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Biological Method for Determination of Glucocorticoid Activity of Crystalline Compounds and Urine Extracts.* (21960)

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During the past decade, several biological methods for assay of compounds exhibiting glucocorticoid activity have been established. These technics have been applied to the assay of urine extracts to measure excretion levels of adrenal cortical hormone by the human. The methods of Reinecke and Kendall(1), Olson, Jacobs, Richert, Thayer, Kopp, and Wade(2), and Pabst, Sheppard, and Kuizenga (3), are based on capacity of glucocorticoids to deposit glycogen in livers of adrenalectomized rats after glycogen stores have been depleted by fasting. These methods use male rats weighing 140 to 180 g and injections of test material are made at regular intervals throughout the assay. The methods are reasonably specific for assay of glucocorticoids and reproducible with a fairly high degree of precision. The disadvantage of the methods lies in their comparative lack of sensitivity. Lack of sensitivity was apparently overcome by using the mouse as assay animal. The method of Venning, Kazmin, and Bell(4) measures deposition of glycogen in depleted livers of fasting adrenalectomized mice, while those of Eggleston, Johnston and Dobriner (5), and of Dorfman, Ross and Shipley(6) measure capacity of adrenal corticoids to prevent loss of hepatic glycogen during fasting. Our experience with the method of Venning, Kazmin and Bell demonstrated the assay to be unreliable with strains of mice available to us. Nissim(7), who conducted a critical study of various methods, believes that precision obtained by Venning *et al.* is dependent upon selection and careful control of a particularly suitable strain of mice bred in their own laboratories.

Existing methods were not suitable for our needs. The methods using young adult rats were not sensitive enough for assay of urine extracts, and uneconomical from the standpoint of animal cost and amount of test material necessary for assay. The mouse methods offered economy both in animals and test materials, but the strains of mice generally available did not lend precision to the assay. It therefore became desirable to develop a method based on satisfactory features of other methods. The method we devised is presented in this communication.

Materials and methods. The animals were 21-day-old male rats of the Holtzman strain weighing 50 to 55 g. These animals were

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placed in a cage, given Rockland Rat Diet and tap water *ad libitum,* to become accustomed to laboratory conditions for a period of 2 days. After this the animals were bilaterally adrenalectomized and returned to the cage. Animals had access to a solution of 5% cerelose in 0.9% NaCl as drinking water until the following morning, then the solution was replaced with one containing 0.9% NaCl only. A good supply of food was available at all times until the evening of the second post-operative day. The animals were then deprived of food. On the morning of the third post-operative day, saline drinking solution was removed, the test compounds injected subcutaneously, and each animal placed in an individual quart Mason jar containing about 2 inches of sawdust. The jars were covered with sealer rings fitted with hardware cloth mesh. It is extremely important that rats be isolated from one another at this point. Occasionally an animal of the control group, or one given an inactive preparation, becames hypoglycemic and subject to convulsions. Such animals tend to fight with other rats if they are caged together. Test materials were administered in 0.1 cc of corn oil per rat. Cortisone was first dissolved in acetone, an aliquot then transferred to corn oil so that the desired dosage was contained in 0.1 cc. and acetone evaporated by vacuum. Six hours after injection of test material, the animals were individually weighed, sacrificed by cervical dislocation, the entire liver quickly removed and transferred to a hot 30% solution of KOH for digestion. A 50 cc pyrex, round-bottom centrifuge tube containing 10 cc of KOH solution proved to be very satisfactory. Small funnels were placed in top of the tubes to serve as condensers and the tubes heated in boiling water bath just previous to autopsy. Digestion process was complete in 30 or 40 minutes, after which the glycogen was precipitated by adding absolute ethyl alcohol (about 1.2 times the volume of the digest) while the digests were still hot. The digests were then brought to a boil, cooled in a cold water bath, again brought to a boil and re-cooled as suggested by Pabst, Sheppard and Kuizenga(3). The KOH-liver digests were placed in refrigerator for at least 2 hours to insure more complete precipitation and coagulation of the glycogen. If necessary, the liver digests may be refrigerated over night. After this, the samples were centrifuged at 2500 rpm for 10 minutes, the supernatant decanted and discarded, and the tubes inverted on absorbent paper and allowed to drain. The precipitated glycogen was then washed with a small amount of absolute alcohol, centrifuged, and again allowed to drain. Glycogen was hydrolyzed by adding 6 cc of 0.6N HCl and placing the tubes in an autoclave at 15 lb pressure for 20 minutes. After cooling to room temperature the hydrolysates were neutralized with 10% NaOH using Phenol Red as an indicator. The solutions were centrifuged, and glucose determined by the method of Reinecke(8). The values obtained were then expressed as glucose per 100 g body weight per rat.

Results. Determination of time for autopsy. In the initial phases it was important to determine the most favorable time at which animals should be sacrificed after injection of cortisone, which was to be employed as the standard. Groups of animals were killed at 2, 4, 6, 8 and 10 hours after injection and amount of liver glycogen deposited was determined. The cortisone in this experiment was 100 μg, a dose which preliminary study indicated would give a good response and yet be somewhat less than maximal. The results of this study are shown in Table I. During the first 2-hour period after injection of cortisone there was no indication of glycogen deposition over and above the level found in oil-treated control animals. At 4 hours the animals had deposited a highly significant amount of gly-

TABLE I. Amount of Liver Glycogen Deposited at 2-Hr Intervals after 100 μg Cortisone Injection.

Interval, hr	No. animals	Glycogen,* avg ± S.E., mg	Increase, mg	"t"
0				
2	7	.66 ± .046		
4	9	10.14 ± .58	9.473	14.013†
6	8	20.25 ±1.52	10.11	6.46 †
8	9	29.02 ±1.46	8.77	4.16 †
10	7	22.76 ±5.25	−6.26	
Oil controls at 6 hr	6	.68 ± .074		

* Glycogen expressed as glucose/100 g body wt.
† P = <0.01.

TABLE II. Glycogen Deposition in Response to Various Doses of Cortisone.

Dose cortisone, μg	No. animals	Glycogen, mg Avg	S.E.	C,* %	"t"
0	25	.68	.074	54	
10	12	2.52	.329	45	7.99‡
30	13	9.69	.925	34	7.1 ‡
50	17	13.8	1.08	32	2.79‡
100	18	22.64	1.07	20	4.9 ‡
200	18	28.67	2.24	33	2.43†
300	19	39.35	1.74	19	3.75‡

* Coefficient of variation; expresses stand. dev. as % of mean response. Avg "C" for all cortisone doses is 30.5%.
† P = <.05.
‡ P = <.01.
Glycogen expressed as mg glucose/100 g body wt.

cogen. Expressed as mg glucose/100 g rat weight, a level of 10.14 mg was found. At 6 hours and again at 8 hours post-injection, highly significant increases in liver glycogen were noted. During the 2-hour period from 6 to 8 hours, deposition of glycogen was somewhat less than that observed during the preceding 2-hour period, and at 10 hours post-injection, the peak glycogen deposition in response to a single injection of cortisone had been passed and glycogen was again being mobilized. It was then decided that 6 hours post-injection was the optimum time for sacrificing the animals. During the period between 2 and 8 hours post-injection, the deposition of liver glycogen progressed as a linear function of time. After 8 hours the concentration of glycogen in the liver decreased. It was considered preferable to select a time during which glycogen was being deposited at a steady rate and before the peak was reached.

Development of a dose-response curve. Cortisone was employed as the standard hormone in development of the assay method, and the data in Table II show that a satisfactory dose-response relationship exists over a relatively large range of doses. Highly significant differences in glycogen deposition were obtained with 20 μg increments in dosage of cortisone in the lower dosage range. A measurable and highly significant response was obtained with as little as 10 μg of cortisone. These results indicate that the assay is much more sensitive than any of the existing methods utilizing the 140 to 180 g rat. In the method suggested by Pabst, *et al.*, a dose of 350 μg of cortisone was necessary to deposit a significant amount of glycogen in the liver, and dose increments of 30 μg were necessary to elicit significant increases in liver glycogen. The *coefficient of variation*(9) has been calculated for each dosage and the average coefficient of variation for cortisone is shown in Table II. Averages for the methods of Olson, *et al.* and Pabst, *et al.*, as calculated by Nissim(7), are 29.5% and 23.25% respectively. The average reported here is 30.5% indicating only slightly higher variation in these animals. The method of Venning (using her strain of mice) is perhaps more sensitive to smaller changes in dosage of cortisone than the method suggested here. She has shown a significant change in glycogen deposition with as small an increment as 10 μg of cortisone, but does not report any liver glycogen values for smaller doses. The 10 μg dose is also in the lower limit for a significant response in our method.

The data in Table II have been used to plot the dose-response curve found in Fig. 1. The mean values of glucose per 100 g weight were plotted against the log.-dose of cortisone and the curve obtained indicated a straight line relationship. From these values the slope,

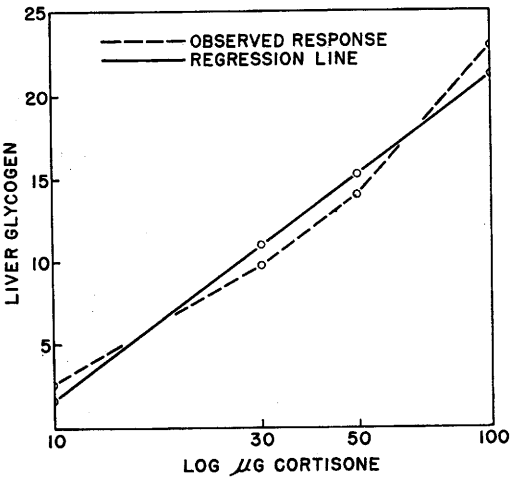

FIG. 1. The mean values for liver glycogen expressed as mg of glucose per 100 g body wt, plotted against the log-dose of cortisone.

"b", which was calculated according to the formula $\frac{\Sigma xy}{\Sigma x^2}$ as discussed by Snedecor(9), was found to be 19.559. The regression line was then calculated according to the formula $\hat{Y} = \bar{y} + b(X - \bar{x})$. The index of precision was calculated according to the formula $\lambda = s/b$ wherein "s" is equal to the average standard deviation about the curve and "b" is the slope of the regression line. According to Emmens(10) the value of λ is a guide to the precision of the assay; a minimal value for λ indicates a steep regression line in relation to magnitude of variance.

It is evident from Table II that as the dose increases above 100 μg of cortisone, there is a sharp rise in the standard error of the mean. Precision indices were calculated using the data from doses a) 0 to 300 μg of cortisone; b) 0 to 200 μg of cortisone; and c) 0 to 100 μg of cortisone. The indices were found to be 0.225, 0.273, and 0.183, respectively. Accepting the fact that for a precise bioassay the value for lambda should be minimal, it is apparent that the range of doses from 0 to 100 μg of cortisone gives the most precise information.

Assay of glucocorticoids in urine. The primary purpose for the development of this assay was to establish a method by which levels of glucocorticoid material could be measured in human urine. Chloroform extracts of urine were prepared according to Mason's modification(11) of an original method for chemical determination of corticoids by Daughaday, Jaffe and Williams(12). Some difficulty was encountered using the crude chloroform extracts of normal urines. Attempts to show a dose-response relationship with normal urine were unsuccessful at the outset. Response to increasing doses of urine extracts was not a linear increase in liver glycogen. In many cases the results showed a decrease in amount of glycogen as the dose of urine extract was increased. This pattern of results suggested that either there was some active principle in urine extracts that was inhibiting glucocorticoid activity, or that there was inactive material, poorly soluble in the volume of oil used, that was interfering with absorption of the glucocorticoids. Many of the urine extracts possessed large amounts of color, all of which appeared to be readily soluble in any of the non-polar solvents. In view of the behavior of crystalline steroids in a benzene-water partition system, as shown by Talbot, Saltzman, Wixom and Wolfe(13), it was believed that this treatment might be beneficial in purifying urine extracts. Talbot, *et al.* found that if the active corticoid compounds were dissolved in benzene and partitioned with water, the only compound that remained in the benzene to any extent was desoxycorticosterone. The more highly oxygenated steroids preferentially moved into the water phase. This procedure was applied to urine extracts and it was found that the pigmented material remained almost entirely in the benzene fraction and that essentially all materials active in liver glycogen deposition moved into the water phase. The experimental data are presented in Table III.

TABLE III. Effect of Benzene-Water Partition of Urine Extracts on Glycogen Deposition.

Extract	Dose equivalent, hr	Glycogen crude extract, mg	Glycogen benz. fract., mg	Glycogen HOH fract., mg
A	3	.646		1.45
	6	1.27	.706	2.15
B	2	30.28	1.62	32.14

Glycogen expressed as mg glucose/100 g body wt.

Benzene-water partitioning considerably reduced the amount of solid material in urine extracts, giving a cleaner preparation that could be dissolved in a small volume of oil for injection. There is some indication that higher levels of glycogen deposition are obtained with the same hourly equivalents of urine extract after a benzene-water partition.

The data obtained from the assay of urine extracts from normal male individuals is shown in Table IV. A dose-response relationship is indicated which shows a highly significant change in glycogen deposition between 200 and 400 ml urine equivalent doses, and a significant change between 400 and 600 ml dose levels. These data are expressed graphically in Fig. 2 together with the standard curve for cortisone for comparison. Re-

TABLE IV. Glycogen Deposition in Response to Doses of Urine Extract from Normal Individuals.

Dose equivalent, ml	No. animals	Glycogen,* mg Avg	S.E.	"t"	Cortisone† equivalent, μg	Cortisone equiv./l, μg
200	6	1.24	.37		9.6	48.0
400	5	6.31	1.18	4.43§	17.5	43.75
600	6	9.09	.51	2.30‡	24.0	40.0

* Glycogen expressed as glucose/100 g body wt. † Determined from stand. curve; Fig. 1.
‡ P = <.05. § P = <.01.

gression lines for cortisone and for urine extract, the slope of which was 16.553, were tested statistically to see if they deviated significantly from parallel. The value "t" was calculated according to the formula: $t = \frac{b_1 - b_2}{\sqrt{\sigma_{d^2}}}$. The value "$b_1 - b_2$" is equal to the difference between slopes and the quantity $\sqrt{\sigma_{d^2}}$ is the standard error of the difference between regression coefficients(14). The value for "t" was 1.633 which showed the two regression lines did not deviate from parallel significantly. Therefore, it can be assumed that urine extracts may be assayed in terms of cortisone. When calculated as μg equivalents of cortisone excreted per liter of urine, as shown in Table IV, it is clear that fairly close agreement in results exists between the dose levels administered.

A comparison of the values for corticoid excretion obtained by this method with determinations made by other investigators also show close agreement. This method shows an average excretion of 44 μg cortisone/l of pooled normal male urine. Schneider(15) has obtained a value of 53.1 μg/l, Zaffaroni (16) reports a range of 20 to 40 μg/l, and Venning(17) reports values of 40 to 80 μg/l of urine of normal individuals. Determinations of Schneider and of Zaffaroni were by means of paper chromatography and Venning's values by biological assay.

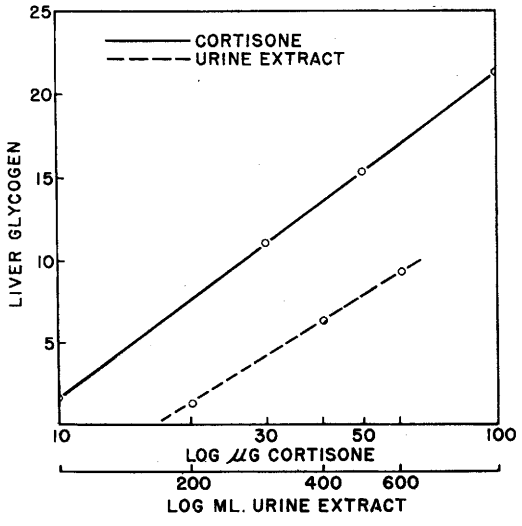

FIG. 2. Regression line for liver glycogen response to urine extracts compared with the regression line for response to cortisone.

Validity of the assay procedure for measurement of variations in corticoid excretion is demonstrated by an experiment wherein a patient was given ACTH intravenously for periods of 3 days interspersed with 3-day control periods. Urine was collected, extracted, and assayed for glucocorticoids by the bioassay method reported here. Table V shows glucocorticoid, and the formaldehydogenic corticoid excretion in response to ACTH stimulation. Results showing an increase in excretion of glucocorticoids during ACTH administration, indicate that this method measures secretion products of the adrenal cortex which influence carbohydrate metabolism, and that the method is sufficiently sensitive to detect fluctuations in excretion of glycogenic steroids in urine.

In Table V the results of both assays are expressed as μg of cortisone excreted per 24 hours. It can be seen that by chemical assay the excretion level is considerably higher than that shown by biological determination. This indicates that chemical determination is measuring formaldehydogenic compounds which do not have glycogenic activity. In the calculation of corticoid excretion the assumption is made that 1 mole of formaldehyde is derived from 1 mole of formaldehydogenic ster-

TABLE V. Daily Corticoid Excretion in Urine during ACTH Stimulation.

Treatment*	Glucocorticoids, μg equiv. "E"/24 hr	FGS,† μg "E"/24 hr
Control	91.2	931.
5 mg ACTH	2380.	3870.
Control	120.	1167.
10 mg ACTH	1298.	3794.
Control	266.	675.
25 mg ACTH	1440.	4125.

* Consecutive 3-day periods.
† Formaldehydogenic steroids.

oid. This method of calculation does not make allowance for differences in glycogen-depositing activity of various formaldehydogenic steroids. It is well known that the formaldehydogenic method measures biologically inactive metabolites of glycogenic steroids as well as active forms. Since the isolation of pregnance-3, 17, 21-triol 11, 20-dione (tetrahydro E) in 1951 by Schneider(15), much investigation into excretion of corticoid metabolites has been conducted.

The introduction of -glucuronidase by Kinsella, Doisy and Glick(18) for hydrolysis of urinary steroids greatly increased the yield of chemically detectable corticoids. Interesting studies on urine and blood levels of corticoids and their metabolites have recently been reported by Baggett, Kuisella and Doisy(19) and Silber and Porter(20). These investigators have also found increased yields of urinary corticoids by enzymatic hydrolysis and, in addition, Silber and Porter showed that the main corticoid in blood is hydrocortisone (Compound F) while in urine it is tetrahydro F, 94% of which is excreted as a glucuronide. Studies by Fukushima, Leeds, Bradlow, Kritchevsky, Stokem and Gallagher, (21) have disclosed 4 new metabolites excreted in urine following ACTH administration. These compounds, designated by the authors as "cortol, -cortol, cortolone, and -cortolone," are not reducing substances and are negative to the Porter-Silber reaction, however, they do yield formaldehyde on periodic acid oxidation.

These increased yields of urinary corticoids and newly discovered metabolites tend to further the understanding of adrenal function and metabolism of cortical secretions to a degree that could not have been arrived at by means of a biological assay alone. It has, however, been shown by Venning(22), Lobotsky, Hannye and Lloyd(23), and Chart(24), that for the routine bioassay of urinary corticoids the hydrolysis of urine with -glucuronidase does not add greatly to the information obtainable by acid hydrolysis. The great increase in yield of chemically detectable corticoids is not reflected by a comparable increase of activity in biological assay procedures. This fact does not negate the usefulness of bioassay procedures or of the enzymatic hydrolysis of urine for determination of corticoid excretion in urine. It is of importance to establish the relative activity of newly isolated metabolites and to quantitate the potency of new synthetic steroids as they become available.

We believe that this bioassay procedure can function as a valuable adjunct to the chemical methods for the evaluation of corticoid excretion as well as being an accurate, simple and economical method for determination of glycogen-depositing activity of crystalline compounds.

Summary. A practical bioassay technic for glucocorticoids is proposed, using the adrenalectomized, 21-day-old male rat as test animal. The method offers the accuracy and precision shown by the use of the young adult rat, and the sensitivity and economy observed in methods using the laboratory mouse. Dose-response curves have been established for cortisone and for the assay of urine extracts. The regression lines for the two substances are parallel, indicating the method is valid for measurement of glucocorticoid excretion. It is suggested that this bioassay method may serve as a valuable adjunct to the chemical methods for the assay of urinary corticoids.

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Colorimetric Study of Absorption and Excretion of 4-(p-Dimethylaminostyryl) Quinoline Methiodide and Related Compounds.* (21961)

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Since 4-(p-dimethylaminostyryl) quinoline methiodide(1) and several related dyes, when administered in the diet, have been found to cause regression of Lymphoma 8 tumors in rats(2-4), the fate of the unchanged dye was studied colorimetrically. The results of this study have led to finding a compound that appears to be unusually active in bringing about regression of Lymphoma 8(3,4).

Methods. The compounds used were prepared in the chemical laboratories of Carson-Newman College by Wilfred Lyke and Joan Wilson, with the financial assistance of a Frederick Gardner Cottrell Grant from the Research Corporation and a grant from the Medical Research Foundation: 4-(p-Dimethylaminostyryl) quinoline methiodide (4M 2M). 4-(p-Dimethylaminostyryl) quinoline methochloride (4M2MCl). 2-(p-Diethylaminostyryl) quinoline ethiodide (2E2E). 2-(p-Dimethylaminostyryl) pyridine methiodide (2M2PM)‡, and 2-(p-Dimethylaminoanil) pyridine methiodide (2M2NPM). The usual method of administering the dyes was to mix them with powdered food of animals, by grinding in a ball mill 8 hours or longer. After offering dyed food for 2 days the animals were given untreated food *ad libitum*, beginning the evening of second day. In a few instances a solution of a dye in a 0.9% NaCl or 5% glucose was administered by a stomach tube or was injected subcutaneously in the flank or intravenously via a tail vein. Urine and feces were collected separately, by conventional apparatus, until 2 days after last dose of dye was administered. The dye was

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* Under contract with the U. S. Atomic Energy Commission.

‡ This compound was included because of the observation by workers at the Midwest Research Institute that it seemed to cause damage to Sarcoma 180 in mice used in tumor necrosis screening tests, although it did not cause conspicuous inhibition of the growth of tumors. Goodson, Lewis H., personal communication.