Western equine encephalomyelitis virus. On the other hand, however, if the St. Louis encephalitis virus did not penetrate all of the nerve endings, in certain of the rats, the subsequently injected Western equine encephalomyelitis virus could reach the brains of these rats over a limited number of nerve fibers and cause a fatal encephalitis. That the Western equine encephalomyelitis virus did reach the brain by passing over a limited number of nerve fibers was suggested by the observation that in those animals which did succumb, the incubation period was longer and the animals survived for a longer period than did controls which had not received St. Louis encephalitis virus before the Western equine encephalomyelitis virus was injected. In the experiments reported here it was found that a single intranasal inoculation of St. Louis encephalitis virus protected 65% of the rats against Western equine encephalomyelitis virus. However, when rats were given 3 inoculations of St. Louis encephalitis virus before the equine encephalomyelitis virus was inoculated a greater degree of protection was noted since 95% of rats so-treated survived. From the findings reported here, as well as elsewhere(1) it would appear that St. Louis encephalitis virus, having passed over the fibers of the olfactory nerve and being present in the brain, "interferes" with the subsequent passage of Western equine encephalomyelitis virus over these fibers and thus prevents the latter virus from reaching the brain. The degree of interference and thus the percentage of animals that survive can be increased if 3 rather than 1 inoculation of St. Louis encephalitis virus precedes the inoculation of Western equine encephalomyelitis virus.

Summary. A single intranasal inoculation of St. Louis encephalitis virus protected 65% of 3- to 4-week-old rats against Western equine encephalomyelitis virus subsequently inoculated by the same route. The percentage of animals protected was increased to 95% if 3 inoculations of St. Louis encephalitis virus preceded the Western equine encephalomyelitis virus.

1. Jordan, R. T., and Duffy, C. E., *J. Inf. Dis.*, in press.

2. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

Received April 22, 1952. P.S.E.B.M., 1952, v80.

A Biological Method for Determining Small Quantities of Sodium Retaining Substances.* (19596)

C. M. KAGAWA, ELVA G. SHIPLEY, AND ROLAND K. MEYER.

From the Departments of Zoology and Medicine, University of Wisconsin, Madison.

Numerous investigations on the retention of urinary sodium for the quantitative estimation of adrenal cortical extracts and desoxycorticosterone have been made in adrenalectomized dogs(1-5), mice(6), and rats(7,8). Several workers(9,10) have taken advantage of the increased potassium excretion in rats treated with desoxycorticosterone as a means of estimating salt-regulating activity. Widespread investigation of the effect of desoxycorticosterone on sodium and potassium excretion has been somewhat restricted by the lack of a practical test, and a satisfactory experimental animal.

This report is concerned with sodium excretion in the adrenalectomized rat in response to various dose levels of desoxycorticosterone acetate with a constant load of administered sodium.

Material and methods. Biological. Male albino rats, obtained from Holtzman-Rolfsmeyer Co. (Madison, Wis.) and weighing from 150-155 g, were adapted to general laboratory conditions for 48 hours in group

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service, and the Wisconsin Alumni Research Foundation.

TABLE I. Sodium Excretion and Urine Volume Changes of Adrenalectomized Rats Given Desoxycorticosterone Acetate.

DCA, μg	No. animals	mg Na exer., avg ± S.E.	Na retained mg	Na retained %	ml urine vol, avg ± S.E.	Urine vol, % change
0	19	2.65 ± .13	—	—	1.21 ± .11	—
1	9	2.58 ± .18	.07	2.5	1.09 ± .13	— 9.9
2	12	1.88 ± .15	.77	29.1	.97 ± .09	—19.8
4	11	1.40 ± .12	1.25	47.2	.87 ± .12	—28.1
6	9	1.09 ± .15	1.56	59	1.34 ± .20	+10.7
12	6	.30 ± .04	2.35	88.7	.75 ± .20	—38

cages, after which time they were further adapted for about 24 hours to individual cages. Following the period of adaptation, the animals were bilaterally adrenalectomized under ether anesthesia. They were fed Rockland Farm rat pellets *ad libitum* both pre- and postoperatively. Tap water given to unoperated animals was replaced by a 5% Cerelose (corn sugar) solution for the adrenalectomized rats. Eighteen and one-half hours after the last adrenalectomy (designated as zero hour), the animals were deprived of food pellets, but were allowed Cerelose solution for an additional 4.5 hour period. Distilled water replaced the Cerelose solution at the 23rd hour, and constituted the sole source of water intake through the remainder of the test. On the 24th hour after the last operation, one hour after removal of the Cerelose solution, each control animal was injected subcutaneously in the shoulder region with 0.05 ml corn oil, followed by a subcutaneous injection of 2.5 ml of a freshly-prepared 0.85% solution of sodium chloride (equivalent to 8.29 mg sodium) divided at 2 sites in the shoulder region. Other animals were similarly injected with 0.05 ml oil solutions containing desoxycorticosterone acetate (DCA) at various dose levels, followed by the salt injection. Three hours later at the 27th hour, a second volume of 0.05 ml corn oil, or oil solution of DCA, was similarly injected into the shoulder region of the respective groups of animals. The total volume of oil, or oil solutions of DCA, injected in 2 stages was 0.1 ml, and the dosage level of the hormone was expressed in terms of total μg of DCA in this volume. One hour following the oil or DCA injections the rats were anesthetized with ether. The urinary bladder was emptied by suprapubic pressure and by the effects of the ether *per se,* but the bladder was not rinsed. While the rat was anesthetized the penis was extruded from its loose prepuce with the aid of forceps and held with a suspended towel clamp. The urethra in the region of the glans penis was ligated twice with No. 10 cotton thread, and after release of the clamp the penis was replaced in its original position. Each animal was returned to its individual cage, without food, but continued with distilled water, for exactly 2 hours. At the end of this period (the 28th to the 30th hours), the animals were sacrificed by cervical dislocation, and the urinary bladder was exposed. The basal portion of the dilated urinary bladder was firmly clamped with a pair of forceps, and the bladder removed by an undercut with a pair of scissors. The bladder, still securely clamped, was rinsed in a beaker of distilled water to remove blood and body fluids, lightly blotted to rid it of excess water, then transferred to a small funnel stoppered with a cotton pellet. The urine was filtered into a 5 or 10 ml burette which contained water at a known level for measurement of small quantities of urine. After measurements of the urine volume by difference, the contents of the burette were emptied into graduated 50 ml centrifuge tubes. The bladder was opened by several cuts with a pair of scissors and the bladder, forceps, scissors, funnel and burette were thoroughly rinsed with small portions of distilled water. The cotton pellet was similarly rinsed several times, alternating flooding and compression. All rinsings were added to the urine sample in the 50 ml centrifuge tube and diluted to the 25 ml mark after protein and phosphate precipitations.

Chemical. The total amount of sodium excreted in each sample was determined colorimetrically(11).

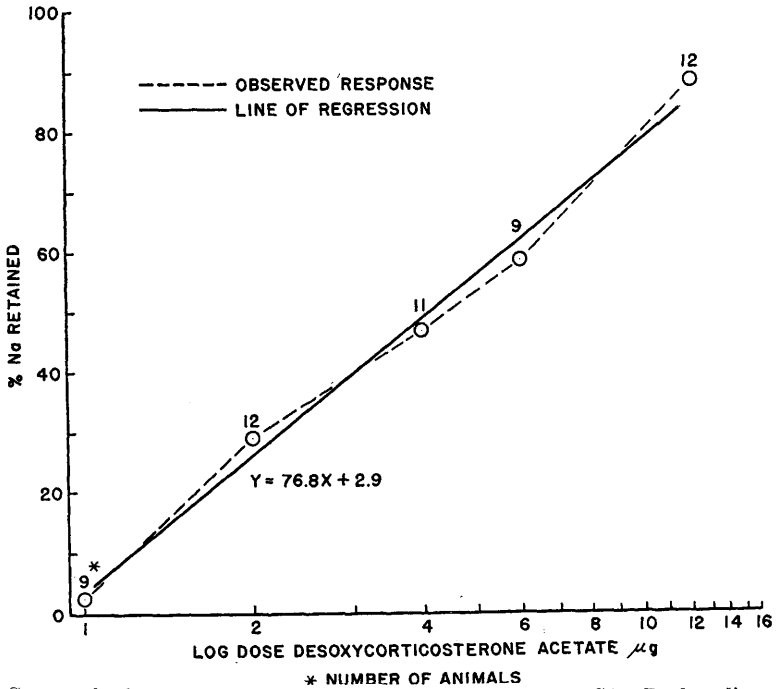

FIG. 1. Curves obtained by plotting % Na retained *vs* log μg DCA. Broken line represents observed response and solid line the line of regression.

Results and discussion. The results of the urine sodium and volume changes in the control and DCA-treated groups of animals are summarized in Table I. An inspection of the table will show that the bioassay of DCA described here, utilizing bilaterally adrenalectomized rats is an extremely sensitive one. Highly significant retention of urinary sodium ($P = <0.01$) was obtained with total doses of 2 μg or more of DCA, as compared to the oil-injected groups of animals. With 2 μg of DCA, sodium excretion was reduced from an average of 2.65 mg in the control group to 1.88 mg. or to the extent of 0.77 mg. Progressive increases in the levels of DCA caused corresponding increases in retention of sodium. Significant changes ($P = <0.05$) in sodium excretion were observed between the doses of 2 and 4 μg of DCA, and between 4 and 6 μg of DCA. Highly significant "t" values of 3.66 and 4.96 were obtained for the differences in sodium excretion between 2 and 6 μg DCA, and between 6 and 12 μg DCA, respectively.

Urine volume change of significance occurred between the oil-injected control and 4 μg DCA treated groups of animals ($P = <0.05$). No significant urine volume changes were observed with other levels of DCA relative to the control group of animals. Differences in the urine volume of animals given the 6 and 12 μg doses of DCA, and between those given 4 and 6 μg DCA, were analyzed statistically, but no significant differences were found.

Plotting the log of the dose of DCA against the percentage sodium retained, a curve with points essentially on a straight line (Fig. 1, broken line) was obtained. The line of regression (Fig. 1, solid line) was determined from the various mean percentages of sodium retained with doses from 1 to 12 μg DCA, by use of the equation $y = y + b(x - \bar{x})$ as discussed by Fisher(12) and by Burns(13), with b representing the slope of the line, y the percentage of sodium retained, $\bar{y}$ the mean value of y, x the log dose of DCA, and $\bar{x}$ the mean value of x. The value of b was calculated from the formula $b = \Sigma y(x - \bar{x})/\Sigma(x - \bar{x})^2$.

Calculation of the index of precision ($\lambda = s/b$, where s is the average standard

deviation in response independent of dose and b is the slope of the curve) indicates a satisfactory degree of precision for the method ($\lambda = 0.256$). The biological technic as presented here is a convenient short-term test which requires a lapsed time of about 30 hours to complete, exclusive of the maintenance of the animals for adaptation, adrenalectomy, and analysis for sodium. With such a short-term test, however, one must observe strict adherence to scheduled procedures. The method of assay as described precludes the requirement for special technical skills, and eliminates the need for special apparatus.

Dorfman, Potts, and Feil(7) reported significant retention of radiosodium with 1 μg of desoxycorticosterone in adrenalectomized male rats. More recently, Deming and Luetscher (8) proposed a bioassay for desoxycorticosterone-like substances sensitive in the order of 10 μg of desoxycorticosterone acetate (DCA) utilizing selected adrenalectomized female rats. The authors indicated, however, that variations in the urinary excretions of sodium in different groups of animals make necessary a preliminary testing with DCA to insure accurate evaluation of a test material. Spencer(6), utilizing adrenalectomized mice, selected on the basis of capacity to excrete 70 to 130% of a sodium load within 6 hours, demonstrated 15% sodium retention in crossover tests with a dose of about 0.5 μg of desoxycorticosterone acetate per 30 g mouse. An inconvenient feature of the biological assays of Spencer(6), and Deming and Luetscher(8) rests on the problem of preliminary selection of animals. The high incidence of adrenal cortical rests in some strains of mice (14) decreases their usefulness as test animals. Simpson and Tait(15) in dose-response studies on the effect of desoxycorticosterone acetate on radiosodium excretion in adrenalectomized rats, observed a decrease in the sodium excretion rate over that of the controls with only 0.5 μg of the hormone. No tests for significance were reported.

Deming and Luetscher(8) reported on the problems of supply, variable activity, measurement of radiosodium, and daily variations in the uncontrolled sodium intake of the animals for the method of Dorfman, Potts, and Feil(7). The problems of technical skill and special apparatus, make the method of Dorfman, Potts, and Feil(7), and of Simpson and Tait(15) less practical from the standpoint of general bioassay procedures than the method presented here. An early study on potassium excretion in rats with the salt-regulating hormone was reported(9). Dorfman(10) observed significant increases in urinary radiopotassium chloride in amounts of 146 to 280 μg (containing radio-potassium) per g of body weight. The indications are, however, that further studies would be required for the development of a quantitative assay of desoxycorticosterone activity.

Summary. 1. A practical micro-bioassay technic for desoxycorticosterone acetate activity is proposed, using albino, urethra-ligated, bilaterally adrenalectomized, male rats of 150-155 g weight. 2. A calibration curve is demonstrated using desoxycorticosterone acetate as the standard hormone and the percentage sodium retained as the measure of its activity. Two μg DCA injected subcutaneously in 2 stages produce a highly significant retention of administered sodium. 3. The proposed bioassay requires 30 lapsed hours and a minimum of special technical skills and apparatus, but a high degree of sensitivity and a satisfactory degree of precision are indicated. 4. No consistent correlation was found between urine volume changes and DCA dosage levels. A significant change in urine volume was observed with 4 μg DCA relative to the control group of animals, but not with the higher or lower doses of DCA studied.

1. Loeb, R. F., Atchley, D. W., Benedict, E. M., and Leland, J., *J. Exp. Med.*, 1933, v57, 775.

2. Harrop, B. A., and Thorn, G. W., *J. Exp. Med.*, 1937, v65, 757.

3. Hartman, F. A., and Spoor, H. J., *Endocrinology*, 1940, v26, 871.

4. Hartman, F. A., Lewis, L. A., and Thatcher, J. S., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 60.

5. Olsen, R. E., Jacobs, F. A., Richert, D., Thayer, S. A., Kopp, L. J., and Wade, N. J., *Endocrinology*, 1944, v35, 430.

6. Spencer, A. G., *Nature*, 1950, v166, 32.

7. Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, v41, 464.

8. Deming, Q. B., and Luetscher, J. A., PROC.

SOC. EXP. BIOL. AND MED., 1950, v73, 171.

9. West, G., *Quart. J. Pharm. and Pharmacol.*, 1941, v14, 26.

10. Dorfman, R. I., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 732.

11. Kagawa, C. M., Shipley, E. G., and Meyer, R. K., To be published.

12. Fisher, R. A., *Statistical Methods for Research Workers*, 1925, Oliver and Boyd, Edinburgh, p117.

13. Burns, J. G., *Biological Sandardization*, 1937, Oxford University Press, London, p26.

14. Jackson Memorial Lab., Bar Harbor, Maine, Personal communication.

15. Simpson, S. A., and Tait, J. F., *Endocrinology*, 1950, v47, 308.

Received March 14, 1952. P.S.E.B.M., 1952, v80.

Production of Pigments Similar to Those from Hair from Amino Acid and Keratin Interaction. (19597)

BERTRAM L. HANNA.* (Introduced by C. R. Moore.)
From the Department of Zoology, University of Chicago.

Arnow(1) found that a red pigment obtained from red human hair by continuous boiling with 0.1 N hydrochloric acid for 3 days or longer has the same absorption spectrum as solutions of oxidized (red) dopa melanin, and concluded that the red pigment is a natural oxidation product of melanin. However, Lee and Penrose(2) obtained a red pigment from the hair of a human albino by the same method and suggested that the color is not of natural origin, but is produced from some colorless chromogen in the hair.

Although soluble pigment is formed during the hydrolysis of the keratin in whole hair with strong mineral acids(3,4), neither isolated hair melanins nor solutions of hair keratin yield a soluble pigment on treatment with acid. This suggests that the soluble pigment may be the product of reactions involving intermediate keratin hydrolysis products. To determine whether keratin may be associated with pigment production from colorless chromogens in the hair, tyrosine and tryptophan were boiled in mineral acids with and without nail keratin and the resulting colors were compared. Tyrosine and tryptophan were used as chromogens since there is evidence that they may be precursors of mammalian epidermal pigments(5,6), and nail keratin because it is pigment free.

Experimental. Washed nail keratin was defatted with ether and weighed amounts were added to known concentrations of *L*-tyrosine and *L*-tryptophan in solution in 0.1 N and 6 N hydrochloric acid and 6 N sulfuric acid. The solutions were heated either continuously or intermittently under reflux for 15 hours or longer and were then allowed to stand in the light for 60 hours, during which time observations were made. Details of the treatments are summarized in Table I. Spectrophotometric readings of the red tryptophan-keratin solutions were made in a Beckman spectrophotometer. All readings were made at least 84 hours after the beginning of treatment. Chromatographic isolation of the tryptophan-keratin pigment was attempted. Adsorption on talc and Celite columns (1:1 by volume) produced sharp bands which were easily eluted with 0.2 N sodium hydroxide.

Results. The results of acid treatment are summarized in Table I. Keratin affects the color potentialities of both tyrosine and tryptophan when these are boiled with concentrated mineral acids (Exp. 2, 3, 5, and 6) and of tryptophan when boiled with dilute HCl (Exp. 2, 5, and 8). Color appears more rapidly in tryptophan solutions in HCl when keratin is present (Exp. 2, 5, and 8). There appears to be a threshold concentration for the production of color from tryptophan alone in 0.1 N HCl (Exp. 2 and 9). Black pigment formation from tyrosine is seen clearly only when tyrosine is treated with 6 N H_2SO_4 in the presence of keratin (Exp. 6) but slight color differences in 6 N HCl solutions (Exp.

* Public Health Service Research Fellow of the National Cancer Institute.