be used to cut sections as thin as 0.1 μ, ours was especially made,* hollow ground on both sides, with a long bevel.

Fixing and Mounting Technics. Several technics have been used to prepare tissues for microtomy. Fixation in about 4% neutral formalin, dehydration through graded alcohols, clearing in xylene, and imbedding in paraffin (85°C melting point) has proved to be one of the most successful. With this method it is not necessary to "double imbed"; that is, to use both celloidin and paraffin.

The process of mounting a tissue section on a grid for electron microscopy varies according to the specimen and the operator, but in general the following technic has been successful. The section is transferred directly from the knife to a glass slide. A dissecting needle with a microscopic point is used to lift and move the sections. The point may be inserted slightly into the edge of the paraffin of the first section of a "ribbon" and the "ribbon" pulled out somewhat. A drop of warm water added to the section on the slide will further "spread" the section. When the water has dried, a drop of xylene may be added, or the whole slide may be immersed in xylene, to dissolve the paraffin from the tissue.

When the tissue is thoroughly dry, the slide is immersed in 2% collodion in amyl acetate which is thin dried in an even film. Lines are scored around the specimen; the slide is breathed upon and immersed gently into water. The section adheres to the collodion film which strips from the glass slide and floats free. A grid is brought up beneath the specimen and both are lifted from the water. Thus the section and the supporting collodion film may be centered on the grid ready for electron observation.

Fig. 2 illustrates some results obtained with the equipment and technics described.

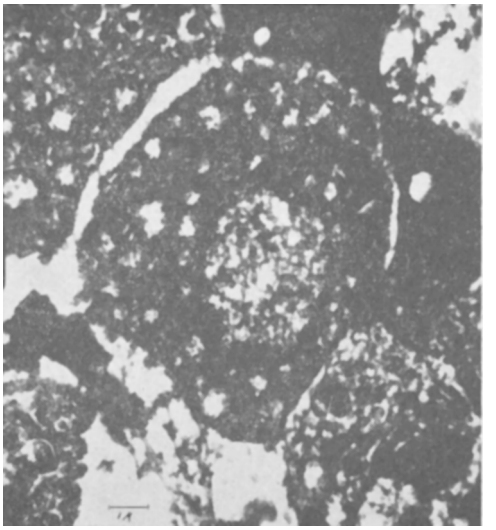

FIG. 2.
Section of rat intestine, sectioned at 0.1 μ. The greater part of the field is filled with a single cell. The nucleus appears less dense than structures seen in the cytoplasm. Individual structures have not been identified. × 13,700.

* Holzheimer, William, Melrose Park, Illinois.

Received July 12, 1949. P.S.E.B.M., 1949, **71**.

17291. The Rate and Total Loss of Body Water on the Survival Time of Adrenalectomized Frogs.*†

CLIFFORD A. ANGERER AND HELENA H. ANGERER.
From the Department of Physiology, The Ohio State University, Columbus.

One aspect of the adrenal problem is the relation of the adrenal cortex to the regulation of water and electrolytes in body fluids. The water content of certain tissues and, more pertinently, of certain cells (eviscerated carcass and liver of rats;[1] skeletal muscles of

* This investigation was aided by the Comly-Coleman Fund of the Ohio State University.

† This work was originally initiated at our suggestion by Martin W. Williams.

[1] Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1933, **104**, 399.

rats[2] and frogs,[3] and non-nucleated erythrocytes of dogs,[4] cats,[5] and rats[6]) is known to increase following adrenalectomy. Water shift in final analysis is the resultant of opposing osmotic forces acting across the plasma membrane of the particular cell in question. Since water shift, and more specifically osmotic pressure, is the variable under consideration, it appears from the very nature of the problem that an aquatic animal, like the frog, is the experimental material of choice. The frog imbibes water continuously through its integument when in an aqueous environment.[7]

The present studies were undertaken to determine the effect of known durations of exposure to a constant dehydrating force on *a*, rate and on *b*, total loss of body water, and on *c*, survival time of adrenalectomized frogs.

Method. Male frogs (*Rana pipiens*) weighing between 20 and 35 g and showing fat bodies on autopsy were used in these experiments. All frogs employed in this work may be conveniently divided into 4 groups (18-22 frogs/group): adrenalectomized frogs whose postoperative body weights were either (1) *uncontrolled,* that is, no attempt was made during the postoperative period to maintain the frog's weight at its preoperative value or (2) *controlled,* that is, the frog's postoperative body weight was maintained to within ± 1.5 g of its preoperative value, and the controls which consisted of both (3) *unoperated* and (4) *renal damaged* frogs.

Adrenalectomy was performed by "cold" cautery. Adrenal insufficiency was determined by the characteristic failure of the individual frog to perform successfully the righting reflex in not less than 3 and not more than 5 successive attempts, after previous observation of its stance and color.[8,3]

Dehydration was effected by placing the desired frog in a closed system (Scheibler desiccator) of constant volume (2230 ml). This chamber was lined, except for the upper surface and a lateral window, with a dehydrating agent (anhydrous $CaCl_2$). The system was continuously flushed during the experiment, except at the time of weighing of the frog, with washed, dried air entering via an 8-mm inlet under a pressure of 4.75 mm Hg. A given frog was placed in a closed wire basket of such size as to prevent excessive movements and of such design as to permit observation of all aspects of the body surface. This basket served both as a scale pan for it was suspended via a separate vent, provided for closure during the experiment, to a superimposed beam of an analytical balance, and as an electrode, for the basket was in circuit with 1 lead of an inductorium. The other electrode was stationary, though in such a position as to contact any portion of the ventral surface of the frog in its movable cage. A frog, after being placed in the desiccating chamber, was immediately weighed to the nearest 0.1 g. The moment of initial weight determination (elapsed time *ca.* 1 min.) was considered zero time. All subsequent weighings were made at half-hour intervals until the animal was declared dead (death point).

The death point was determined by the persistent absence of rhythmical heart beats. A very effective preliminary index, less arduous and thus less time-consuming, was the disappearance of skeletal muscle reflexes on faradic stimulation of the ventral integument with a pointed exploratory electrode, and also the less consistent, but more readily observed, buccal respiratory movements. The heart and buccal movements tend to accentuate as body volume decreases with continued dehydration. This favorable circumstance tends to offset the enfeebling of these movements prior to the terminal stage.

Autopsies were performed on all frogs immediately upon termination of an experimental run in order to confirm the continued

[2] Crismon, J. M., and Field, J., 2nd., *Am. J. Physiol.*, 1940, **130**, 231.

[3] Angerer, C. A., and Angerer, H. H., *Fed. Proc.*, 1942, **1**, 3.

[4] Harrop, G. A., *Bull. Johns Hopkins Hosp.*, 1936, **59**, 11.

[5] Hegnauer, A. H., and Robinson, E. J., *J. Biol. Chem.*, 1936, **116**, 769.

[6] Gonzalez Q., J., and Angerer, C. A., *Am. J. Physiol.*, 1947, **149**, 502.

[7] Adolph, E. F., Physiological Regulations, p. 110, The Jaques Cattell Press, Lancaster, Pa., 1943.

[8] Maes, J., *Arch. Intern. de Physiol.*, 1937, **45**, 135.

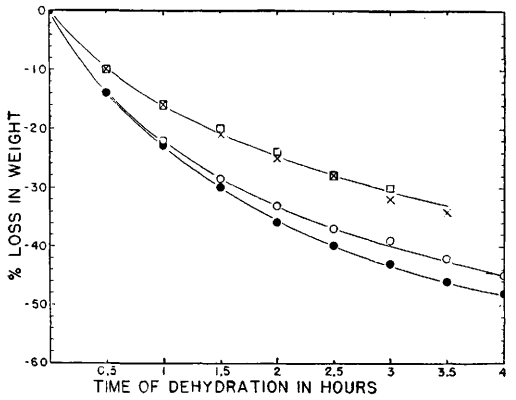

FIG. 1.

Percentage loss in body weights (water loss) of 4 groups of frogs plotted as functions of respective times (readings at ½ hour intervals) of exposure to a constant force of dehydration. The characters used to delineate the curves for each group are as follows: adrenalectomized frogs whose postoperative body weights were either uncontrolled (X), or controlled (□) to within ± 1.5 g of their respective preoperative values, and the control frogs—renal damaged (○) and unoperated (●). Volume of dehydrating system = 2230 ml, dry air current + 4.75 mm Hg, temp. 18-22°C.

absence of heart action and presence of fat bodies. In only 3 frogs of the 4 groups studied were feeble heart beats observed on autopsy which were not detected immediately before. These hearts failed to survive the half-hour interval following the close of the experimental run. These data are not included in the results presented.

Results. The various mean values for the percentage loss in body weights for the 4 groups of frogs are plotted in Fig. 1 as functions of time of exposure in hours to a constant dehydrating force. The means of the various data have been statistically compared for significance by "Student's" method and are presented together with their respective "t" values in Table I. The figure and table are self-explanatory after recourse to their accompanying legends.

When data from the 4 groups of frogs are analyzed for *a,* rate of loss and *b,* total loss of body weight, and *c,* duration of survival, the following orders of statistical significance are indicated (see Table I): the 2 groups of controls (renal damaged and unoperated) show no significance for the foregoing items *b* and *c,* though *a* is probably significant. For reasons to be discussed, the renal damaged frogs are considered the controls for all subsequent comparisons. A comparison of data from the adrenalectomized frogs gives no significance for the aforementioned items *a, b,* and *c.* However, when either group of adrenalectomized frogs are compared with their controls, a high degree of significance is found for items *a* and *b,* and for item *c* as it affects the controlled but not the uncontrolled adrenalectomized frogs.

Discussion. The assumption is made here that any loss in body weight during the relatively short period of exposure to a constant force of dehydration is due to loss of body water.

The only significant difference between data from the 2 groups of controls (unoperated and renal damaged) lies in the respective rates of water loss. This difference is interpreted as due to the reduction in the total effective dehydrating surface of operated frogs arising from the encroachment of the thickened integument at the line of suture (4-5 cm) and of the loss in integument in effecting this suture. For this reason, the renal damaged frogs must be regarded as the true controls.

Since the difference between the respective means for data obtained from the 2 adrenalectomized groups is not significant, it remains to compare both of these groups with their controls. It has been shown that uncontrolled adrenalectomized frogs undergo postoperatively a progressive increase in body weight, so that, *e.g.,* on 7 and 12 days the mean body weight has increased by 28 and 33% respectively.[9] The 2 groups of adrenalectomized frogs when compared with the control show a highly significant decrease in the rate and in the total loss of body water; they differ between themselves in that the survival time is probably significant ($P < 0.02$) for the controlled but not for the uncontrolled ($P > 0.05$) adrenalectomized frogs. Were, possibly, the increase in osmotic pressure, resulting from forced dehydration, the underlying cause of this difference, then the uncontrolled, having the greater initial water load

[9] Angerer, C. A., unpublished data.

TABLE I.
Summary and Comparison (t-test) of Mean Values for All Data (Line 3) Obtained from the Various Groups of Frogs Subjected to a Constant Force of Dehydration.

Frogs	Adrenalectomized						Control					
Experimental groups	1 Wt uncontrolled			2 Wt controlled			3 Normal			4 Renal damaged		
Statistical classes	*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr	*a* %	*b* %	*c* hr
Mean	24.2	32.2	3.3	24.6	28.4	2.7	35.8	48.0	4.0	32.9	43.4	3.8
S.E. ±	0.7	1.9	0.3	1.2	1.4	0.4	1.0	1.0	0.1	0.7	1.2	0.1
S.D. ±	2.3	6.6	1.0	4.0	4.7	1.4	3.4	3.5	0.4	2.3	4.0	0.5
t-test 2	0.30 NS	1.63 NS	1.19 NS									
t-test 4	9.32 HS	5.07 HS	1.55 NS	6.20 HS	8.43 HS	2.54 S	2.46 S	1.53 NS	1.09 NS			

Any given letter among the following (line 3) indicates the same variable studied in any group of frogs (lines 1 and 2) and this meaning is used throughout the text:
a = % loss of body weight at the end of 2-hour period of dehydration;
b = % total loss of body weight at death-point;
c = Duration of dehydration in hours until death-point.
S.E. = Standard error, and S.D. = Standard deviation of respective mean.
t-test = Statistical comparison of ratio of difference between an indicated pair of means/estimated standard error of this difference.
Statistically: NS = Not significant (P >0.05); S = Significant (P 0.05-0.01); HS = Highly significant (P <0.01).

in comparison with the controlled adrenalectomized frogs, not only should survive longer but also should suffer the greater rate and the greater total loss of body water. None of these postulates is met on comparing the 2 groups of adrenalectomized frogs; but on comparing the latter groups with their controls the reverse tends to be true. The conclusion reached is that the adrenalectomized frogs can tolerate relatively slight loss in body water before lethal effects are encountered. This is not due to any decrease in permeability of the integument of adrenalectomized frogs, for all evidence points to an increase in permeability[10] in general and for frog skin[9] in particular.

The decreased rate of dehydration found in both groups of adrenalectomized frogs may be interpreted as due to the immediate decrease in replenishment of body fluid at the body surface. This condition may arise from the hemoconcentration and hemostasis known to occur in peripheral vessels as a result of adrenocortical insufficiency or ablation.[11] Physiologically, forced dehydration has much in common with sweating but without benefit of the concomitant vasodilatation arising from the increase in environmental temperature. Sweating, and dehydration, like adrenocortical insufficiency, leads to a further decrease in plasma volume.[12] Thus, a decreased plasma volume, together with an increased blood viscosity and osmotic pressure, has a deleterious effect on an already weakened heart action[13] which follows in the wake of adrenalectomy.

That mammals deficient in adrenal cortical hormone are less able to withstand various forms of stress (certain types of drugs, poisons, toxins, infections, variations in environmental temperature, barometric pressure, and traumatic procedures) is too well-known to require elaboration.[14] Thus, an increase in osmotic pressure induced either by a re-

[10] Hartman, F. A., *Endocrinology*, 1942, **30**, 861.

[11] Swingle, W. W., Vars, H. M., and Parkins, W. M., *Am. J. Physiol.*, 1934, **109**, 488.

[12] Adolph, E. F., Physiology of Man in the Desert, p. 170, Interscience Publishers, Inc., New York, N. Y., 1947.

[13] Nicholson, W. M., and Soffer, L. J., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 236.

[14] Swingle, W. W., and Remington, J. W., *Physiol. Rev.*, 1944, **24**, 89.

duction of free-moving particles or by a possible increase of osmotically-active particles, is to be considered as another example of stress with which the adrenalectomized organism fails to cope.

Summary. All frogs employed in this study may be conveniently divided into 4 groups (18-22 animals/group): the adrenalectomized frogs whose postoperative body weights were either controlled, to within ± 1.5 g of their individual pre-operative values, or uncontrolled; and the controls, both renal damaged and unoperated frogs. The individuals of each group were subjected to a constant dehydrating force and the resulting data were statistically analyzed with respect to the following points: *a*, the rate of loss and *b*, the total loss of body weight (water) and *c*, the duration of survival on exposure to a constant force of dehydration.

1. A comparison of the difference between respective means of the 2 groups of adrenalectomized frogs shows no significance as regards the foregoing items *a*, *b*, and *c*. 2. Comparison between the 2 groups of controls (renal damaged and unoperated frogs) gives no significance with respect to items *b* and *c*, though it does for *a*. 3. A comparison between the respective means for either group of adrenalectomized frogs and their controls (renal damaged) produces a significance for *a* and *b*, and for *c* as it affects the death point of the controlled but not of the uncontrolled adrenalectomized frogs. 4. On the basis of the known cardiovascular embarrassment subsequent to adrenalectomy, it is suggested that the increased osmotic pressure resulting from the forced water loss and the attendant decrease in peripheral circulation brings an increased osmotic stress to bear on an already weakened heart action. It is suggested that this stress is the deleterious factor in affecting the physiological points raised.

Received July 5, 1949. P.S.E.B.M., 1949, **71**.

17292. Use of Antitryptic Agents in Tissue Culture. I. Crude Soybean Trypsin-Inhibitor.*†

JOSEPH F. MORGAN AND RAYMOND C. PARKER.

From the Connaught Medical Research Laboratories, University of Toronto.

Substances that inhibit the proteolytic activity of trypsin have been found in serum and plasma,[1] in egg white,[2] in navy beans and soybeans,[3] and in extracts of pancreas.[4,5] Because of the great activity of these antitryptic agents, it seemed of interest to investigate the possibility of using them in tissue culture as a means of preventing the digestion of the plasma coagulum that frequently occurs during the growth of cells *in vitro*.[6-8] The soybean antitrypsin,‡ which has been

* This investigation was supported, in part, by grants from the National Cancer Institute of Canada, and the Ontario Cancer Treatment and Research Foundation.

† Grateful acknowledgement is made to Miss M. Ogilvie, Mrs. C. J. Porter, and Mr. C. J. MacFayden for technical assistance.

1 Grob, D., *J. Gen. Physiol.*, 1943, **26**, 405.

2 Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, 1934, **106**, 409.

3 Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 139.

4 Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

5 Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., *J. Am. Chem. Soc.*, 1948, **70**, 3034.

6 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

7 Losee, J. R., and Ebeling, A. H., *J. Exp. Med.*, 1914, **19**, 593.

8 Santesson, L., *Acta path. et microbiol. Scand.*, 1935, Suppl. **24**

‡ It is interesting to note that Fischer has just reported (Fischer, A., *Science*, 1949, **109**, 611) a series of experiments with crystalline soybean trypsin inhibitor supplied by Kunitz.[9] His results are in complete accord with those reported here.

9 Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.