in the regard. If such a reaction regularly took place at the beginning of a flight, its traces would be seen immediately after brief one-day flights in particular. In fact, after such flights, as after water immersion, 17-oxycorticosteroids are within ordinary limits or only /177 slightly increased. As we have already pointed out, it may be concluded that the reactivity of the adrenal cortex is slightly reduced after returning from space but this is not fatigue caused by previous hyperfunction.

Could aldosteronism be a symptom of nonspecific hormonal reactions? There is no clear answer to this question. We only know that the administration of large doses of ACTH stimulates the secretion of mineralocorticoids [162, 222]. It seems completely unlikely that aldosteronism would develop during the flight as a result of excretion of ACTH but that there would simultaneously be no increase in the excretion of 17-oxycorticosteroids.

The changes in the state of the sympathetic-adrenal system described by the Americans are more difficult to explain. Of course, we cannot exclude the possibility that the increase was the result of the action of nonspecific factors, but there is another viewpoint possible, based on an analogy with certain physiological situations.

TABLE 45. EXCRETION OF ACID-LABILE CONJUGATE ALDOSTERONE WITH URINE DURING A 30-DAY SIMULATION EXPERIMENT WITH BED REST (IN MICROGRAMS PER DAY)

Series	Before experiments	Before the experiment, days			After the experiment, days	
		10th	20th	30th	3rd	4th
4th						
B.	22,4	42	46	31	—	33
Nk.	19	27	24	35	—	17
Sh.	9	18	18	50	—	54
Average	17	29	29	39	—	34
5th						
Ne.	19	20	20	47	25	—
K.S.	22	27	21	29	9	—
S.	26	24	21	16	35	—
Average	22	24	21	31	23	—

Commas indicate decimal points.

The normal reaction of both man and animals to hypoxia is excretion of adrenaline and noradrenaline. Recently, R. V. Beleda and G. N. Kassil' showed that the ability of man to tolerate hypoxia corresponding to an altitude of 5 km above sea level is a function of the ability of his organism to secrete noradrenaline. If there is not an adequate increase in the excretion of this substance, a collaptoid state will develop during the stay in the rarefied atmosphere of the pressure chamber. A similar situation has been described by Ye. I. Chazov [146] in the case of an acute myocardial infarct: cardiogenic shock developed while in this case, i.e., a sharp decrease in blood pressure, excretion of noradrenaline with the urine was less than in those cases when the blood pressure was kept at a constant level.

It is possible that in both examples the mobilization of noradrenaline reserves by the organism is necessary to maintain the tone of the vessels. In hypoxia, the reason is obvious — local oxygen insufficiency acts as a stimulus that provokes a local increase in blood flow. When the entire organism is simultaneously exposed to hypoxic conditions and all the vessels are dilated, collapse may occur. The increase in noradrenaline secretion is intended to compensate for this vasodilatory reaction. Here the activation of the sympathetic-adrenal system is not simply one component of the "stress" reaction, but has a completely separate mechanism and significance.

Following the completion of a spaceflight, as after a prolonged stay in bed, there is a decrease in the orthostatic stability, i.e., the ability of the organism to maintain vascular tonus upon transition from a horizontal to a vertical position. This is usually explained by the fact that the venous system /178

is too dilated and so the blood can accumulate in it [41]. Without going into a discussion of the concrete mechanisms of the hemodynamic problems in orthostasis following flights, we can merely mention that the analogy between this situation and hypoxia or cardiogenic shock is striking, so that we cannot completely exclude the specific component in the adrenaline reaction following completion of a flight.

Currently we do not have sufficient data to solve the problem of the role of endocrine changes in postflight orthostatic disturbances, however the indirect indications are that these problems cannot be viewed separately. The first item is a regular reaction of aldosterone and renin to a change in body position. Do these play any role in protecting man from orthostatic collapse? Can aldosteronism which develops during flight or a simulation experiment involving bed rest affect these reactions in some way? The second is the ability of water immersion, like spaceflight, to cause a depression of the function of the adrenal cortex and to provoke orthostatic instability. But it is precisely hydrocortisone which is used to draw patients out of serious traumatic, postoperative or cardiogenic shock, i.e., those states in which a decrease in vascular tone is characteristic. It may be that both of these parallels are a simple random coincidence, but perhaps weightlessness, which disturbs the ordinary regular workings of hemodynamics, causes such a marked "regrouping" of the endocrine relationships that everything does not simply fall into place following return to Earth.

In summing up our knowledge of the functional status of the endocrine glands during spaceflight, we must point out their limitation. The most reliable information on the decrease in glucocorticoid function and the increase in mineralocorticoid activity were obtained during the flight of the American spacecraft "Gemini VII". Although they are supported by certain observations of ours, obtained during the spaceflights or simulation experiments, on the whole they require checking. There is still some doubt surrounding the role of the hormonal systems during the readaptation period.

Processes in Bone Tissue During Flight

In a paper delivered at the COSPAR symposium in Warsaw in 1962, the famous expert on bone biochemistry, the American scientist V. Newman [248], directed attention to possible problems in calcium metabolism during a prolonged stay in weightlessness. The dangers which he cited were based on the well-known fact that a decrease in the degree of mineralization of the bones takes place in man with advancing age, and there is a corresponding increase in brittleness. This process can be accelerated by decreasing the load on the supporting apparatus. Similar fears were expressed by the Soviet scientist P. A. Korzhuyev /179 in 1964 [75], in discussing the question on a wider evolutionary basis.

Later, the problem of mineral metabolism in the bones attracted the interest of journalists who discussed it in the popular press but in many instances had not had the opportunity to study the problem in sufficiently deep detail and to give it the correct scientific emphasis. In our laboratory, this problem has not been studied experimentally, so that many of the general ideas led to the conclusion that under the conditions of the actual durations of flights by

cosmonauts there was no threat of demineralization of the bones, so that this section, in contrast to the other parts of the present book, was not written on the basis of our own experimental research but exclusively on the basis of the data in the literature. Therefore, what were the actual facts that provided a basis for these fears, and how justified were they?

In order to answer this question, we must deal briefly with the physiology of bone tissue and the methods we have available for studying its functional state. Most of the mineral substance of the bone consists of microscopic crystals which resemble oxyapatite in structure and which have approximately the following chemical composition:

$$[Ca_9^{++}(H_3O^+)_2(PO_4^{\equiv})_6(OH^-)_2][Ca^{++}Mg_{0,3}^{++}Na_{0,3}^{+}CO^{=}\ (citrate)]_{0,3}^{\equiv}].$$

The formation of these crystals occurs only on a matrix of protein substance that is rich in collagen; therefore, the processes oxyproline metabolism may have a direct relationship to the synthesis of bone tissue [270].

In children, whose bones are growing, polypetides which contain oxyproline are excreted with the urine; after bone development is complete, their content drops off sharply. On the other hand, in the case of acromegaly, when incorrect growth of bones occurs in adults, oxyproline again appears in the urine.

The basic functional unit in bone is the Haversian system or osteon, which is composed of several rows of bone lamellae, arranged in the form of concentric circles around a blood vessel. During the entire human lifetime, old osteons are being destroyed and new ones formed, thus enabling the bone to change its structure depending on the load. However, this change takes place slowly, sometimes even requiring years.

The derivation of the activities of the ions of phosphorus and calcium in the blood in an adult human being is about 0.89×10^{-7}, in other words close to their derivative of solubility. This means that the fluids in the organism constitute a solution which is practically saturated with the calcium salt of phosphoric acid. Inasmuch as the extent of the Haversian canals is very great, the surface at which the bone system is in contact with the fluids of the organism can be measured in hundreds of thousands of square meters. This makes /180 possible a rapid transfer of the salts from the tissues to the blood and vice versa.

In the organism of an adult human being there is about 1 kg of calcium, of which about 99% is bone. Most of it is metabolically inert, and this is because during an interval of time which is measured in days only a few grams of calcium from the organism can get into equilibrium with a calcium isotope introduced into the organism, or strontium, which is similar to it in chemical structure. According to the data of Lichwitz, V. V. Parlie [235], in 24 hours this value is about 6.3 ± 0.2 grams, while other sources claim that it is less [276]. With increased ossification, as in the breakdown of bone — osteolysis, these amounts increase, reaching 15 to 30 grams, so that the breakdown of the bones and their increased synthesis promotes the acceleration of metabolic

processes, but in any case the massive bone tissue, which is inert in a metabolic sense, is many times more physiologically active.

The crystals of oxyapatite have a basic nature, so that their absorption is associated with the alkalinization of the fluids in the organism, while osteosynthesis is accompanied by acidification. Hence, the bones participate in the maintenance of acid-base balance and vice-versa: any factors which influence this equilibrium affect the excretion of calcium from the organism.

Calcium is taken in with the food, and is not only absorbed in the intestine but also excreted there, so that most of this substance is lost from the organism with the feces (approximately 4/5). Regardless of this fact, the changes in calciuria have a definite diagnostic significance, since in the norm they are relatively constant: the average value is about 200 mg/day (10 meq/ /day), and the variation from one day to the next usually does not exceed 40 mg (2 meq).

According to Lichwitz and Parlie [235], bone diseases can be roughly divided into three groups:

- osteoporosis, in which the pathological process occupies the protein matrix; this disease is characterized by a decrease in the total mass of the bone lamellae;

- ostemalation when the protein matrix is almost undamaged but the content of mineral substance is reduced;

- osteolysis, which is characterized by a violent breakdown of bone tissue.

The organism is threatened not only by demineralization of bone but also the reverse process: excess deposition of salts which can lead to the formation of kidney stones, calcification of the tendons and disruption of mobility in the joints. As a rule, calcification is a symptom of disease, although sometimes (for example, in tuberculosis) the petrification focus serves as a symptom of a favorable culmination of the pathological process. As it applies to the conditions of spaceflight, there has been frequent mention of the danger that /181 loss of calcium from the tissues of the skeleton leads to excessive deposition in other organs.

Although there is no doubt that the bone system actually undergoes a change of structure during the entire lifetime of an organism, the time required for such important changes in mineral saturation to occur are measured in time intervals that are much greater than the anticipated duration of spaceflights. This problem was dealt with in great detail by roentgenologists, particularly A. I. Kurachenkov [86], who studied skeletal changes in athletes. According to his data, even children, in whom all processes are more mobile, hypertrophy of the bone apparatus occurs only after several years of systematic training. All athletes rapidly lose their form if they do not train constantly and the cardiovascular system and muscles can no longer work at maximum loads. However, the increased density of bone is retained for many years [86].

This is in agreement with the observations of clinicians [235] that in women, following the onset of menopause, osteoporosis does not develop immediately but only after several years, i.e., a considerable time interval is required for the endocrine changes to have a real effect on the strength of the bones.

In many observations, prolonged limitation of mobility has led to negative calcium balance and an increase in the Cu content in the urine. Such observations were published by Widen [285], who, in 4 healthy persons subjected to 6-7 weeks of immbolization, observed a total loss of up to 14 grams of calcium for the entire period of the experiment. The most detailed experiments were performed by Lynch and Johnson et al. [238] who provide data on the total loss of calcium for a 4-week limitation of mobility in subjects with various composition of a gas medium:

Gaseous medium	Number of subjects	Average values		Limits of variation	
		g	eq	g	eq
Terrestrial	22	3.1	0.155	1.47-5.24	0.073-0.262
Corresponding to an altitude of 10,000 feet (3,050 meters)	19	2.2	0.11	0.85-3.92	0.042-0.186
Corresponding to an altitude of 12,000 feet (3,650 meters)	9	0.96	0.046	0.15-1.69	0.007-0.085

In other studies, particularly those of M. S. Seregin, I. G. Popov et al. [134], in the case of 100 days of hypodynamia, it was not possible to produce a pronounced negative calcium balance although the excretion of Cu during the experimental period was higher than in the controls.

We know that even with the most serious damage to the bones, for example in osteomalation caused by malignant tumors, daily calciuria reaches 1 gram. /182
The kidneys are simply unable to excrete a larger amount [235].

Hence, none of the model experiments succeeded in showing any calcium losses that were commensurate with the total content in the organism, which in an adult man is about 1 kg. According to calculations presented in a paper by S. A. Gozulov and N. I. Frolov [45], a regular decrease in bone strength can occur only with a decrease in the mineral saturation by 10%, i.e., if the total negative balance is about 100 grams. This is almost 10 times more than the values which were observed in the model experiments. Excretion of calcium with the urine was studied during the flights of the cosmonauts aboard the "Soyuz-9" and "Gemini-VII" spacecraft. The results are shown in Tables 11 and 12.

As we can see from this data, A.G.N. showed a marked increase in calcium excretion only once — on the 18th day of the flight. In one of the crew members

of "Gemini VII" the calcium losses from the organism during the flight corresponded precisely to the intake of the substance into the organism, while in another the balance was even positive, i.e., there was an accumulation of calcium in the organism during the flight. Since most of this substance is excreted from the organism with the feces, during the flights in the Apollo program Brodzinski, Rancitelli, Haller and Dewey [171] studied the calcium content in the feces. According to these data, the authors were able to perform some rough calculations and determine the state of balance, assuming that the excretion via the intestine accounted for 80% of the total losses. The following are the results of these studies:

Craft	Astronaut	Intake with the diet during the entire flight, mg	Excretion with the feces during the entire flight, mg	Balance, mg
Apollo 7	Average for 3 men	836	1,140	-590
Apollo 8	G.D.R.	427.2	1,150	-1010
Apollo 9	G.D.R.	562.5	1,190	-930
Apollo 9	L.M.P.	494.3	1,100	-880
Apollo 9	C.M.P.	489.0	2,260	-2340
Apollo 10	Average	832.9	730	-80
Apollo 11	Average	1000.3	1,090	-360
Average		767.7	1,120	-635

As we can see from these data, although the results of the study of balance in all flights gave negative values, they were small — in one case the organism lost about 2 grams after 14 days — an amount which is negligible in comparison with the total amount. These results have led the authors to conclude that demineralization of bones does not constitute a realistic threat to the health of cosmonauts.

All of the above indicates that we can forget completely about the danger /183 that increased excretion of salts from the organism during a flight lasting several weeks or even 2 to 3 months would have any kind of an effect on the mechanical strength of the bones. However, at least theoretically, one can imagine that there could be a transfer of salts from one bone to another without the total amount of calcium in the organism changing. This idea came up because in many studies that were performed using the x-ray method it was possible to detect a change (reduction) in the density of the shadows of individual bones. These studies were mainly performed in the USA by Mack et al. [239, 240], and in the Soviet Union by I. G. Krasnykh [88]. The essence of the method consists in the fact that some part of the skeleton is recorded once on x-ray film, usually the calcaneus or one of the bones of the phalanges of the fingers. At the same time, the shadow from a metal wedge is recorded on the film. The density of both is measured with the photometer and the mineral saturation of the bone tissue is expressed conditionally in units of thickness of the metal wedge. In publishing the results of such measurements, the authors for some reason preferred to express their data in relative rather than absolute units, i.e., the density of the shadow in a given subject changed

in a certain way relative to certain original values. This method made it possible to find a decrease in the bone density in the majority of the stand tests, as well as in several spaceflights. On the basis of the data that were obtained, Stabbs even constructed a theory which holds that a change in the load leads to the development of a piezoelectric effect which in turn causes absorption of the mineral substance [273].

The method of x-ray densitometry did not generate considerable faith from the very beginning, since an inhomogeneous medium was being subjected to photometry and certain parts allowed more radiation to pass through than others. During measurement, these differences were averaged out without taking into account the fact that according to the rules of photometry it is necessary to average not the values themselves but the extinctions, i.e., the logarithm of the amount of light absorbed. The error associated with these differences is difficult to eliminate and it makes the method very approximate.
Several years ago the method of x-ray photometry of bones was subjected to a very extensive and convincing criticism by Cameron, Jurst, Sorenson and Maresa [175]. These authors feel that the error can reach 20 to 30% due to the fact that a non-chromatic x-ray beam is used and also because it is very difficult to obtain good reproducibility and proportionality between the degree of blackening of the film and the intensity of the radiation. From their point of view, much better results are provided by the method of photon absorption, when the absorption of gamma radiation from some radioactive preparation is measured by means of an ionization chamber. /184

Hence, it is theoretically impossible to imagine that the organism or cosmonaut would lose an amount of calcium in several months that would really have an effect on the strength of the bony skeleton, and the metabolic studies that have been conducted in simulation experiments and during flights have not revealed losses that were anywhere near large enough as far as the amount of this element was concerned to have a biological effect. At the same time, x-ray study methods, which have yielded results that justify consideration of the demineralization of bones, are not very reliable from the methodological standpoint. All of this indicates that the problem of demineralization of bones as a result of the action of weightlessness is not a primary problem in the practical sense at the present stage as far as limiting the length of stay in man in space is concerned although theoretically this problem cannot be completely forgotten.

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PART III

THE MECHANISM OF WATER ABSORPTION IN CERTAIN OSMOREGULATORY ORGANS

Constancy of the concentration of osmotically active substances in the /195 fluids of the internal environment of vertebrate animals is ensured by the osmoregulatory system. This system makes it possible to regulate the separate and independent excretion from the organism of both water and salts. When there is excess water in the organism, the walls of the kidney tubules become impermeable to water and the latter is excreted. On the other hand, when there is a shortage of water the permeability of the walls of the distal tubules with respect to water increases and resorption increases [1, 31]. The same ability to change permeability to water is possessed by the skin and urinary bladder of frogs and toads [5, 8, 16, 19, 20]. Due to the relative simplicity of the structures involved, many studies have been performed on these biological objects, devoted to researching the mechanism of water and sodium salt transport.

During the last 15 years there has been intensive concern with the problem of the movement of water through various membranes. We can now consider it firmly established that the movement of water in the osmoregulatory organs, both in the presence of antidiuretic hormone (ADH) and without it, takes place only passively with the osmotic gradient [21, 30]. It has been found that the diffusion of water labelled with deuterium or tritium through the wall of the urinary bladder under the influence of ADH is increased 1.5-2 times, while the flow of water with the osmotic gradient can increase by 100 fold or more [16]. The lack of correspondence between isotopic and osmotic permeability was attributed by Ussing [19] to the assumption that ADH increases the diameter of the limited number of pores through which the water loss occurs.

There are two hypotheses as to where these pores are located. One of them was suggested by Ussing [19] and is developed in the works of Leak et al. [20, 21]. According to this viewpoint, ADH increases the permeability of the apical membrane of the cell, so that the flow of water that occurs in the presence of ADH passes through the bodies of the cells. Facts that have been accumulated /196 in recent years concerning the differences between the transport pathways of water and sodium under conditions of action of ADH have led to an elaboration of this hypothesis, whose authors suggest that there is a double row of barriers in the apical membrane of the cells, one of which controls permeability with respect to ions and the other with respect to water [23].

An alternative hypothesis was suggested in 1958 by A. G. Ginetsinskiy [2]. In his opinion, ADH stimulates the secretion of hyaluronidase by the cells of the distal portions of the kidney tubules, increasing the permeability of the acid mucopolysaccharides of the intercellular substance and facilitating the movement of water with the osmotic gradient [1]. This hypothesis was based on two groups of facts: excretion of hyaluronidase with the urine increased

following administration of ADH, and depolymerization of the hyaluronic structures occurred in the interstitial tissue of the internal cortical layer of the kidney. Recently, new facts have been learned which support this viewpoint; beneath the electron microscope it has been possible to observe a sharp increase in the intercellular distances under the influence of ADH [4, 10] and it has also been found that water transport under the influence of the osmotic pressure differential is increased when hyaluronidase is added [3].

Without entering into the very active dispute concerning the location of the pores, which developed in the 1960's [1, 7, 20], it must be emphasized that there is no unanimity of opinion concerning the question of the pathways for water transport at the present time. Likewise, the physical mechanism which forms the basis of this phenomenon is still insufficiently clear, although in recent years a theory of this phenomenon has been developed on the basis of the principles of the thermodynamics of irreversible processes [11, 12].

The goal of the present work consisted in examining the molecular mechanisms for the movement of water through tissue membranes and studying (in experiments on the urinary bladder of frogs) the ratio between isotopic and osmotic permeability. In experiments whose results have been discussed previously, the ratio between the movements of D_2O, ThO and H_2O was determined. However, the differences in isotopic and osmotic permeability could be explained by the different rates of movement of the hydrogen ions and the water molecules. Therefore, in the present paper, for an experimental analysis of this problem, we measured the movements of D_2O and H_2O^{18} through the membrane of the urinary bladder of the frog.

Theory of Osmotic Fluid Transport Through a Semipermeable Membrane

Let us consider an ideal fluid whose molecules (we will call them the molecules of the solvent) are elastic, practically incompressible spheres which are constantly in motion. In order for these spheres to be able to move, there must be free space between them which we shall call "the cavity."[1] /197
The total volume of all the cavities in a unit volume of fluid is dependent only on the hydrostatic pressure — when it increases, the volume of the fluid decreases exclusively because of the decrease in the volume of the cavities. The coefficient of compression of the fluid (β) is that value by which its volume decreases when the pressure increases by 1 atmosphere. Among the molecules of the solvent there may be molecules of dissolved substance which also are in the form of elastic spheres. Unlike the molecules of the solvent, the molecules of the dissolved substance cannot pass through a certain plane which we shall call the ideal semipermeable membrane. We will likewise assume that the membrane is ideally thin.

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[1] Instead of the word "cavity," other terms can be used such as "slit" or "hole."

In the fluid, each molecule, regardless of whether it is a molecule of the solvent or of the dissolved substance, can move only if there is a free space or a cavity next to it. Of course, the volume and shape of each cavity will not correspond to the volume and shape of the molecule; it will be a broad spectrum of cavities whose nature we do not know. Any movement of the molecule is equivalent to a like movement of the cavity in the opposite direction. Therefore, the total path traveled by all of the cavities is equal to the total paths traveled by all the molecules in the same time. If we assume that the coefficient of diffusion of the cavities (Δ) is proportional to the total distance traveled by all the cavities, and accordingly that the coefficient of diffusion of the molecules (D) is proportional to the total distance traveled by all the molecules, then

$$D(1 - A) = \Delta \cdot A,$$

where A is the fraction of the total volume which is occupied by the cavities and 1 - A is the fraction which is occupied by the molecules. This means that the cavities, which occupy a comparatively small part of the total volume, will move around rapidly, and we cannot exclude the possibility that the molecules will move around in fixed associations or complexes which will move as a whole, so that each individual molecule will move a short distance and the mutual positions of the molecules will be only slightly changed. If we assume that more cavities pass through some imaginary plane in the fluid in one direction than in the other, we will see that the fluid moves as a whole and the mutual positions of the molecules will be insignificantly disturbed.

The total volume of the cavities can be evaluated on the basis of the following considerations. We will imagine, as we did in Figure 34, that the solution is located on the right hand side of a semipermeable membrane and a /198 pure solvent is located on the left side. Then, if there is a cavity located to the right of the membrane, a molecule of solvent which is located opposite this cavity, on the opposite side of the membrane, will have the same probability of occupying the position of the cavity as any other molecule located next to it. A different situation exists if the cavity is located next to the membrane but on the left side, i.e., on the same side as the solvent. This cavity can move through the membrane only if there is a molecule of solvent opposite it. However, if a molecule of the dissolved substance is located there, which under these conditions cannot pass through the membrane, the cavity will not be able to pass through the membrane either. In this case, we will say that the cavity is reflected from the membrane.

It is natural to assume that the movement of the cavities through the membrane is proportional to the fraction which they occupy near the surface of the membrane, i.e., the value A. Therefore the movement of cavities (i.e., the amount which pass through a unit surface of the membrane in a unit time) will be represented by kA, where k is the coefficient of proportionality. The movement of the cavities in the reverse direction, i.e., from the solvent into the solution, will be somewhat less than kA $(1 - \alpha)$, since some of the cavities are reflected from the molecules of the dissolved substance. Here α is the molar fraction of molecules of dissolved substance. Therefore, we see that

more cavities move from the solution into the solvent than in the opposite direction. This is equivalent to the fact that the fluid crosses over from the solvent into the solution, and a process takes place which we shall call osmosis. In order to stop osmosis, it is necessary to decrease the total volume of the cavities in the solution, so that the flows through the membrane /199 in both directions become equal. This can be done if we compress the solution, producing a hydrostatic pressure P in it. Then the volume of the cavities will decrease by a value of βP where β is the coefficient of compression of the fluid and the level of the movement of cavities from the solution into the solvent will be k(A - βP). If the level of the hydrostatic pressure is equal to the osmotic, the fluid will not be able to pass through the membrane. In this case, the flows will be as follows:

$$(1-\alpha)kA = k(A-\beta P) \quad \text{and} \quad A = \frac{\beta P}{\alpha}.$$

From this equation we can calculate the total volume of the cavities A. For an isoosmotic solution of blood, we will assume that P equals 8 atm,

$$\beta = 50 \cdot 10^{-6}; \quad \alpha = 1/166.$$ Hence, A = 6.6%.

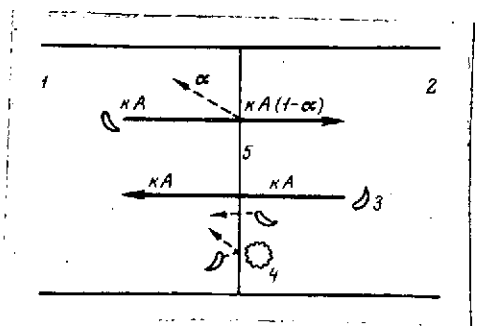

Figure 34. Diagram Explaining the Movement of Cavities Through a Semipermeable Membrane. 1, Solvent; 2, Solution; 3, Cavities; 4, Molecules of Dissolved Substance; 5, Semipermeable Membrane.

In order to calculate the level of the osmotic flow of the fluid through an ideal semipermeable membrane, we must know the value of k. This can be done if we proceed on the basis of the following considerations. Let us see what happens when the pressure is distributed in a fluid that fills a long rigid tube. For the sake of simplicity we shall assume that the cross-section of this tube is equal to unity. We will measure the pressure relative to that part of the fluid in the tube where it has not yet been increased. In this case, the entire volume of the tube can be divided into 2 parts, in one of which the pressure is 0 and in the other P_1 (see Figure 35). The boundary between these sections moves in the direction of the region with the low pressure at the speed of sound (v), and the unit of time is the volume in which the pressure P_1 is increased by v. (We will assume that the cross-section is equal to unity). In order to accomplish this, a volume equal to $\beta P_1 v$ must exist in the tube.

Now let us look at this from the standpoint of the previously developed concepts regarding the diffusion of cavities. The movement of cavities from the low pressure region to the high pressure region is Ak, the movement of

cavities in the reverse direction is $(A - \beta P_1)k$, and the difference in the movements is P_1k. This means that a volume of cavities equal to P_1k moves in a unit time from the low pressure region to the high pressure region, or, what amounts to the same thing, an equal volume of molecules moves in the opposite direction. If we make these two values equal to each other, we will obtain

$$\beta P_1 k = \beta P_1 v; \qquad k = v.$$

Now it is possible to calculate the osmotic flux. It is equal to the difference between the movements of the cavities from the solution into the solvent and from the solvent into the solution $Ak - Ak(1 - \alpha) = \alpha Ak$. Substituting the values found for k and A, we will have /200

$$\alpha Ak = \alpha \frac{\beta P_1}{\alpha} v = \beta P_1 v.$$

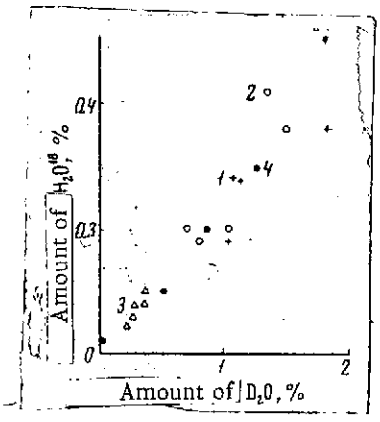

Figure 35. Motion of the Boundary Between Areas with Different Pressure Levels in a Rigid Tube. 1, Direction in which the interface between the particles with different pressures moves; 2, Direction of movement of the fluid.

Substituting known values for osmotic pressure, speed of sound in a fluid and the coefficient of its compression, we will find that the value of the osmotic transport of the fluid with a difference in osmotic concentrations of 0.3 osmols/liter (corresponding to an osmotic pressure of 8 atm) will be

$$5 \cdot 10^{-6} \cdot 8 \cdot 1 \cdot 10^{5} = 40 \text{ см}^3/\text{sec} \cdot \text{см}^2.$$

Even if the volume of those areas through which the water diffusion takes place is about 1% of the total surface of the tissue membrane [16], the flow of water as calculated for 1 cm^2 of surface of urinary bladder of the frog will be 0.4 ml per second or more, i.e., much greater than the values which are actually observed. We must keep in mind that the value used here refers to an ideal semi-permeable membrane, which does not interact with the molecules of water and therefore has no resistance to their motion. Real molecules, however, unavoidably display this resistance. Moreover, we have assumed that the concentration of osmotically active substance in the entire solution is the same, which may not be the case, since the movement through the membrane must necessarily reduce it.

Study of the Permeability of the Urinary Bladder of the Frog to Water

Materials and Methods

The object of this study was the isolated urinary bladders of winter frogs, *Rana temporaria*. The hollow space in the urinary bladder was filled with hypotonic solution (Ringer's), whose osmotic concentration was about 10 times less than that of the isoosmotic blood in these animals. This solution contained in addition about 15% D_2O and 1.8% H_2O^{18}. These bladders were incubated for 30 minutes in 5 ml of aerated normal Ringer's solution for cold-blooded animals. Then the bladders were transferred to a new batch of the same solution, to which 50 med of pituitrin P had been added, and left there for another 30 minutes. After the conclusion of the experiment, the fluid that remained in the bladder was added to 5 ml of Ringer's solution. Before the experiment, after changing the solution, and at the end of the experiment, we weighed the bladders. The osmotic permeability was measured on the basis of the weight loss while the isotopic permeability was determined on the basis of the amount of isotopes that had been excreted. The method of determining the osmotic permeability of the urinary bladder in the frog has been described in detail previously [6].

To measure the deuterium in a hermetically sealed vessel, 0.3 ml of the solution to be investigated was added to 1-2 g of calcium carbide. The partially deuterized acetylene given off was transferred to a gas cell with an optical path length of 100 mm and windows of sodium chloride. The infrared spectra of absorption were measured in the 2500-2800 cm^{-1} range using a Zeiss UR-10 instrument. In this range, the deuterized acetylene shows 2 series of absorption bands which appear on the spectral curve as 2 broad peaks with maximum absorption at 2600 cm^{-1}. There is also a sharp peak at 2670 cm^{-1} which is characteristic of hydrogeneous acetylene. The value of the ratio is proportional to the content of deuterium in the water: /201

$$\frac{E_{2600} - E_{2500}}{E_{2670} - E_{2500}} .$$

The calculation was performed using a calibration curve which must be plotted for each series of determinations, since the absorption peaks of the gases are very sharp and therefore small changes in the nature of the functioning of the apparatus have a considerable influence on their size.

The H_2O^{18} content was determined by means of mass spectroscopy. For this purpose, 1% sodium bicarbonate and 1% human blood were added to a sample of the solution to be tested (the blood acted as a source of carboanhydrase). The tests were stored at room temperature for at least a week. During this time, isotopic equilibrium was established between the oxygen in the HCO^-_3 ion and the water. If there was no carboanhydrase in the solution or it was inactivated (this can be determined on the basis of the change in color of the hemoglobin,

indicating its denaturing), equilibrium was not established in this period of time. For analysis on a mass spectrometer, carbon dioxide gas was extracted from 0.1 ml of the sample, adding a drop of hydrochloric acid. Sulfuric acid is unsuitable for this purpose since the $S^{30}O^{16}$ ion has the same mass as $CO^{16}O^{18}$ and disturbs the determination. The water present in the sample is frozen out with dichloromethane, cooled with liquid nitrogen to the freezing point. The analyses were carried out on the MI-1305 mass spectrometer, and the O^{18} content was determined by the ratio of the peaks of masses 46 and 44.

For electron microscopic examination, pieces of tissue from the urinary bladder were fixed at 0° in 25 minutes using a 2% solution of OsO_4, dehydrated, and cast in methacrylate or araldite using the normal methods. Ultrafine sections were studied by means of the GEM-5 electron microscope at 80 kV. This part of the work was carried out jointly with an associate at the USSR Academy of Sciences Institute of Cytology, L. N. Vinnichenko, to whom the authors would like to express their deep appreciation.

Results of the Experiments

In 1952, Everr [3] pointed out that the permeability of the walls of the urinary bladder of the toad, *Bufo marinus*, with respect to water increases sharply under the influence of ADH, so that this tissue can serve as a model for studying the processes of facultative reabsorption of water in the kidneys [1, 8, 20]. It was later established that the same properties are possessed /202 by the urinary bladders of other amphibians, particularly the frog, *Rana temporaria* [5]. Evidently in these animals the urinary bladder serves as a special water reservoir in which it is stored when there is an excess of it and from which it can be absorbed when needed.

Permeability of the tissue membrane with respect to water is studied in two ways. The first is called the isotopic method and consists in a certain amount of water labelled with its isotopes (deuterium, tritium or O^{18}) being added to a solution on one side of the membrane and measurements being performed to determine the rate at which this isotope appears on the other side of the membrane. The second method is called the osmotic method and consists in a solution of osmotically active substance being placed on one side of the membrane and not passing through the membrane, while on the other side is pure water or a less concentrated solution of the same substance. In this case, the water will pass from one solution into the other under the influence of the difference in osmotic concentrations. For example, if we determine the figure by weighing one of them, we can get an idea of the amount of water that has passed through the membrane.

Even very early work showed that permeability of frog skin and urinary bladder to water measured by the osmotic method changes sharply under the influence of pituitary hormones while when it is measured by the isotopic method it shows very little dependence on them. This problem was carefully studied by Ussing, Coford-Johnson [19] and Leaf [20]. According to the data

on isotopic exchange, in experiments with D_2O and ThO in the urinary bladder of the toad, unidirectional flow of water increased under the influence of ADH from 338 to 543 microliters per cm^2 per hour, while the transport of water with the osmotic gradient at 170 ml per cm^2 per liter increased from 4 to 209 microliters per cm^2 per hour [22]. The osmotic movement of water under the conditions of action of ADH was a function of the steepness of the osmotic gradient [22]. Hence, in the absence of ADH, regardless of the high diffusion permeability and the intensive isotopic exchange through the wall of the urinary bladder of the toad, the flow of water with the osmotic gradient is very small: ADH sharply increases this osmotic flow of water.

The difference between osmotic and isotopic permeabilities becomes clear in the case of synthetic 2-layer lipid membranes. Thus, according to the data of Thompson and Wang, for an artificial film of phosphatidylcholine (H-21), the isotopic permeability was 4.4 and the osmotic permeability was 17.3 microliters per second [32]. According to the data of these researchers, the osmotic permeability is always higher than the isotopic, but this difference in the preparations of the membranes from different sources is not the same.

One of the most likely explanations for this difference consists in the fact that the hydrogen ions and the isotopes of this substance are transported through the membrane according to laws other than those obeyed by a complete molecules of water. To study this possibility, we made an attempt to measure /203 the value of the permeability to water of a membrane from the urinary bladder of a frog, using water that was labelled with 2 isotopes — deuterium and O^{18}.

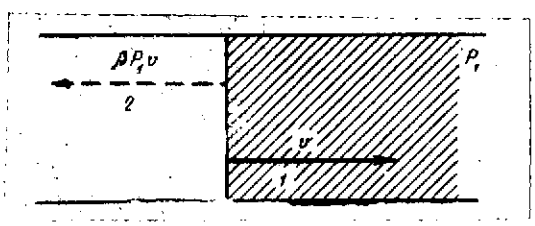

Figure 36. Relationship Between the Amounts of Water Labelled with Deuterium or the Oxygen Isotope, Excreted from the Urinary Bladder of a Frog. On the axes of the coordinates we have plotted the content of D_2O or H_2O^{18} in an external solution whose amount was the same in all the experiments: 1, Without the hormone; 2, In the presence of ADH; 3, Remaining in the urinary bladder (total volume added, as much as 5.5-6.0 ml); 4, Different dilutions of the original solution.

Results of the experiments are shown in Table 46 and Figure 36.

As we can see from the figure, the ratio between concentrations D_2O and H_2O^{18} in the papers studied showed little difference from their ratios in the tests of the solution which was administered inside the bladder. This indicates that the water, labelled with either hydrogen or oxygen, passed through the wall of the bladder at the same rate (within the limits of accuracy of the experiment involved). The only exception is experiment 3 in which /204 relatively more deuterium oxide passed through during the first 30 minutes and later, more water labelled with oxygen. However, these differences can in no way explain the different results which were obtained when measuring permeability by the isotopic and osmotic methods.

TABLE 46. QUANTITIES (IN MG) OF H_2O, D_2O AND H_2O^{18} EXCRETED FROM THE URINARY BLADDER OF A FROG IN THE PRESENCE OF ADH (a) AND WITHOUT IT (b).

Substance Investigated	1		2		3		4		5	
	a	b	a	b	a	b	a	b	a	b
H_2O	8	257	22	441	3	80	1	139	14	586
D_2O	53,5	40	90	66	51	35	55	51	90	74
H_2O^{18}	6,5	4	8,5	10	4	4,5	6,5	4,5	12,0	8,5
	Excreted in 30 minutes (in moles calculated on the basis of $1 \cdot 10^{-4}$ M of initial concentration):									
H_2O *	4,0	128	11	220	1,5	40	0,5	70	7	293
D_2O	4,9	7,4	7,1	10,4	3,7	5,0	4,4	7,4	7,1	11
H_2O^{18}	5,4	7,6	5,8	11,2	2,9	5,4	5,4	7,5	7,8	12,3
Original content of water in the bladder, mg	982		1417		752		1050		1414	

Commas indicate decimal points.

Note: A solution containing approximately 15% D_2O and 1.8% H_2O^{18} and salts at a concentration about 10% less than Ringer's saline solution for cold-blooded vertebrates was placed inside the urinary bladder. Outside there was ordinary Ringer's solution for cold-blooded animals, whose concentration was about 0.2 osmol.

* The number of moles of excreted H_2O calculated on the basis of $1 \cdot 10^{-4}$ osmols of Ringer's solution.

These differences are very clear from the data in the tables. If we calculate the amount of water excreted from the bladder, D_2O and H_2O^{18} in 30 minutes of the experiment as calculated per 1 mole of the original difference in concentration, we will find that in the absence of the hormone these values will be very similar for all 3 substances. When we calculate the transport of water we assume that the difference between its concentrations on the two sides of the wall of the bladder is equal to the concentration of salts in Ringer's solution for cold-blooded animals — 0.22 osmol. When the bladders are immersed in a solution that contains pituitrin, the osmotic permeability increases sharply, while the isotopic permeability shows almost no change. The figures listed in this table may only roughly characterize the values of permeability, since the isotopes accumulate in the external solution during the experiment and the concentration gradient is reduced. In order to exclude this error, we had to calculate the value of the coefficient of diffusion accordingly, calculating the actual difference in concentrations for each moment in time. This calculation is easily performed for the first half of the experiment, during which the volume of the bladder remains practically constant. However, during

this period of time, the concentrations of the isotopes within the bladder was so high that the correction was insignificant. During the second half of the experiment, this calculation is practically impossible to perform, since 2 processes are going on simultaneously — isotopic transfer of labelled water and the participation of these isotopes in the general movement of the osmotic transfer. Therefore, we do not know how uniformly the osmotic transfer took place at that time, since practically speaking there is no possibility of any kind of practical correction that can be made for back diffusion. From these considerations, it is better to operate directly with experimental data, especially since they are quite obvious.

The experimental data presented above completely support the results of work carried out previously using only hydrogen isotopes. The differences in the values of osmotic and isotopic water transport in tissues treated with ADH cannot be explained by the characteristics of transport of any particular water isotope.

Mechanism of Water Transport Through the Wall of the Urinary Bladder

The experimental data which have been obtained are easily explained if we assume that special water-transport channels open up in the walls of the urinary bladder under the influence of ADH, that such channels are filled with /205 water, and that they separate the cavity of the bladder from the interstitial or intracellular fluid. These channels have the form of tubules, and both ends of each tubule are permeable to water and impermeable to salts. The number of cavities which emerge from such a tubule in a unit time through each of its ends is a function of the osmotic concentration of the surrounding solution. The higher this concentration, the fewer cavities will emerge, because some will be reflected from the molecules of the dissolved substance. Since the number of cavities that enter the channels is always maximum (we will assume that there is pure water in them, so that each cavity from the surrounding solution can be replaced by a molecule of water from the channel), we will find that fewer cavities emerge from the channels than enter them. As a result, the concentration of the cavities in the channels will increase, i.e., the pressure in them will become negative. It will decrease to the point where the concentration of the cavities will be so high that the numbers of cavities that pass through the membrane in both directions will be the same. If the osmotic concentrations in the solutions wetting both ends of the tubes are not the same, a pressure will be established in the tubes (i.e., a concentration of cavities) such that the cavities will enter through one end of a tube and leave at the other. This will mean that the water will pass from one solution into the other through the channels. The rate of this transfer is determined exclusively by processes that occur in the two semipermeable membranes at the ends of the channels. As we have shown in the theoretical section of this paper, the rates of these processes may be very high. Inside the tube, however, the water moves in a continuous flow, i.e., not according to the laws of diffusion but according to the laws of hydrodynamics. If the water-carrying channel was filled not with pure water but with a saline solution, it could not carry out its function, since the salts, influenced by the overflow of liquid, would accumulate at one end, eliminating the difference in osmotic concentrations. The existence of

these channels has no influence on the ability of each molecule of water to diffuse. Each labelled molecule must make its way among the other molecules according to the same laws in the presence of ADH and without it. This explains why the osmotic permeability changes under the influence of ADH so significantly, and the isotopic permeability shows almost no change. Thus, we can assume that water passes through the wall of the bladder through special channels that are highly organized structures.

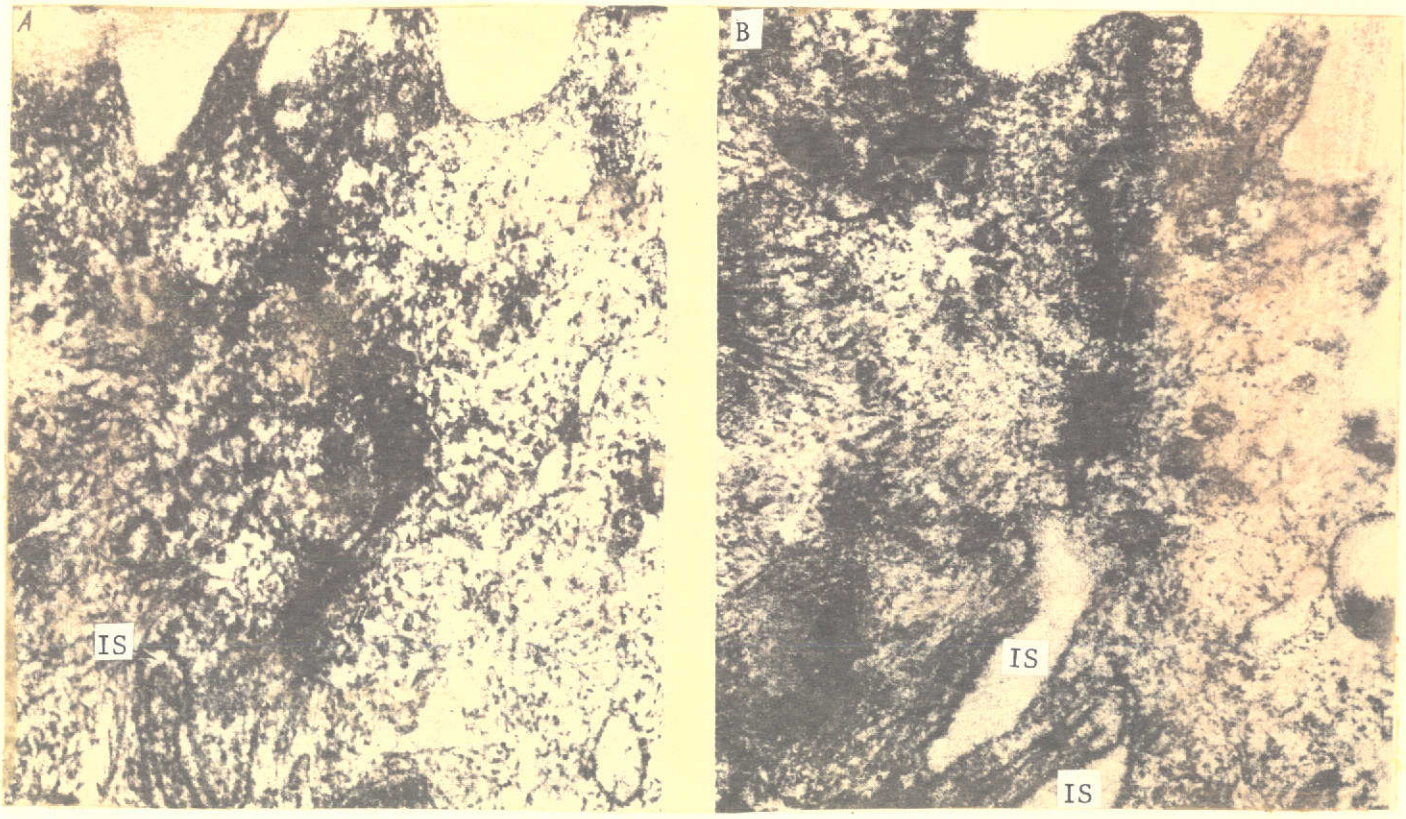

Figure 37. Electron Photomicrographs of the Wall of the Urinary Bladder of the Frog. A, In the absence of the hormone; B, under the influence of pituitrin (50 mled/ml), one can see the dilation of the intercellular spaces (IS) following the first desmosome (D).

Many attempts have been made to localize the ADH effect. None of the electron microscopic efforts dealing with this problem has succeeded in revealing any changes in the ultrastructure of the apical membrane of the cells of the urinary bladder of the toad and frog [4, 10, 25, 27]. However, in both the kidneys [15] and the urinary bladder [4, 10, 25] changes have been observed /206 in the intercellular substance. The study of the region of the intercellular connection of epithelial cells of the urinary bladder of the frog shows that following the action of ADH under conditions when there is a movement of water with the osmotic gradient, only the most apical part of the region of the

intercellular junction remains unchanged; below the first desmosome there is a considerable increase in intercellular space (Figure 37). The change in the structure of the intercellular substance has also been observed with pronounced absorption of fluid in the gall bladder [18, 33]. However, the question of whether ADH acts primarily on the permeability of the intercellular substance or the cell membranes remains in doubt.

We must take up the question of the possible ultrastructure and localization of the water-permeable channel. It may be located in the apical membrane of the cell, or at the boundary between two cells. At the present time, there are 3 hypotheses concerning the location of membranes whose permeability changes when ADH is added. A number of investigators feel that the apical membrane of the cell consists of 2 layers — an outer one which is dense and an inner porous one located beneath it. In the opinion of Lichtenstein and Leaf [23], the dense layer is always permeable to water while the permeability of the porous layer is increased by ADH. This conclusion is based on data from experiments using amphoteritsin, which increases the permeability of the membrane to sodium but not to water [23]. Hayes, on the other hand, suggests that ADH increases the permeability to water of the external dense layer of the membrane [17]. Finally, there may still be another possibility, namely, the influence of ADH has something to do with the change in permeability of the intercellular substance. Previously we presented some electron microscopic studies indicating that under conditions of action of ADH and the intensive movement of water with the osmotic gradient the ultrastructure of the interface between two cells on the side of the cavity of the urinary bladder remains unchanged and it is only below the first desmosome that the separation of cells from one another takes place, indicating a change in the state of the intercellular substance (see Figure 37).

As a working hypothesis, we can assume that this zone of intercellular juncture, formed by the external membranes of the cell, is a water-permeable channel. It is well known that a characteristic of membranes of the apical part part of the cells of the epithelium of the distal segment of the kidney tubule, the urinary bladder of the frog and several other organs which excrete hypotonic fluids is their resistance to the action of solutions with a reduced osmotic concentration. At the same time, however, the membrane of the basal portion of these cells, like the membranes of the other cells, is permeable to water and when it is in contact with hypotonic solutions there is a swelling of the cells. Hence, if the protruding membrane of the cell of the epithelium of the mucous membrane of the urinary bladder is also permeable up to the first desmosome with respect to osmotically free water in the direction of the cytoplasm, like the apical membrane, this region may contain a formation of water transporting channels which are always permeable to water but will not allow dissolved substances to pass through. The movement of water along this channel /207 will be governed by the state of the intercellular substance which is located beneath the first desmosome. When the acid mucopolysaccharides are in a polymer state, the movement of the water will not take place; after depolymerization, conditions are created for the movement of water with the osmotic gradient.

There is a great deal of data to support this assumption. Mucopolysaccharides are probably missing only in the most apical areas of contact

between the kidney cells [28]. The movement of water with the osmotic gradient in the direction from the mucous to the serous membrane takes place at a rate that is nearly twice as great as in the opposite direction [9]. An analysis of this phenomenon on a model has indicated that this effect is caused by the passage of water through 2 layers that have different properties [26]. In this case, we can consider the water-permeable channels as one of the layers while the other is represented by the acid mucopolysaccharides of the intercellular substance. The rate of movement of water with the osmotic gradient will be determined by the polymericity of the mucopolysaccharides. In model experiments, it has actually been found that the movement of the fluid through hyaluronic acid depends on its polymericity [24]. In the kidney, we have observed a decrease in the polymericity of the acid mucopolysaccharides under the influence of ADH [1]. Proof of the correctness of this hypothesis may be found in the presence of channel-like structures in the region of the closed zone of the intercellular connection. When working with lanthanum to treat pieces of liver and heart muscle, an extracellular space was found in those zones [29], but in the urinary bladder treatment with lanthanum failed to reveal any such structures. The authors of some electron microscopic papers consider the zone of the most apical region of intercellular contact to be impermeable to either solutions of dissolved particles or water [14]. The facts and ideas which we discussed earlier indicate that the idea of impermeability ("tight junction") for water is not a general rule.

Summing up the above, we must conclude that the question of the location of water-permeable channels requires further study, although all of the data presented previously indicate the extremely great importance of intercellular substance. Assuming for the sake of a model that the water moves along a channel measuring tens of angstroms and narrow jets are ejected into the saline solution, washing the surface of the cell, we can assume that good conditions are created for movement of fluid near the mouth of the channel, so that the concentration of salts will always be high there.

In the theory developed above for osmotic pressure, we considered the /208
molecules of water to be elastic spheres and did not assume the fluid had any internal structure. Such an assumption was sufficient to explain a number of effects but it still does not mean that this kind of model actually reflects the real relationships between water molecules. However, we can assume that more complex models, which take into account the actual shape and forces of interaction between water molecules in a solution, will not require revision of the previously developed concepts. In this connection, we must stress yet another fact: if a solution isotonic with respect to blood is located both inside and outside the urinary bladder, the hydrostatic pressure in such a channel would be 6-7 atm (i.e., at the level of the osmotic pressure of blood) less than atmospheric. It is quite possible that it would affect the structure of the water. The influence of the walls of the channel is also of some importance.

Hence, the motion of free spaces between molecules (cavities) having thus been discussed, we can construct a theory of osmotic pressure which links the value of the osmotic pressure, the coefficient of compression of the fluid, the

volume of free space between molecules, the degree of osmotic transport of fluid through the semipermeable membrane and the rate of propagation of sound in a fluid. The differences between osmotic and isotopic permeability of the wall of the urinary bladder of the frog with respect to water cannot explain the relatively rapid movement of the hydrogen ion in comparison with the movement of the water molecule. Osmotic transport of water through the wall of the urinary bladder of the frog can be clearly explained if we assume that there are special water-carrying channels. In electron microscopic study of the action of ADH, no changes were found in the apical membrane of the cells, but there was an increase in the difference between the cells located beneath the first desmosome.

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