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MECHANISM OF INCREASING OSMOTIC RESISTANCE OF ERYTHROCYTES IN RATS ADAPTED TO HYPOXIA

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ABSTRACT. On the basis of experimental data, it is determined that the increase in the osmotic resistance of rats adapted by hypoxia is an adaptive reaction which forms a part of the general mechanism of increasing the resistance of the organism.

In the preceding work (Barbashova, 1963), we showed that in case of an increase in the resistance of an organism brought about by training for hypoxia, an increase is noted in the osmotic resistance of erythrocytes. Several interpretations of these phenomena are theoretically possible. It is known that the stability of the erythrocytes of adult animals increases in the case of blood losses, burns, in the case of poisoning by hemolytic poisons, and in case of the action of cobalt (Shustrov, Vlados, 1920; Gitel'zon, Terskov, 1960, 1961) and as we have shown, in case of the prolonged effect of hypoxia, i.e., in all cases in which the stimulation of erythropoiesis is brought about. The considerable intensification of the new formation of red blood is, as a rule, accompanied by the entry of less mature erythrocytes into the blood stream; this is expressed first of all in an increase of the quantitative content of reticulocytes and the appearance of their young forms. /1385*

It has been shown by numerous investigations that young forms of erythrocytes possess great resistance to the action of hypotonic solutions of salts, acids, hemolytic poisons, and a number of other agents (Gitel'zon, Terskov, 1960, 1961; Vorob'yev, Brilliant, 1961, et al.). Consequently, in the first place, it may be considered as an assumption that the reason for the increase of the osmotic resistance of the erythrocytes of rats adapted to hypoxia is an enrichment of the blood by more resistant forms of red cells. If this is so, there should be a strict correlation between a change in the composition of red blood and the resistance of the erythrocytes.

In the second place, as confirmed by numerous observations, erythrocytes vary with respect to their resistance not only according to their own age, but also according to the age of the animal. Thus, the erythrocytes of embryos and newly born animals possess a greater osmotic resistance than do the erythrocytes of adult animals (Shustrov, Vlados, 1920; Zakulenko, Pavlova, 1927; Hornung, 1927, et al.); they are also more resistant to hemolytic poisons (Shustrov, Vlados, 1920). On the other hand, their resistance to the action of alkali is less than that of adults (Trincher, Gintsburg, 1963). Consequently, if a change in the composition of red blood, brought about by the

* Numbers in the margin indicate pagination in the foreign text

prolonged action of hypoxia, is combined with the appearance of some properties that are characteristic of the blood of a newly born animal, then it follows, that along with an increase of osmotic resistance and resistance to hemolytic poisons, there should also be observed a lowering of the resistance to the action of alkalis.

Finally, it may be assumed that the increase of the osmotic resistance of erythrocytes in rats adapted to hypoxia is connected not with the "rejuvenation" of the blood and not with the appearance of some kind of embryonic /1386 properties of the blood, but with a unidirectional shift in the resistance of the entire mass of erythrocytes, including the mature ones, which is a function of a change of the physical and chemical properties of the blood. The possibility of such a mechanism of change of the resistance of erythrocytes is indicated in the works of Gitel'zon and Terskov (1961). If the increase of the osmotic resistance of the erythrocytes of rats adapted to hypoxia has this particular mechanism, then a strict correlation between the value of the osmotic resistance and the composition of the red blood is not at all necessary.

The task of the present work has been experimental verification of the correctness of the expressed assumption concerning the possible causes of the increase in the osmotic resistance of the erythrocytes in the blood of rats adapted to hypoxia, which has been demonstrated by us. With this aim there was determined the osmotic, the saponinic, and the alkaline hemolysis of erythrocytes, as well as the quantitative and the qualitative composition of the red blood of white rats trained for hypoxia.

PROCEDURE

Serving as the object of the investigation were white rats of the Vistar line (males), with a weight of 200-250 g. Adaptation to hypoxia was attained by means of training in a barometric chamber with a daily increasing rarification of the atmospheric air up to 308 mm Hg, which corresponds to an altitude of 7,000 m. Exposure in the barometric chamber lasted 4 hours a day, 6 times a week. Two groups of rats were trained: the first group (22 rats) for one month, and the second group (10 rats) for three months. Twenty-four hours after termination of the training period in the barometric chamber, resistance to acute hypoxia was determined in order to evaluate the degree of adaptation. With this aim, the trained rats and the controlled rats were placed one by one under the bell of a vacuum pump, in which an air rarification corresponding to an altitude of 12,000 m was created in the space of 40 seconds. Maximum exposure at this "altitude" was 5 minutes. If before the end of this period there commenced an acute convulsive attack, or if a prolonged cessation of breathing was noted, the rats were immediately "lowered" by means of the rapid intake of air under the pump bell. Observations were made of the frequency and character of breathing, the general motor activity, the state of the muscle tone, and the nature of the convulsive tremors of individual groups of muscles or of the entire body. The method of training in the barometric chamber and the method of evaluating the overall resistance of the organism of the experimental animals to hypoxia have been described earlier

(Barbashova, 1963). Not earlier than 24 hours after such a test (in the overwhelming majority of cases - later), the rats adapted to hypoxia were taken, inevitably paired with control rats, for determination of the blood composition and the resistance of the erythrocytes.

The osmotic resistance of the erythrocytes was determined by the conventional method, according to the degree of blood hemolysis in solution with a decreasing concentration of sodium chloride (Barbashova, 1963).

The alkaline resistance of the erythrocytes was studied by means of the method described by K. S. Trincher (1959) - according to the rate of hemolysis of a suspension of erythrocytes in an isotonic alkaline buffer. Blood in the amount of 0.1 ml was mixed with 0.5 ml of a solution consisting of 3 parts physiological solution and 1 part 5% solution of sodium citrate. An amount equal to 0.01 ml of this (5-times diluted) blood was mixed with 5 ml of an isotonic borate buffer with pH 9.97 and was kept at a temperature of 20 or 37° C. The moment of mixing of the blood with the alkaline buffer was noted at the beginning of the action of the alkaline medium upon the erythrocytes. At specific intervals of time, the optical density of the mixture was measured by means of a photoelectrocolorimeter (with a wavelength of 534 mm) for determination of the degree of hemolysis. The hemolysis was considered to be completed when the values of the optical density attained the lowest constant values corresponding to the density of a transparent hemoglobin solution.

The rate of hemolysis of the erythrocytes under the action of the hemolytic poison, saponin, was determined by a similar method. To this end, 0.05 ml of the same (5-times diluted) blood was mixed with 15 ml of physiological solution, and the initial optical density of 4 ml of this mixture was determined by means of a photoelectrocolorimeter. Then directly into the cuvette with the blood, standing in the photoelectrocolorimeter, there was rapidly added 0.75 ml of 0.1% solution of saponin, dissolved in a physiological solution. After rapid and thorough mixing every 20 seconds, a reading was made of the value of the optical density of the mixture. A constant temperature of 26° C was maintained in the photoelectrocolorimeter. The hemolysis was considered to be completed with stabilization of the values of the optical density and their equalization with the density of a transparent hemoglobin solution, just as in the determination of alkaline hemolysis.

The composition of the red blood was determined by the conventional method: the quantity of hemoglobin was measured in a Sali hemometer; the number of erythrocytes was counted in a Goryaev chamber; the number of reticulocytes was counted on the basis of four groups, according to the classification of I. A. Kassirskiy and G. A. Alekseyev (1955).

RESEARCH RESULTS /1387

1. Characterization of the overall resistance of rats to hypoxia in the case of an "ascent" to 12,000 m. A rapid "ascent" to 12,000 m showed that all 22 rats that were trained in the barometric chamber for the duration of a month adapted well and acquired an increased resistance to hypoxia. They all survived the 5-minute exposure in satisfactory condition; even by the time the "altitude" of 12,000 m was reached, the respiration was rapid

(from 100 to 130 per minute) and rhythmical; no acute motor excitation was noted during the first minute at the "altitude"; the muscle tone was disturbed to a moderate degree, and the rats retained the capacity of assuming their conventional position. After the "descent" the animals could immediately move about independently. Concerning the 10 rats that were trained for three months, their condition while at 12,000 m testified to poor resistance to hypoxia. Just as the 24 control rats, at the moment the "altitude" of 12,000 m was attained, they manifested acute motor excitation; convulsions were observed in individual parts of the body; as a rule, the respiration ceased; after brief convulsive attacks came pauses of deep atony, when the animals lay on the floor of the chamber, spread out on the stomach. In cases when the animals nevertheless recovered their respiration and could remain at the "altitude" for some time (sometimes even during the entire 5-minute exposure), the respiration was slow (from 12 to 70 times per minute), and arrhythmic; phase pathological disturbances of the character of the breathing were observed, with the appearance of gaspnoitic dyspnea. The condition was so severe that after the "descent" the rats lay on their sides and began to move about only after several minutes. The obtained results give us the right to consider that only the month-long training period in the barometric chamber yielded positive results, and that only this group of rats may be considered to have adapted successfully and to have raised its overall resistance. On the other hand, in the case of the rats whose training lasted three months, adaptation had broken down; apparently the training regimen turned out to be too severe with such a duration. Subsequently we shall call this group of rats overtrained. It should be noted that rats of the Vistar line are generally distinguished by a lower resistance to hypoxia. This forced us to reduce the acuteness both of the training regimen (7,000 m instead of 7,600 m, as was the case in our previous studies) and of the conditions in the testing of the overall resistance to acute hypoxia (12,000 m instead of 13,000 m).

2. The red blood pattern. The month-long training in the barometric chamber was carried out with two groups: 15 rats were trained for hypoxia in the spring of 1963 and 8 were trained in the fall of that year. Due to a number of considerations which will become clear with the further exposition of the material, the data on the composition of the red blood will be presented for each group separately. As can be seen from the data of the tables, one-month training for hypoxia brought about perceptible stimulation of blood formation both in the spring group of rats and in the autumn group. The blood was enriched with hemoglobin on the average from 15.3 to 19.2g % in the spring and from 15.3 to 19.3g % in the autumn; the number of erythrocytes rose from 9.03 to 10.21 million in the spring and from 8.58 to 10.55 million in the autumn; the number of reticulocytes increased from 25 to 62% in the spring and from 23% to 33% in the autumn. Here a shift to the left in the composition of the reticulocytes is expressed with identical clarity.

Concerning the group of overtrained rats (trained in the spring for three months), in spite of the fact that they were unadapted and in the majority of them the number of erythrocytes did not differ from the controlled values, nevertheless 6 animals out of 10 had a higher hemoglobin content (an average of 17.1g % for the group), and a perceptible shift to the left in the composition of the red blood was noted. Not only was the number of reticulocytes /1388

TABLE

AVERAGE VALUES OF QUANTITATIVE BLOOD COMPOSITION

Quantity	Control rats		Adapted to hypoxia		Over-trained
	Spring	Autumn	Spring	Autumn	
Rats	15	9	14	8	10
Hemoglobin (in g %)	15.3 ± 0.10	15.3 ± 0.16	19.2 ± 0.44	19.3 ± 0.35	17.1 ± 0.39
Erythrocytes	9.03 ± 0.14	8.58 ± 0.19	10.21 ± 0.3	10.55 ± 0.3	8.83 ± 0.33
Reticulocytes (in %)	25 ± 1.33	23 ± 0.87	62 ± 3.40	33 ± 1.44	41 ± 3.50
Including	Hor	None	0.1	None	0.6
I Group	4	7	18	12	16
II Group	7	6	16	10	12
III Group	14	10	28	11	12
IV Group					

in these six rats increased, but there was a high content even of such immature cells as nuclear normoblasts (Group 1). The leftward shift of the cells in the red series testifies to the intensification of the erythropoietic function of the red sprout of the bone marrow in the majority of the rats in this group.

3. The osmotic resistance of the erythrocytes. Just as in our previous study (Barbashova, 1963), the osmotic resistance of the erythrocytes of rats adapted to hypoxia was higher than in the controlled animals. The hemolytic curves were exactly identical with those in the previous study; therefore we shall not cite them here. We shall merely indicate that in the present study, a 50% hemolysis of the erythrocytes in the controlled animals took place on the average at a sodium chloride concentration equal to 0.58%, with fluctuations in individual experiments from 0.54 to 0.62%, whereas, in the rats adapted to hypoxia the 50% hemolysis took place at lower dilutions of the salt solutions - on the average at 0.49%, with fluctuations in individual experiments from 0.45 to 0.54%.

If the values characterizing the red blood composition in each control rat and adapted rat are compared with the values of the osmotic resistance of the erythrocytes (assuming as the resistance indicator the sodium chloride concentration at which 50% of the erythrocytes were hemolyzed), it appears at first glance from Figure 1, A, B that there is a direct relationship between these values. The higher the erythropoiesis (Figure 1, B, I, II), the greater the number of reticulocytes in the blood (Figure 1, B, III), the greater is also the osmotic resistance (Figure 1, B, IV). However, such an impression is an erroneous one. Analysis of the obtained results in greater detail brings out a number of facts which force one to consider that there is no real correlation between the height of the osmotic resistance of erythrocytes and the extent of the leftward shift in the red blood composition. Actually, it turns out that the highest resistance of erythrocytes in the rats noted in Figure 1, B, IV, by numbers 1, 2, 3, 4, in no way correlates with the degree

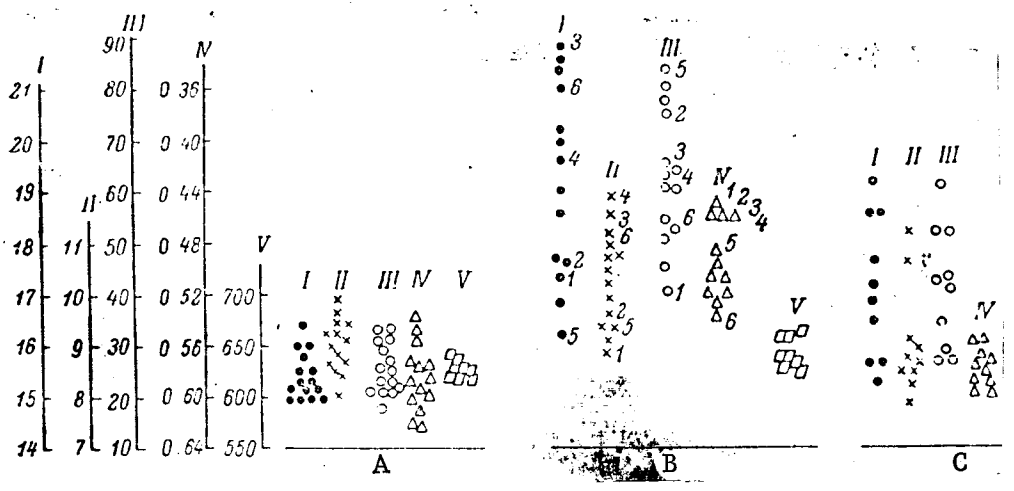

Figure 1. Comparison of the Resistance of Erythrocytes to Hemolysis with the Red Blood Composition of Rats.

Key: I - amount of hemoglobin (in g%);
II - number of erythrocytes (million);
III - amount of reticulocytes (in percent);
IV - sodium chloride concentration (in percent) at which 50% hemolysis takes place;
V - time (in minutes) at which the degree of alkaline hemolysis corresponds to an optical density of 0.50 (at 20° C).

A - control: B - adapted to hypoxia; C - overtrained rats.

Each mark stands for a value obtained for an individual rat.

of blood "rejuvenation", i.e., an increase in the number of reticulocytes in the same animals (Figure 1, B, III, 1, 2, 3, 4). There is also no relationship between the osmotic resistance and the hemoglobin content in the blood, nor with the total number of erythrocytes (Figure 1, B, I, II, 1, 2, 3, 4). There is also no correlation when the values of osmotic resistance of erythrocytes in the rat designated by the numberal 5 (media resistance) or in rat 6 (lowest resistance) are compared with the content of reticulocytes, erythrocytes and hemoglobin in the same rat. Therefore, the change in the osmotic resistance in the erythrocytes of animals adapted to hypoxia depends upon something else.

The certainty of this is reinforced to an even greater degree upon examination of the results obtained for the group of overtrained rats (Figure 1, B). In spite of the fact that in the majority of the rats of this group the number of reticulocytes is greater (Figure 1, B, III) and a perceptible shift to the left is noted in their series, the osmotic resistance of the erythrocytes (Figure 1, B, IV) differs in no way from the values obtained for the /1389

control animals (Figure 1, A, IV); in their case, 50% hemolysis took place on the average at a sodium chloride concentration of 0.58%, just as for the control animals. The conclusion invites itself that an increase in the osmotic resistance is not a consequence of increased erythropoiesis which brings about "rejuvenation" of the blood.

4. Resistance of erythrocytes to saponin. The rate of saponinic hemolysis was determined only for the autumn group of rats, only for animals well adapted to hypoxia and for the control group corresponding to them. A total of 8 pairs of animals were examined. In all 8 of the rats adapted to hypoxia, the saponinic hemolysis curves were very similar to one another. They also did not differ from the hemolysis curves of the control rats. Therefore, in Figure 2 we cite only statistically reliable average values. As can be seen from Figure 2, all the phase variations of the optical density of the erythrocyte suspension after the addition of saponin took place in the same manner in the experimental and in the controlled rats. At first, for about one /1390 minute a small drop was noted, and then an increase in the optical density was observed, associated with a period of erythrocyte spherulations. Approximately 2 and 1/2 minutes after this, a sharp decrease in the values of optical density was observed, testifying to the rapidly increasing hemolysis of a constantly growing number of erythrocytes, which was then succeeded by a smooth decrease in the optical density - the phase where the last, most resistant cells are hemolyzed. Approximately by the sixth minute the hemolysis process in both groups terminated, as was testified to by the stable values of optical density, equal to the density value of a transparent solution of hemoglobin.

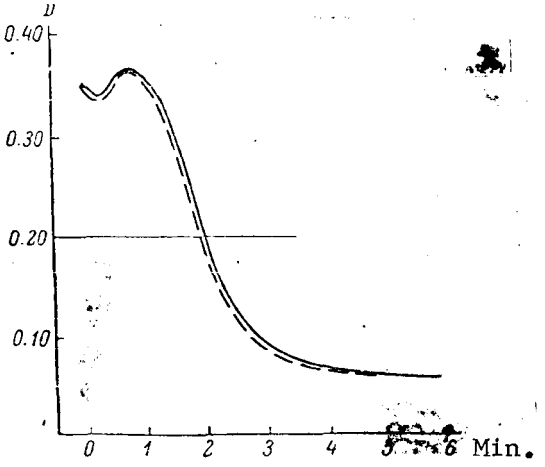

Figure 2. Rate of Saponinic Hemolysis of Rat Erythrocytes.

Along the ordinate - the optical density of the erythrocytes suspension; along the abscissa - time (in minutes). Broken line - controlled animals; solid line - animals adapted to hypoxia.

If the rate of saponinic hemolysis is expressed in terms of the time of the onset of hemolysis corresponding to an optical density value of 0.20 (approximately 50% hemolysis; Figure 2), and if these values are compared for each rat with the composition of its blood, the following will be obtained (Figure 3): the identity of the resistance of erythrocytes to saponin in the case of adapted (Figure 3, B, IV) and controlled rats (Figure 3, A, IV) in no way correlates with the considerable intensification of erythropoiesis and the leftward shift of the composition of the red blood which takes place in the case of adaptation to hypoxia (in Figure 3, compare B, I, II, III with A, I, II, III).

5. Resistance of erythrocytes to alkali. The rate of alkaline hemolysis was determined in the blood both of the spring and the autumn group of animals.

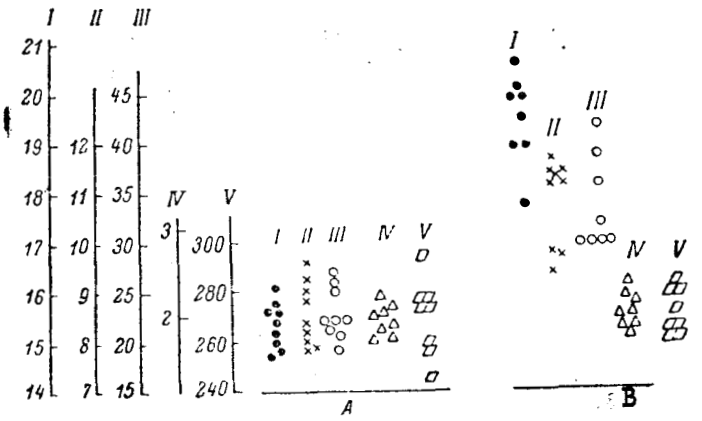

Figure 3. Comparison of the resistance of Erythrocytes to Hemolysis with the Red Blood Composition of Rats.

IV - time (in minutes) at which the degree of saponinic hemolysis corresponds to an optical density of 20°.
V - Same as Figure 1, but at 31°.
Remaining designations are the same as in Figure 1.

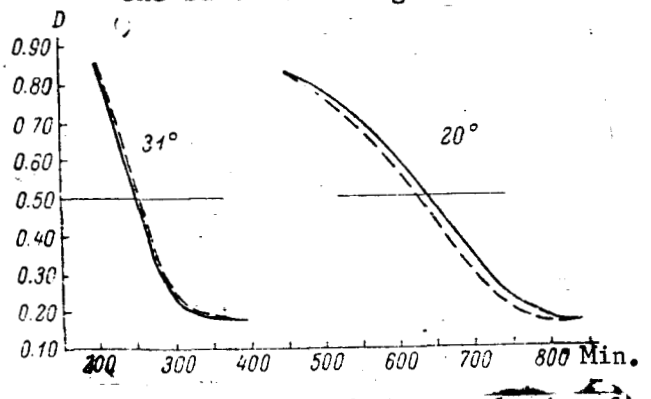

Figure 4. Rate of Alkali Hemolysis of Erythrocytes in Rats.

Designations are the same as in Figure 2.

Figure 4 shows the average values of the hemolysis rate of erythrocytes under the effect of an isotonic alkaline borate buffer with a pH value of 9.97 in control rats and in those adapted to hypoxia. As can be seen from Figure 4, at a room temperature of 20° (spring group) and at a temperature of 31° (autumn group) the hemolysis rate in all stages of its course is the same for both groups of small animals. The small difference in the hemolysis curve at 20° is statistically unreliable. /1391

Figure 1, V and Figure 3, V show experimental data for alkaline hemolysis for each animal. As a criterion for the rate of alkaline hemolysis there was adopted the time (in minutes) from the beginning of the action of the alkali until the values of the optical density fall to 0.50, which corresponded to the onset of approximately 50% hemolysis. It can be seen from Figures 1 and 3 that at both temperatures the hemolysis rate in all the experimental animals, the control group as well as those adapted to hypoxia, were similar. We cannot consider the small increase in the resistance of adapted rats of the spring group (Figure 1, B, V) to be reliable, since the scatter zones of the values overlap strongly, and although P is less than 0.05, it is greater than 0.03, which with such a number of experiments as we have had cannot be a criterion of reliability. To speak of a tendency toward an increase of stability would be stretching things considerably.

Thus, the resistance of erythrocytes in rats adapted to hypoxia did not change with the action of saponin and alkali, although in these animals there are clearly manifested not only an increase in hemopoiesis and erythropoiesis, but also a shift to the left in the composition of the cells of the red series.

CONCLUSION

It is first of all necessary to reject the assumption that the reason for the increase in the osmotic resistance of erythrocytes in rats adapted to hypoxia is the enrichment of the blood by more resistant new forms of erythrocytes, in connection with a leftward shift in the composition of the red blood. Such an assumption is contradicted in the first place by the absence of an increase in osmotic resistance of erythrocytes in overtrained rats, i.e., those not adapted to hypoxia, although in the case of these animals the hemopoiesis was higher and a perceptible leftward shift was noted in the composition of the red blood. In the second place, this assumption is contradicted by the fact that, in spite of a considerable increase in the number of young cells of the red series in rats adapted to hypoxia, the rate of saponinic hemolysis did not increase, but remained unchanged.

It is also necessary to decline the assumption that "rejuvenation" of the composition of peripheral blood is accompanied by the return of some properties that are inherent in the organism in the early stages of ontogenesis, which cause a change of resistance of the erythrocytes. If this were the case, a reduction in the resistance of erythrocytes to the action of alkalis would be noted. In our experiment, however, both at a temperature of 20° and a temperature of 31°, the rate of alkaline hemolysis in rats adapted to hypoxia coincided completely with the hemolysis rate in the controlled animals.

There remains in force the assumption that the cause of the increase of osmotic resistance of erythrocytes is a unidirectional shift of the resistance of the entire mass of erythrocytes, which is connected with some changes in physical and chemical properties of the blood. It should be noted that the change in the resistance of erythrocytes noted by us is of a specific character, since it is manifested only with a change of the osmotic pressure in the medium and is not detected in case of the action of an alkali or of saponin.

Since the rate and the character of saponinic and alkaline hemolysis of the erythrocytes of animals adapted to hypoxia remain constant in spite of the fact that a higher content of hemoglobin, erythrocytes, and reticulocytes in the blood was noticed, with a leftward shift in the reticulocyte series, the conclusion may be drawn that not every "rejuvenation" of the composition /1392
of the red blood is accompanied by an increase in the overall hemolytic resistance of the erythrocytes.

The fact of the increase of the osmotic resistance of erythrocytes is characteristic only for rats that are well adapted to hypoxia, and is lacking in animals overtrained in the barometric chamber; in the latter animals, although a leftward shift in the composition of the red blood is noted, the resistance of the organism as a whole is not increased. This phenomenon coincides completely with the analogous observations in our preceding study (Barbashova, 1963). Apparently the increase in the osmotic resistance of erythrocytes in animals adapted to hypoxia is an adaptive reaction, and is a part of the general mechanism of increasing the resistance of the organism. It remains to be investigated what the physical and chemical shifts in the blood are which lead to a change in the character of the water-salt exchange of erythrocytes.

If the increase in the osmotic resistance of erythrocytes is really a component of the general mechanism of increasing the resistance of the organism in training for hypoxia, and its decrease testifies to an unfavorable course of the training process, then special attention should be devoted to the question of whether it is possible to use the osmotic resistance of erythrocytes as a test for determination of the overall resistance of the human organism with various kinds of training that are connected with the origination of a hypoxic state of the organism. This could provide the physicians and the physiologists in the field of sports with an additional possibility in checking the correctness of carrying out various kinds of training.

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