NATIONAL AERONAUTICAL AND SPACE ADMINISTRATION
GRANT NGR 14-005-050

THE PHYSICAL AND CHEMICAL PROPERTIES OF HUMAN SWEAT AND FACTORS AFFECTING THE WATER BALANCE IN CONFINED SPACES

FINAL REPORT

July 1, 1965 - June 30, 1968

from the

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I. PERSONNEL ASSOCIATED WITH NASA GRANT NGR 14-005-050

Table I.1 in Semi-Annual Report No. 4 (1 January 1967 - 30 June 1967) lists all persons who worked under this grant from July 1, 1965 to June 30, 1967. Table I in Semi-Annual Report No. 5 (1 July 1967 - 31 December 1967) lists all persons who worked under this grant during this period. Following is a list of all persons who worked under this grant from January 1, 1968 to June 30, 1968.

Name and Title	Period of Association	Percentage of Salary Paid From this Grant
R. E. Johnson, Professor	1 January - 30 June 1968	0
F. Sargent II, Professor	" "	0
Juan C. Fasciolo, Visiting Professor	1 January - 15 January 1968 15 January - 30 June 1968	100 0
Robert Buffington, Supervising Clinical Lab. Technologist	1 January - 30 April 1968 1 May - 30 June 1968	24 0
Mrs. Frances Robbins, Research Assistant	1 January - 31 January 1968 2 February - 15 June 1968	17 25
GRADUATE ASSISTANTS:		
Paul Molé	1 January - 29 February 1968	42
Mrs. Becky Johnson	1 January - 30 June 1968	0
Mrs. Maliha Ramadan	" "	0
TECHNICAL PERSONNEL		
Brent C. Seager, Captain USAF (BSC)	1 January - 30 June 1968	0

SUBJECTS: Hourly wage - working intermittently

Marvin Turbow, Janice Beare, Carrol Percak, Paul Shales, Carol Fogle, Greg Totel, Ken Browning, Joe Velazquez, Mike Velazquez, Jim Anderson, Steve Pearl

II. DISCUSSION OF RESEARCH COMPLETED UNDER NASA GRANT NGR 14-005-050

The grant was in effect from July 1, 1965 through June 30, 1968. Three main lines of research were followed: first, methods; second, the physico-chemical properties of human eccrine sweat; and third, some physiological aspects of the human eccrine sweat gland.

Five useful methods were developed. First, a versatile and simple system for collecting, storing and analyzing expired air utilizes bags made of metallized polyethylene. These are impermeable to CO_2 for at least 12 hours at 25°C. The air is drawn through a paramagnetic O_2 meter and a thermal conductivity CO_2 meter in tandem. Second, a reliable system for analyzing the three fractions of sulfur in urine and sweat by turbidimetry has been developed from the relation:

Total S = Inorganic S + Acid hydrolysable S + Neutral S.

Third, all steps in the estimation of nitrogen by nesslerization have been scrutinized, and a comprehensive, sensitive system for total N, urea N and ammonia N developed. Fourth, one Fortran program is now usable for calculating the metabolic mixture (protein, fat, carbohydrate, energy produced, and metabolic water) from the three primary measurements urinary N, O_2 consumed and CO_2 produced. Another is available for calculating the water balance either by the balance equation (fluid in minus fluid out) or the Peters-Passmore equation (water balance a function of solids and gases in and out). Finally, a volumeter to measure the volume of the human body by air displacement has been developed. It is based on the gas law: PV = nRT. A gas tight chamber of known volume is used. A known volume of air is injected and changes in pressure and temperature read off both when the subject is in the chamber and when the chamber is empty. The volume of the body can then be calculated. It is not necessary to measure the residual volume of the lung. Body weight is known, and the density is calculated from the formula: Density = Volume/Weight. The body fat can then be calculated from the Pace-Rathbun formula. A K^{40} counter is used for estimating total body potassium and the lean body mass. Thus the major body compartments can be calculated from the formula: Total Body Mass = Water + Fat + Dry Lean Body Mass.

Three published studies were made on the physico-chemical properties of sweat. First, the viscosity of sweat is temperature related. It decreases with increased temperature. At about 27°C there is a discontinuity, suggesting a polymerization system. Second, sweat is an ordered acid-base system. In the acid range, ammonia becomes important; in the alkaline range, bicarbonate. Amino acids may play buffer roles at all acidities. Neither phosphate nor keto acids are present in appreciable amounts; lactate, though present in high concentrations is not in its buffer range. Third, the sum of the solutes as measured by the total dry solids is greater than the sum of the constituents Na, K, NH_3, Ca, Mg, chloride, lactate and urea. To account for this "unknown solute" there must be present a substance (or substances) not ordinarily recognized or measured. It appears to be in the residual nitrogen fraction (total N minus urea N minus ammonia N). Amino acids account for at least part of it, but not all.

Two aspects of the physiology of eccrine sweating were studied. First, correlations were made between the rate of sweating and the concentration of the various constituents. As judged by its freezing point eccrine sweat is almost always hypotonic to blood plasma. Nevertheless, with respect to the individual solutes, there is a wide disparity. Some like calcium, magnesium, sodium and chloride are hypotonic; others like lactate and ammonia are strongly hypertonic; some, like urea and potassium are almost isotonic; and some, like ketone bodies, creatinine and phosphate, are present in plasma but hardly at all in sweat. With increased rate of sweating some, like sodium and chloride, increase in concentration; others, like urea, magnesium and calcium decrease; and others, like potassium and lactate show little or no change. No present model fits all of the changes known to occur. Second, studies were made of the effects of daily cholinergic stimulation at a single area of skin in the forearm. When injected daily mecholyl caused a "training effect", i.e. an increased rate of sweating for a given dose. By contrast, when daily iontopheresis was used to deliver the drug to the site, mecholyl and pilocarpine both showed a diminution in the rate of sweating, i.e. the reverse of "training". In the iontopheresis experiments, a diminished rate of sweating was accompanied by a paradoxical increase in the concentration of sodium.

These investigations have practical applications in situations when man is working in confined spaces. The astronaut in a small closed ecological system is an example. There are three groups of present or potential practical applications. First, rapid reliable methods are available for estimating total nitrogen, urea, ammonia, total sulfur, inorganic sulfate, acid hydrolysable sulfate and neutral sulfur. These are important metabolic products found in sweat, urine or both. The collecting, storage and analysis of expired air for O_2 and CO_2 have been greatly improved. Metabolic calculations have been accelerated by means of computer programs. A pneumatic body volumeter simplifies the process of measuring or calculating body volume, body fat and lean body mass. These change with nutritional stress. Second, much has been learned about the physico-chemical properties of sweat and the solutes therein. Under some conditions of physical work and thermal stress, the skin is far more important than the kidney in the body's economy of water, minerals and nitrogen compounds. Finally, a start has been made on elucidating some of the physiological control systems related to the rate of sweating. Thus it may become possible to manipulate and guide the activity of the largest organ in the body, the skin, toward maximum survival potential.

III. GRADUATE THESES, PUBLICATIONS, AND MANUSCRIPTS RESULTING FROM CONTRACT NASA NGR 14-005-050

As a means for enabling graduate students to pursue research, and thus earn the degree M.S. or Ph.D., research grants are of fundamental importance for science in the United States. The present contract has aided in the production of several M.S. research reports, published papers, and manuscripts in preparation. Following is a list of these.

M.S. RESEARCH REPORTS

Blase, Barrie W.
Title: The Density and Viscosity of Human Sweat.
Dated: Urbana, Illinois, 1966.

Johnson, Becky
Title: Training of the Eccrine Sweat Glands by Acetyl-β-Methylcholine.
Dated: Urbana, Illinois, 1966.

Naman, Ramzia K.
Title: The Role of Calcium and Mucopolysaccharides in the Mechanism of Eccrine Sweating.
Dated: Urbana, Illinois, 1966.

Ramadan, Maliha
Title: Validation of Methods for Studying Calcium and Magnesium in Sweat.
Dated: Urbana, Illinois, 1968.

PUBLISHED PAPERS

Bernauer, E. M., Mole, P. A., and Johnson, R. E.: Calculation of metabolic mixture and water balance using fortran programs. Metabolism 16: 899-909, October, 1967.

Blase, B., Morimoto, T., and Johnson, R. E.: The viscosity and density of human eccrine sweat. Federation Proc. 26: 445, Mar.-Apr. 1967.

Morimoto, T., and Johnson, R. E.: Ammonia and the regulation of acidity in human eccrine sweat. Nature 216: 813-814, November 25, 1967.

Johnson, Becky, Hertig, B. A., Gifford, David, and Sargent, F.: Sweating responses of males and females at rest in warm baths. The Physiologist 9: 213, Aug., 1966.

Johnson, Becky, Gincauskas, A. T., Lloyd, R. W., and Sargent F.: Electrolyte values in sweat during training with acetyl-β-methylcholine: Fed. Proc. 26: 445, March-April, 1967.

Johnson, R. E., Robbins, Frances, Schilke, R., Mole, P., Harris, Janet, Wakat, Diane: A versatile system for measuring oxygen consumption in man. J. Appl. Physiol. 22: 377-379, February 1967.

PAPERS IN PRESS

Johnson, R. E., Morimoto, T., and Robbins, Frances: The excretion of solutes in human eccrine sweat. Exocrine Gland Symposium, International Congress of Physiology, August 1968.

PAPER SUBMITTED FOR PUBLICATION

Johnson, R. E., and Robbins, Frances D.: Nitrogen by nesslerization.

THE VISCOSITY AND DENSITY OF HUMAN ECCRINE SWEAT. B. Blase*, T. Morimoto* and R. E. Johnson. Dept. of Physiology and Biophysics, Univ. of Illinois, Urbana, Ill.

Very few studies have been reported on the physical properties of human eccrine sweat. We collected sweat in impermeable arm bags from young men walking in a hot dry room. The viscosity was measured with the Cannon-Fenske modification of Ostwald's viscometer and the density with a pycnometer. For 11 samples at 24 C the average density was 1.00176 g/ml, the average absolute viscosity was 0.9197 cp, and the average relative viscosity was 1.0128 compared with water. In a study of temperature dependence in the range 24 to 29 C the density and relative viscosity of sweat showed unexpected discontinuities. With rising temperature the density and viscosity of water and solutions of sodium chloride decreased linearly, whereas at about 28 C, the density and relative viscosity of sweat rose abruptly. We conclude that the viscosity and density of sweat are not the same as for water, as is commonly assumed in calculations of evaporative heat loss. (Supported by a grant from NASA.)

Federation Proc. 26:445, Mar-Apr 1967

SWEATING RESPONSES OF MALES AND FEMALES AT REST IN WARM BATHS.

Becky Johnson*, B. A. Hertig, David Gifford* and F. Sargent, II. Department of Physiology and Biophysics, and Physical Environment Unit, University of Illinois, Urbana, Illinois.

Difference in sweat rates between men and women is not significant in dry heat, while in humid heat the difference is significant (Morimoto et al., submitted to J. Appl. Physiol.). Brown and Sargent (Arch. Environ. Health, 11:442, 1965) have reported that in humid heat females develop hidromeiosis (decrement in rate of thermally induced sweating) more readily and to a greater extent than do males. We have extended these observations by immersing four male and four female unacclimated subjects in the bath, after the technique of Hertig et al. (J. Appl. Physiol. 16:647, 1961). Differences were observed in sweating response between the sexes, particularly (a) threshold skin temperature for sweating at rest: males av. 35.6°C (range 35.1-35.9), females av. 36.4 (range 36.0-36.8); (b) total sweating for two hour exposures at 0.6°C above threshold: males 164 g/m^2, females 133 g/m^2; and (c) time course of hidromeiosis: males 1st hr sweat 117 g/m^2, 2nd hr sweat 59 g/m^2, females 79 and 62 g/m^2 respectively. The temporal and quantitative sex differences in hidromeiosis are most likely related to sensitivity to the effect of skin wetness, these differences being greatest in the saturated conditions of the bath. Supported in part by Grant GB-4374 from the National Science Foundation.

ELECTROLYTE VALUES IN SWEAT DURING TRAINING WITH ACETYL-β-METHYLCHOLINE.

Becky B. Johnson,* Aurelia T. Gincauskas,* and Robert W. Lloyd,* F. Sargent II. Univ. of Ill., Urbana, Ill.

K. J. Collins (1963) demonstrated training of the sweat glands with intradermal injections of acetyl-β-methylcholine. In our experiment his observations were confirmed. 5 men and 5 women served as volunteer subjects. 2 intradermal injections of acetyl-β-methylcholine (100 μg/0.5 ml of saline each) were given at a selected site on the forearm for either 10 or 18 consecutive days. Sweat samples were collected on pre-weighed filter papers in an aluminum capsule fixed to the forearm. Analyses were made for sodium and chloride ions. There was a marked increase in sweat rate, sweat sodium, and sweat chloride. Both electrolyte concentration and sweat rate increased for the first 10 to 12 days and then leveled off at an elevated value which was maintained through the 18th day. Sweat rate increased by a mean value of 60%, sodium, 120%, and chloride, 270%. No sex differences were observed. The response of eccrine sweat glands to this sudorific drug was different from that to temperature, for in acclimation the electrolyte concentration of sweat declines rather than increases. (Supported by a grant from the National Science Foundation.)

IV. PROCEDURES FOR ESTIMATING INORGANIC, HYDROLYZABLE AND TOTAL SULFUR

PRINCIPLE

Inorganic sulfate forms a precipitate with Ba^{++}, and is measured turbidimetrically. Acid hydrolysable sulfate is converted to inorganic sulfate by acid and heat. Neutral sulfur is converted to sulfuric acid by oxidation in an atmosphere of pure O_2. The various fractions are calculated from the following equations:

Total S = Inorganic S + Hydrolysable S + Neutral S

Hydrolysable S = S after hydrolysis - Inorganic S

Neutral S = S after oxidation - S after hydrolysis

1. Inorganic Sulfate in Urine

A. Reference: F. Berghind and B. Serbo, Turbidimetric Analysis of Inorganic Sulfate in Serum, Plasma and Urine, Scand. J. Clin. and Lab. Invest. 12: 147-153, 1960.

B. Procedure:

1. Prepare Na_2SO_4 standards with concentrations of 0.1 to 0.4 mM/L. $(NH_4)_2SO_4$ standards can also be used.

2. Prepared urine by diluting it to give a concentration in the cuvette of approx. 0.2 mM/L SO_4. Lowest cuvette conc. is about 0.1 mM/L. (Usually 1:25 for urine already diluted to 3ml/min).

3. Pipette 2 ml aliquot of urine, H_2O (for water blank) and Na_2SO_4 standards (0.1 to 0.4 mM/L) into plastic tubes.

4. Add 0.1 ml of 10 N HCl. Mix well by flicking tube c̄ fingers. (Final acid conc. in cuvette should be approx. 0.2 N HCl.)

5. Add 1.9 ml H_2O (2.0 ml can be added instead) to each tube. Mix well as in step 4.

6. Add 1.0 ml barium gelatine reagent (BGR) to each tube.

7. A urine blank must also be prepared by following the above procedures except in step 6, 1.0 ml H_2O is added in place of 1.0 ml BGR.

8. Cap each tube and mix by inverting each ten times.

9. Allow tubes to stand at room temperature for a minimum of 15 minutes, but no longer than 30 minutes.

10. Uncap tubes, place in spectrophotometric cuvettes and read each against distilled water at 2 or 360 mμ.

11. Obtain ΔOD_{360} by substracting water blank OD from OD of standards, urine, etc.

12. Further correct urine ΔOD_{360} by substracting urine blank OD from ΔOD obtained in #11.

13. Plot standard ΔOD_{360} vs. standard conc. of Na_2SO_4 to obtain standard curves.

14. Using ΔOD_{360} for urine, obtain SO_4 conc. from the standard curve.

15. Obtain original conc. of sulfate in urine by multiplying this conc-tration by dilution factor (usually 1:25 dilution of 3 ml/min. urine).

C. Precautions:

1. Method is pH dependent, therefore the 0.1 ml of 10 N HCl must be delivered as carefully as possible. Further, this must be mixed well immediately after each addition of HCl.

2. Use volumetric pipettes in each instance, if possible. Note the 0.1 ml 10 N HCl is added using a blow out pipette.

3. The turbidity developed is time and temperature dependent as well as pH dependent. Therefore each of these must be controlled. As long as the room is air conditioned at about 75°F, incubation can be accomplished by allowing prepared tubes to stand in the rack. The minimum time is 15 minutes and the maximum is probably 1 hour. It is recommended that 30 minutes be routinely used.

4. Urine has color which absorbes at 360 mμ, therefore, a blank for each new urine specimen must be prepared by replacing 1 ml BGR with H_2O.

5. Store all reagents in refrigerator.

D. Reagents:

1. Na_2SO_4 Standards: (MN = 142.05)

Stock Soln. = 10 mM/L = 1.4205 gm/L

Working Soln.=	0.1 mM/L	1 ml of 10 mM/L diluted to 100 ml
	0.2 mM/L	2 ml of 10 mM/L diluted to 100 ml
	0.3 mM/L	3 ml of 10 mM/L diluted to 100 ml
	0.4 mM/L	4 ml of 10 mM/L diluted to 100 ml

2. Barium Gelatine Reagent (BGR)

a. 2 gm gelatine (B & A, from Allied Chemical, U. S. P. Powder, code 1797)
b. 0.2 gm thynol (Merck)

c. add a & b above to 400 ml H_2O
d. heat in H_2O bath and stir until dissolved
e. cool to room temperature and filter through #42 whatman paper
f. add 4 gm $BaCl_2 \cdot 2H_2O$ c̄ rapid stiring
g. store in refrigerator.

3. 10 N HCl

a. conc. HCl = 12N = 440 gm/L
b. Take 830 ml 12 N dilute to 1000 ml to give 10 N HCl.

E. Characteristics of the Method:

1. Sensitivity: concentration = 0.04 mM/L
Absolute amt. = 19.2 μ gm SO_4

2. Reproducibility: S.D. = 0.14 mM/L
C.V. = 2.57%

3. Recovery of added Na_2SO_4 = 100.6%, i.e. $\frac{0.179}{0.178}$ mM/L x 100 = 100.6%

4. Water Blank OD_{360} = 0.045 to 0.060

5. Standard OD_{360} 0.1 mM/L = 0.125 mμ
0.3 mM/L = 0.260 mμ
0.4 mM/L = 0.320 mμ

6. Urine (1:25 dil) OD_{360} = 0.230 mμ

2. Hydrolysable Sulfur (Inorganic plus ethereal sulfur) in Urine

A. Reference: no reference used specifically for this part of the method. Assay same as for inorganic sulfate part.

B. Procedure:

1. Prepare urine as given in 1B2.

2. Pipette 2 ml aliquot of urine, H_2O, one Na_2SO_4 Standard (0.2 mM/L) and one indoxyl sulfate standard (0.2 mM/L) into plastic tubes.

3. Add 0.1 ml 10 N HCl to each tube, mix well by flicking each tube with the fingers. (Need HCl conc. ≅ 0.5 N)

4. Cap each tube and mix well by inverting ten times.

5. Place tubes in wire rack into water bath set at 95°C (80°C may also be used).

6. Allow to incubate for 2 hours minimium.

7. After 2 hours, remove tubes from water bath and allow tubes to cool to room temperature.

8. During this time the Na_2SO_4 standards and urine samples for estimating inorganic sulfate can be prepared up to step #4.

9. Once hydrolyzed tubes have cooled to room temperature, proceed with preparation of tubes as outlined for inorganic sulfate starting with step #5.

10. The indoxyl sulfate standard, if run, requires a correction for color. Therefore, duplicate tubes must be prepared, one for turbidity and the other for color development. The tube for color is prepared as for a urine blank. That is, the 1 ml BGR is replaced with 1 ml water.

11. The ΔOD_{360} for urine is obtained by substracting the OD_{360} for the water blank. The ΔOD_{360} for indoxyl sulfate standard is obtained by first correcting the OD for the indoxyl color blank by substracting the water blank from it, and then, substracting this ΔOD_{360} for the color blank from the OD_{360} of the indoxyl sulfate standard.

12. The ethereal sulfate in urine is obtained by substracting the estimated inorganic sulfate value from that value estimated after hydrolyzing the urine sample. This difference is the estimated ethereal SO_4 fraction. $\therefore$ (Hydrolyzable SO_4) = (ethereal SO_4).

C. Precautions:

1. The hydrolysis of esterified sulfur in urine requires both heat and an acid solution.

2. The temperature needed for hydrolysis is between 80 and 95°C.

3. The duration for incubation is a minimum of 2 hours. Note that up to 4 hours did not change the estimation of ethereal sulfur in urine.

4. The HCl concentration in the cuvette for hydrolysis should be approx. 0.5 N. Using 0.1 ml of 10 N HCl in 2.1 ml total volume gives concentration of HCl of 0.48 N. After delivering 1.9 ml H_2O and 1 ml BGR. The concentration is approx. 0.2 N HCl (final).

5. If a high final concentration is used, the absorbancy is reduced. Therefore, 0.2N HCl appears to be the max. conc. for hydrolysis of ethereal sulfur with this method.

D. Reagents:

1. Na_2SO_4 Standards (see inorganic method)

2. BGR (see inorganic method)

3. 10N HCl (see inorganic method)

4. Indoxyl Sulfate (K salt) M. W. = 251.30 gm/mole

Stock Soln. = 10 mM/L = 2.513 gm/L

Working Soln. = 0.1 mM/L 1 ml diluted to 100 ml $\bar{c}$ H_2O

as (SO_4) 0.2 mM/L 2 ml diluted to 100 ml $\bar{c}$ H_2O

0.3 mM/L 3 ml diluted to 100 ml $\bar{c}$ H_2O

0.4 mM/L 4 ml diluted to 100 ml $\bar{c}$ H_2O

E. Characteristics of Method:

1. Sensitivity: Concentration in cuvette = 0.04 mM/L
   Absolute amount = 19.2 μ gm SO_4

2. Reproducibility: S.D. = ± 0.056 mM/L
   C.V. = 7.5%

3. Recovery of indoxyl
   Sulfate added to urine = 95.5 - 106.6%
   $\bar{X}$ = 101.1%

3. <u>Total Sulfur (Inorganic + Ethereal + Neutral Sulfur) in Urine</u>

A. Reference:

1. Alisino, J. F., The Determination of Sulfur in Organic Compounds, Microchem. J. 2(1): 83-90, 1958.

2. Analytical Chemistry 33: 11, 1961.

B. Apparatus:

1. Oxygen flask infrared igniter, Thomas-Ogg Model 6471-B.

2. Thomas-Ogg Combustion Flask, Model 6471-P10
   a. ground glass stopper (6471-R25)
   b. platinum sample carrier (6471-Q10)
   c. sample wapper, black paper (6471-Q25)
   d. flask clamp

3. Commerical tank of 100% oxygen

C. Procedure:

1. 0.1 to 0.2 ml undiluted urine (to give final conc. in test tube of approx. 0.2 mM/L) is added to the black paper with a 0.1 or 0.2 ml blow-out pipette.

2. The black paper is folded and hung on a drying rack before sample is pipetted onto it.

3. The sample is allowed to dry (0.1 ml sample usually dries in about 1 hour) in drying box which is covered.

4. Into a Schoneger combustion flask is pipetted 10 ml H_2O (blow-out pipette) and 0.5 ml 1:4 diluting 30% H_2O_2.

5. The sample is folded, placed into the platinum wire basket and the basket attached to the hook of the ground glass stopper.

6. The ground glass stopper joint is lubricated with glycerine. The glycerine acts as a sealer to prevent the lose of the gascous SO_2 and SO_3 formed after combustion. A sealer must be used. Lubriseal was found to work as well.

7. The stopper is then put into place and rotated so that one glycerine is spread about the joint uniformly.

8. Next, the flask is gased a few seconds with pure oxygen, the stopper is replaced quickly and secured with the flask clamp which is screwed tightly in place.

9. The prepared flask is then placed on the platform in a up-right position in the oxygen igniter.

10. The door is closed and the hood shield is lowered.

11. The infrared lamp is then turned on for a short time so that the flask can be moved to align the black paper wick with the infrared light.

12. The IR light should be so aligned that it hits the black paper wick and not the platinum carrier.

13. The IR lamp is then turned on until the sample begins to burn.

14. Allow the sample to combust completely before removing the flask from the igniter.

15. Remove the flask and gentely invert and rotate the flask repeatedly until the internal walls of the flask and the stopper stem are completely washed.

16. Allow flasks to stand for approx. 1 hour (A minimum standing time of 30 minutes may be adequate). The repeat step #15.

17. With a volumetric pipette, remove 2 ml aliquot and place into plastic tubes and cap for latter analysis of inorganic sulfate.

18. Duplicate samples of urine should be run along with a blank. (The blank is 2 ml of the combusted solution with all reagents except 1.0 ml replaces the 1 ml of BGR).

19. The samples at this point are stable for at least 24 hours if stored in the refrigerator.

20. The samples are then prepared further for analysis of inorganic sulfate starting with step 1B4.

21. The ΔOD_{360} for urine is obtained by substracting OD_{360} for water and urine blank from the OD_{360} for urine.

22. The conc. of sulfate is obtained from the Na_2SO_4 standard curve which is a plot of ΔOD_{360} vs. Na_2SO_4.

23. The actual concentration of total sulfate in urine is obtained by multiplying this value by the dilution factor.
    0.1 ml sample into 10.5 ml total volume = 105
    $\therefore$ (Cuvette conc. SO_4) x 105 = original total SO_4 conc.

24. The neutral sulfate in urine is obtained by:
    (Total sulfate) - (hydrolyzable sulfate) = (neutral sulfate)

D. Precautions:

1. The dilute H_2O_2 solution must be prepared fresh daily, because it readily decomposes and results in an under estimation of inorganic sulfate.

2. The joint between the combustion flask and the glass stopper must be sealed with glycerine or lubriscal and further sealed by securing the stopper to the flask with the flask clamp.

3. The flask must be thoroughly rinsed with the solution immediately after combustion is completed and again one hour later (just before the 2 ml aliquot is removed). This is necessary because of gaseous sulfur condenses on the internal surfaces of the flask. If this washing procedure is not followed an underestimate of sulfur will result.

4. A standard neutral sulfur sample should also be analyzed daily to check the accuracy of the method. Therefore, pipette 0.1 ml of 27.39 mM/L cysteine-HCl (dried) onto filter paper and proceed as outlined above. This standard also requires a blank (i.e., BGR replaced with H_2O).

5. There is a possibility that the combustion could be explosive, resulting in breaking the flask. Therefore, the combustion procedure should be accomplished under a hood with the hood shield between the investigator and the igniter.

6. The flask stopper and platinum carrier should be thoroughly washed with top water, demineralized water and distilled water, in that order.

E. Reagents:

1. Na_2SO_4 standard (see inorganic sulfur method)

2. BGR
3. 10NHCl
4. H_2O_2 (1:4 dil 30%)
   25 ml 30% H_2O_2 diluted to 100 ml with H_2O
5. Cysteine - HCl standard
   M.W. (dried) = 157.63

$$\frac{215.84\text{mg}}{50\text{ ml}} \times \frac{1\text{ mmole}}{157.63\text{ mg}} \times \frac{1000\text{ml}}{\text{L}} = 27.3856\text{ mM/L}$$

$$\text{conc. in flask} = 27.39\text{ mM/L} \times \frac{0.1\text{ml}}{10.5\text{ml}} = 0.26082\text{ mM/L}$$

F. Characteristics of Total Sulfur Method:

1. Sensitivity = concentration in cuvette = 0.04 mM/L
   absolute amount in cuvette = 19.2 μg SO_4

2. Reproducibility = S.D. = ± 0.45 mM/L
   C.V. = 1.9%

3. Recovery of added
   indoxyl sulfate in urine = 104.0%
   indoxyl sulfate in H_2O = 105.7%
   cysteine-HCl in urine = 103.8%
   cysteine-HCl in H_2O = 101.1%
   L-cystine in H_2O = 96.8%
   Composite (indoxyl sulfate cysteine-HCl) soln in H_2O = 100.1%

4. <u>Sulfur Analysis in Human Sweat</u>

A. Inorganic sulfate

The average inorganic sulfate in 21 samples from 6 young men is 0.117 ± 0.076 (range = 0.043-A325). Since the lower limit of sensitivity for the method is about 0.04 μ moles/ml of SO_4^{2}, raw sweat can be used for the estimation of inorganic sulfate without first concentrating it. However, sweat as collected from the skin has considerable turbidity, and therefore, it must be first filtered to remove the turbidity. This can best be accomplished by using the Swinny Filter Holder (No. xx30 01200) with the millipore filter (No VCMP - 01300) attached to a suitable sized syringe.

1. The sweat is prepared for estimation of inorganic sulfate and acid hydrolyzable sulfate in the same manner as urine.

2. However, as noted above it must be first filtered with a millipore system to remove the turbidity.

3. For preparation start with step #3 of the inorganic sulfate in urine procedure.

4. It is recommended that the standard Na_2SO_4 concentrations used for the calibration curve be as follows.
   0.05 mM/L
   0.10 mM/L
   0.15 mM/L
   0.20 mM/L

5. Precautions

   a. The same precaution outlined for urine also apply for sweat.

   b. In addition, sweat has turbidity and must be filtered before it can be analyzed.

C. Total Sulfur

1. Under the conditions used for the est.of lot .S in urine, approx. 2 + mM SO_4/L must be in the solution.

2. Since sweat SO_4 normally is not that concentrated, it needs to be concentrated for it can be analyzed for total .S.

3. Sweat must be concentrated from 10 to 50 times. This can be accomplished by lyopholyzation or by low heat drying in an oven overnight.

4. 0.2 ml of conc. sweat is added to black filter paper and dried at 90°C for 1 hour. Note: water is held more strongly by sweat. Therefore, the heating conditions must be stronger in order to assure complete drying of the sample placed on the paper. Otherwise the paper will not combust completely.

5. After combustion, take 4 ml aliquot instead of 2 ml.

6. Add 0.1 ml 10 N HCl and 1 ml BGR.

7. Proceed as outlined for the inorganic sulfate method.

8. Characteristics

   a. sensitivity 0.04 mM/L or 192 in cuvette

   b. reproducibility SD = 0.016 mM/L C.V. = 5.6%

   c. recovery: indoxyl SO_4 = 103.6%
      L-cysteine = 96.4%

9. Precaution

   a. sweat must be concentrated

   b. sweat must be completely dried on paper for complete combustion

V. CHOLINERGIC TRAINING OR DENERVATED SWEAT GLANDS

The literature pertaining to this research deals with two main problems: 1) acclimatization and training and the ionic composition of sweat produced during these processes, and 2) the responses of denervated sweat glands to cholinergic stimulation.

During the course of acclimatization the volume of sweat produced in response to a standard level of heat stimulus increases (Robinson and Robinson, 1954). This change occurs not only in response to total body heat exposure, but can also be brought about by localized heat stimuli. Fox et al. (1962) found that repeated daily heating of only a portion of the body increases the sweating response of the heated area.

The observed changes could result from either of two phenomena. Each sweat gland could produce more sweat, or a greater number of sweat glands could become active. Data taken during acclimatization experiments indicate that the number of active sweat glands remains constant, thus each gland must secrete a greater volume of sweat (Sargent et al. 1965).

Several factors could act to produce the observed changes in the activity of the sweat glands. Changes in the sensitivity of the central nervous system to thermoregulatory stimuli may be a factor. Experimental evidence indicates that there is a quicker onset of sweating at lower body temperatures after acclimatization (Fox et al. 1963). These changes account for only part of the process of acclimatization, however.

A second possible factor in the sweat response is local skin temperature. It has already been noted that localized heat produces training of the sweat glands. The relative contributions of skin temperature and sweat gland activity have been elucidated in further experiments. Fox et al. (1964) inhibited sweating in one arm by local cooling while the remainder of the body was exposed to heat. The uncooled arm and total body sweat rates rose by 425% and 130% respectively. The sweat rate of the cooled arm did not increase. It therefore appears that the increased sweat rate associated with acclimatization is dependent on the increased secretory activity during acclimatization.

In order to eliminate the factor of low skin temperature of the previous experiment, Brebner and Kerslake (1962) inhibited sweating centrally by direct cooling. Subjects were immersed to the waist in water at 10°C. The oral temperature was elevated to 37.6° by heating the trunk with heat lamps for 110 minutes on ten consecutive days. Irradiation in warm baths produced profuse sweating while it did not in cold baths. The mean skin temperature was similar in both situations. The forearm sweat rate increased only slightly in the cold bath exposures. In the warm bath the forearm sweat rate increased substantially.

The response of sweat glands to direct repeated peripheral stimulus under cool conditions was studied using intradermal injections of acetyl-β-methylcholine (Mecholyl) sufficient to maintain sweating for two hours. An enhancement

comparable to that in heat acclimatization was brought about by this direct stimulation of the glands without any rise in core temperature (Collins, 1963).

In experiments that extended those of Collins we continued training beyond the ten day period that he used. We found that the sweat rate increased until the fourteenth or fifteenth day after which it plateaued at an elevated level.

In summary, the changes in the quantity of sweat produced in response to a thermal stimulus are apparently dependent on increased activity of the sweat gland and are due to a local training phenomenon.

As the volume of sweat produced per unit time changes, the composition of the sweat is also altered. Two types of phenomena, short term and long term changes, have been observed.

As the sweat rate increased during active sweating the chloride concentration of the sweat increases (Kawahata, 1950). Similarly the concentration of sodium also rises with increasing sweat rate (Schwartz and Thaysen, 1956). These changes occur in a matter of seconds or hours and are short terms in contrast to the changes accompanying acclimatization.

Reports on the long term changes in sweat sodium and chloride levels are confused. This is due in part to such variables as dehydration,, hormonal changes, salt depletion, and rectal and skin temperature which have been handled differently or totally neglected in various studies. In this work attempts will be made to control these factors or at least take them into account.

Johnson et al. (1944) gave evidence that dehydration tends to increase the salt concentration of sweat. In contrast, Ahlman et al. (1953) reported that the sweat concentration of men who had gone without water for 18 hours before sweat experiments was not different from that of men who were liberally prehydrated. Robinson and Robinson (1954) reported that long term changes associated with acclimatization result in a decline in both sodium and chloride concentrations. This change was brought about as a result of total body exposure to heat, so any number of factors could have produced the observed results.

We measured the sodium and chloride concentrations in sweat produced during training and found that as sweat rate increases, the sodium and chloride concentrations also increase. Once isotonicity is approached both sweat rate and ionic concentration ceased to increase and plateaued at the elevated level. Thus increased acitivty alone will not result in the production of a more dilute sweat. The possibility remains that there are temperature dependent local changes in the sweat gland that do result in decreased sodium and chloride concentrations.

Cannon (1939) noted that when the afferent neurons to a target tissue are destroyed "an increased irritability to chemical agents develops in the isolated structure of structures". This has since been known as Cannon's Law. Several authors have reported that the sweat gland is an exception to this law. Janowitz and Grossman (1951) observed the reaction of denervated sweat glands to parasympathomimetic drugs as early as 30 hours after denervation. The local sweating induced by intracutaneous injections of pilocarpine, physostigmine,

prostigmine, and A Ch was noted to disappear in from 30 hours to 7 days after sympathectomy. The sweat glands did respond to radiant heat, but the amount of heat necessary also caused local erythema and blistering. Similarly, Shelley and Florence (1960) reported that intradermal injections of pilocarpine and A Ch had no effect on the sympathectomized areas. Earlier reports contradict these findings, however. Lewis and Landis (1929) reported that in one case 1/7 grain of pilocarpine nitrate given subcutaneously produced free salivation and "very little" sweating on the 15th day following sympathectomy. In another case 1/5 grain of pilocarpine nitrate given subcutaneously on the 21st day produced profuse sweating over the entire body.

Wilson (1935) reported that "in man pilocarpine may cause sweating on the face after degeneration of all types of nerves in this area".

Guttman (1939) also reported that "sweat secretion induced by pilocarpine was noticable on the denervated side, especially in the face but it was diminished". The experiment was carried out four weeks after ganglionectomy. In several other cases in which the pilocarpine test was performed two to four weeks after the operation the subject showed "hypohydrosis of the denervated side . . ., although the sweat secretion began first in this area".

Some of the discrepancies in the data reported in the literature may be due to the techniques used. The same method is generally used to determine areas of denervation. Total body heat stimulus is usually used and the areas that sweat are made visible by the method of Silverman and Powell (1945) or a similar method. It is unlikely that the discrepancies are due to incorrectly identifying denervated areas.

The dosage of drug used may be the source of differences. Lewis and Landis (1929) note that "It seems probably that when pilocarpine has been reported as failing to produce sweating in an area deprived of its sympathetic nerve supply, that such sweating as has occurred on the remainder of the body may actually have been induced reflexly, or that, with a dose of pilocarpine not wholly adequate, the preexisting tone of the nerves to the sweat glands on the innervated side has sufficed to tip the balance and thus bring the glands on this side to visible activity. In this connection a frequent clinical custon to apply heat and pilocarpine simultaneously is to be noted".

As for the more recent work of Janowitz and Grossman (1951) and Shelley and Florence (1960), the comparatively small dose of drug used may have been too low to stimulate the denervated glands. In each case where sweating in denervated areas was reported, 1/7 to 1/5 grains or .0092 to .013 grams of pilocarpine was injected subcutaneously. In contrast, only 0.1 cc of 1:1000 pilocarpine was injected by Janowitz and Grossman. Shelley and Florence (1960) injected 1% pilocarpine intradermally. No volume was given, but 1/2 cc is a fairly large intradermal dose.

In the cat paw, Burn (1922) found that four to six weeks after denervation there was a diminished response to an injection of 0.5 mgm of pilocarpine. However, the denervated areas would respond to larger doses. If similar changes in sensitivity occur in man, a large dose of pilocarpine may be required to elicit a response. Thus the question of the ability of denervated sweat glands to respond to cholinergic drugs remains open.

An interesting possibility is that the sensitivity of sweat glands to cholinergic stimuli may depend on the prior stimulus level. If so, repeated daily stimulation might increase the sensitivity of denervated sweat glands to cholinergic drugs. In previous training experiments the glands have started at some "normal" level of sensitivity and their sensitivity has increased. Possibly training could begin at zero function, or greatly reduced sensitivity, and increase from there. This problem will be investigated.

The objectives of this research will be as follows:

1. To determine the response of denervated sweat glands to a single dose of pilocarpine.

2. To determine whether pilocarpine will cause training when applied repeatedly for several days.

3. To observe the responses of denervated sweat glands to repeated cholinergic stimulation.

4. To observe the time course of training by pilocarpine and/or methacholine.

In all of the experiments a consistent procedure must be followed in the preparation of the skin prior to collections.

Although this problem has recieved little attention by many workers, it is of great importance. Since the skin can be a source of contamination of sweat samples, it is generally cleansed prior to collection. However, the method of cleansing is not clearly defined. If the skin is scrubbed, this will result in some local trauma and erythema. Different soaps, especially medicated ones, contain substances that may have different effects on the sweat glands. The effects of these substances have never been evaluated.

The water vapor pressure of the air above the skin may also result in alterations of sweat rate and/or composition. Brown and Sargent (1965) showed that in a humid environment a decline in sweating occurs. This has been termed hidromeiosis. In another study using hot baths this phenomenon was also demonstrated (Johnson et al. 1966). Although Segar et al. (1965) found that high water vapor pressure under a water-proof patch did not alter the sweat collected this factor may be of importance when larger areas are involved.

In addition to the treatment of the skin, other factors must be controled during collection periods. The state of hydration, salt intake, and metabolism will be standardized.

The technique of iontophoresis has been sufficinetly developed so that pilocarpine can be administered by this method. A second problem in subjects that are only partially sympathectomized is to map out the areas that are denervated. This is done with the technique of Silverman and Powell (1945). A tincture of ferric chloride in ethanol is applied to the skin and allowed to dry. Tannic acid powder is applied to the dry area. Upon contact with sweat the tannic acid reacts with the ferric chloride to form a black stain that sharply outlines areas of sweating activity.

Once the areas that are denervated have been identified, the sweating response of a denervated area to pilocarpine will be studied. A normally innervated area will be used as a control. Sweat samples will be collected on filter papers in a sweat capsule.

The second group of experiments will determine whether repeated daily stimulation with pilocarpine results in training. If training is dependent only on activity of the sweat glands, as Collins proposes (1963), then one would expect pilocarpine to promote training. The response could be specific for acetylcholine or its derivatives, however.

If training can be accomplished with pilocarpine, this drug will be applied by iontophoresis and used to attempt training of denervated sweat glands. As before, normally innervated areas will serve as controls.

Previous findings are consistent with the hypothesis that the response of a sweat gland to a standard dose of stimulus is dependent on the previous stimulus level. Thus when a small area of skin is stimulated locally by a drug or heat, this stimulation is added to the normal "background" level from the central nervous system. The sweating response to a standard dose of drug subsequently administered will be greater than it would have been prior to the training. This ability to increase sweat production does seem to have an upper limit. In previous experiments there has been a plateauing of sweat rate once isotonic sweat is produced (Johnson et al. 1967).

The question that now arises is whether denervated sweat glands will resume production when once again exposed to a cholinergic stimulus. Can training begin at a level of zero function as well as from some normal state? In order to answer this question training of denervated areas with repeated applications of a cholinergic drug will be attempted. If pilocarpine is found to be suitable for this purpose, it will be used. If this is not the case, methacholine, which has been shown to evoke training, will be used. This would present a problem in the administration of the drug. Previously intradermal injections have been used, but this method has several drawbacks. One of these is a focus of high drug concentration in the area of the wheal and a gradient of decreasing concentration further from the injection site. A second problem is the repeated trauma to the skin in the area of the injection site. Possibly methacholine could be introduced by iontophoresis, but there are few references to this in the literature and further study of this method would be necessary.

In his training studies, Collins (1963) found that two hours of active sweating daily were sufficient to bring about training. This period of time has been used in our training experiments. It is possible that the rate of training could be increased either by increasing the length of the daily period of active sweating, or by stimulating more frequently than once a day. We plan to extend the period of stimulation to three hours daily in one series of experiments. In another we will stimulate for two hours twice daily. Clearly the time requirements for these studies pose a problem when human subjects are used.

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