Comparison of Chemical and Biological Methods for the Estimation of Urinary Corticoids.

Thesis

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ANDREW PATERSON KENNY, B.Sc.(Lond.), A.R.I.C.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Chapter I

I N T R O D U C T I O N

Much valuable information has already been accumulated on the profound physiological action of the adrenocortico-:trophic hormone and of cortisone-like drugs and with the increasing use of these compounds in therapy it is desirable to continue to add to our knowledge on this important sub-:ject in order to make diagnosis and control of treatment of still greater value to the clinician and ultimately to the patient. At present the chief means of assessing the functional state of a patient's adrenal glands is to estim-:ate the level of excretion of 17-ketosteroids and corti-:coids in urine. Further information can be obtained by repeating these tests during a course of adrenal stimula-:tion with adrenocorticotrophic hormone as is done in the Thorn test (Thorn, Forsham, Prunty and Hills, 1948; Renold, Forsham, Waisterrena and Thorn, 1951), and such tests have proved a distinct advance since it has been found that in a certain proportion of cases suffering from adrenocortical dysfunction, the excretion of the above mentioned steroids may fall within the range of accepted normal values, yet the patients fail to show a response to exogenous ACTH (Nabarro, 1954).

More recently attention has been directed to changes

in/

in blood corticosteroid levels, but these can still be re-:garded as at the investigational stage and the finding may be complicated by renal or hepatic disease (Bongiovanni and Eberlein, 1955 a), or by diurnal variation (Reddy, Abu Haydar, Laidlaw, Renold and Thorn, 1956).

For the estimation of 17-ketosteroids the Zimmermen reaction (1935) has for long been recognised as the basis of all reliable methods, but for the estimation of urinary cort-:icoids many different methods have been employed utilising different principles. One aspect of the work which has re-:seived scant attention is the comparison of the chemical and biological methods of assay, in particular in relation to the conjugated fraction of the corticosteroids of the urine.

It is therefore proposed in this investigation to study the degree of correlation between the results found by one particular chemical method for the determination of urinary corticoids, and the assay of the same material by a biologic-:al method in order to obtain a fuller understanding of the significance of the conjugated material

The urines examined have been obtained from normal sub-:jects and from patients suffering from Simmonds' disease, Cushing's syndrome and from cases of pemphigus and nephrosis undergoing courses of treatment with cortisone and ACTH.

Discussion of Methods./

Discussion of Methods.

The main advantage of the biological methods of assay in this field lies in the fact that certain specific physiological responses can be separately studied. Examples of the effect of active adrenocortical steroids are the anabolic action as measured by growth and increase in body weight (Grollman, 1941), electrolyte balance and renotrophic action as assessed by certain renal function tests and balance studies (Hartman and Spoor, 1940), gluconeogenesis and carbohydrate control by glucose tolerance tests and the accumulation of glycogen in the liver (Britton and Silvette, 1932). Biological tests of a more general character such as cold survival tests (Dorfman, Shipley, Schiller and Forwitt, 1946), and capacity for work (Ingle, 1940), have been used, and again the decrease in circulating eosinophiles has been used as an index of corticoid activity (Spoerl and Meyer, 1949 ; Rosenberg, Cornfield, Eaton and Anderson, 1954).

One important consideration with regard to the biological test is that the structure of the compound need not necessarily be known and that assays can be made on relatively crude extracts and amorphous material, but such assays suffer from the disadvantage that relatively larger amounts of material are required and moreover they involve the investigator/

investigator in considerably more work, and usually the end results are statistically less significant than a corres-:ponding chemical method.

For the chemical methods on the other hand a knowledge of the structure of the compound is a necessary preliminary, since these tests depend wholly upon the differential par-:titioning between solvents followed by the application of known chemical reactions for certain groups and linkages in the steroid molecule, but in the general steroid field a surprising number of what could be termed specific reactions have been elaborated for both qualitative and quantitative work.

Results of prime importance in the qualitative and semi-:quantitative sense have been obtained by methods employing paper partition chromatography (Zaffaroni, Burton and Keutman, 1950; Bush, 1952;) and an entirely new aspect of adrenocortic-:al steroid chemistry leading to the isolation and identifi-:cation of aldosterone (Simpson, Tait, Wettstein, Neher, von Euw & Reichstein, 1953), was in large measure dependent upon the use of this technique.

The first method employed for the chemical determination of urinary corticosteroids was that of Fieser, Fields and Lieberman (1944), who used periodic acid and lead tetra-:chloride as oxidising agents to oxidise the side chain of the/

the molecule and leave a 17-ketosteroid residue. The method had the limitation that it involved working by difference since 17-ketosteroids had to be determined before and after oxidation, and since only the free fraction was being deter-:mined in urine extracts, this difference was relatively small. The same principle has been reintroduced by Brooks and Norymberski (1952) who used sodium bismuthate, a more powerful oxidising agent, to determine what are now termed "17-ketogenic steroids". The method of Talbot, Saltzman, Wixam and Wolfe (1945), depended upon the ability of the α-ketol side chain of the isolated steroid to reduce alkaline copper reagent in a manner analogous to the sugars. This latter method on which was based many of the valuable early observations has been largely superseded. The method of Heard, Sobel and Venning (1946) which used the same principle, the reduction of phosphomolybdate, served more as a screening test and is now in the same category.

Lowenstein, Corcoran and Page (1946) introduced a method based upon the ability of periodic acid to yield formaldehyde from the C_{20}-ketol or glycolene side chain. The formalde-:hyde was isolated by distillation and estimated by a specific reaction with chromotropic acid, a reaction discovered by Eegrieve (1937) and adapted for the quantitative estimation of/

of formaldehyde by MacFadyen (1945). These three methods in their original form estimated only free corticoids.

Again the method of Reddy, Jenkins and Thorn (1952) based on the Porter and Silber reaction (1950) which used phenylhydrazine in sulphuric acid as the chromogenic agent has been introduced for the estimation of corticosteroids which have a 17-hydroxyl group, and in virtue of the fact that butanol is used as the solvent, it has the advantage that it is capable of extracting glucuronic acid steroid glycosides and as a consequence is capable of estimating the total corticosteroids without a preliminary enzymic hydroly-:sis. The degree of pigmentation of the extracts limits the value of the method, particularly in the estimation of free corticoids, but efforts have been made to reduce this by raising the pH at which extraction takes place and by passing the extract through a magnesium silicate column prior to colour development (Glen and Nelson, 1953).

It was early recognised that the severe acid hydrolysis necessary for the liberation of urinary steroids from their conjugates could result in profound changes such as dehydra-:tion, stereoisomeric transformations and chlorine substitu-:tions, in the case of 17-ketosteroids (Bitman and Cohen, 1951), and/

and it was shown that boiling with dilute mineral acid could result in almost complete loss in the case of certain corticosteroids (Heard, Sobel and Venning, 1946; Tompsett, 1953) and until recently nearly all the information available was on that fraction which was free in urine. It has been shown however (Tompsett, 1953; 1955) that desoxycorticosterone and certain adrenocortical steroid metabolites, including those which have a 21, 20, 17-triol side chain, are acid stable under prescribed conditions and this property has been suggested as a means to their estimation.

Enzymic hydrolysis for the release of conjugated steroid was first used to liberate oestrogen from urine (Cohen and Marrian, 1935) and rat liver preparation of crude β-glucuronidase have been used for the hydrolysis of C_{19} and C_{20} steroid glucuronides (Talbot, Ryan and Wolfe, 1943).

By far the greater part of the conjugated material was shown Cohen (1951) and Cox and Marrian (1951) to be combined with glucuronic acid, although Norymberski has contended that up to half of the total 17-ketogenic steroids as estimated by his method may exist as sulphate. According to Marrian and Paterson (1951) there appears to be in urine at pH 1.0, an acid stable and acid labile fraction and there has/

has been considerable controversy regarding the best method of extraction. These apparent fluctuations in yield with respect to pH and time of extraction are no doubt related to some of the observations listed above in connection with acid stability and necessitate a strict set of conditions during extraction if comparable and reproducible results are to be obtained.

The scheme recommended by Bayliss (1952) involves the incubation of the urine for 48 hours with β-glucuronidase at pH 4.5 in order to liberate the glucuronic acid conjugates which are then extracted with chloroform. The same specimen is then re-extracted without delay after the urine has been taken to pH 1.0, to be followed by a third extraction 24 hours later and probably gives a more complete yield of corticoid material than any other method so far devised. Using β-glucuronidase enzyme, Cohen (1951) was able to obtain an eighteen-fold increase and in certain cases up to a fifty-fold increase in yield of corticoids from urine as compared with extraction at pH 1.0, and this author also found that boiling the urine before incubation with the enzyme often resulted in higher yields and suggested that the urine contained either a glucuronidase inhibitor or a steroid destroying/

destroying agent or both.

β-glucuronidase inhibitors have been studied by Levvy (1953) who showed saccharic acid to be a powerful inhibitor, but later found the saccharo - 1:4 lactone to be the most active of the substances he studied. There is little likelihood of saccharic acid accumulating in the urine under ordinary conditions since it is normally metabolised.

Because of previous satisfactory experience it was de-:cided to employ the method of Corcoran and Page (1948) in this investigation and to consider mainly the fraction which occurred free in the urine and was directly extractable with chloroform and that part of the corticoid material which could be subsequently liberated by β-glucuronidase and further extracted with the same solvent.

Chapter II

CHEMICAL ASSAY OF URINARY CORTICOIDS.

1. PREPARATION AND STANDARDISATION OF β-GLUCURONIDASE ENZYME.

(a) Preparation of Enzyme. Solutions of ox spleen * β-glucuronidase were prepared according to the method of Mills (1948) and taken as far as Stage C. This product was considered sufficiently pure for the purpose of hydrolysing the urinary glucuronic acid conjugates. It was found that preparations of acetone dried and de-:fatted ox spleen could be stored without significant loss of activity and further that the preparation ob-:tained after addition of ammonium sulphate could be air dried on filter paper and stored, to be dialysed at con-:venience, but the most satisfactory course was to freeze dry the final dialysed solution and store in a refriger-:ator until required. This allowed a stock of enzyme material to be maintained, at different stages of prep-:aration, which could be quickly processed for use.

The activity of each preparation was determined, prior to use, by the method of Talaley, Fishman and Huggins (1946) using phenolphthalein monoglucuronide as the/

* The enzyme will hereafter be referred to simply as glucuronidase.

the chromogenic substrate.

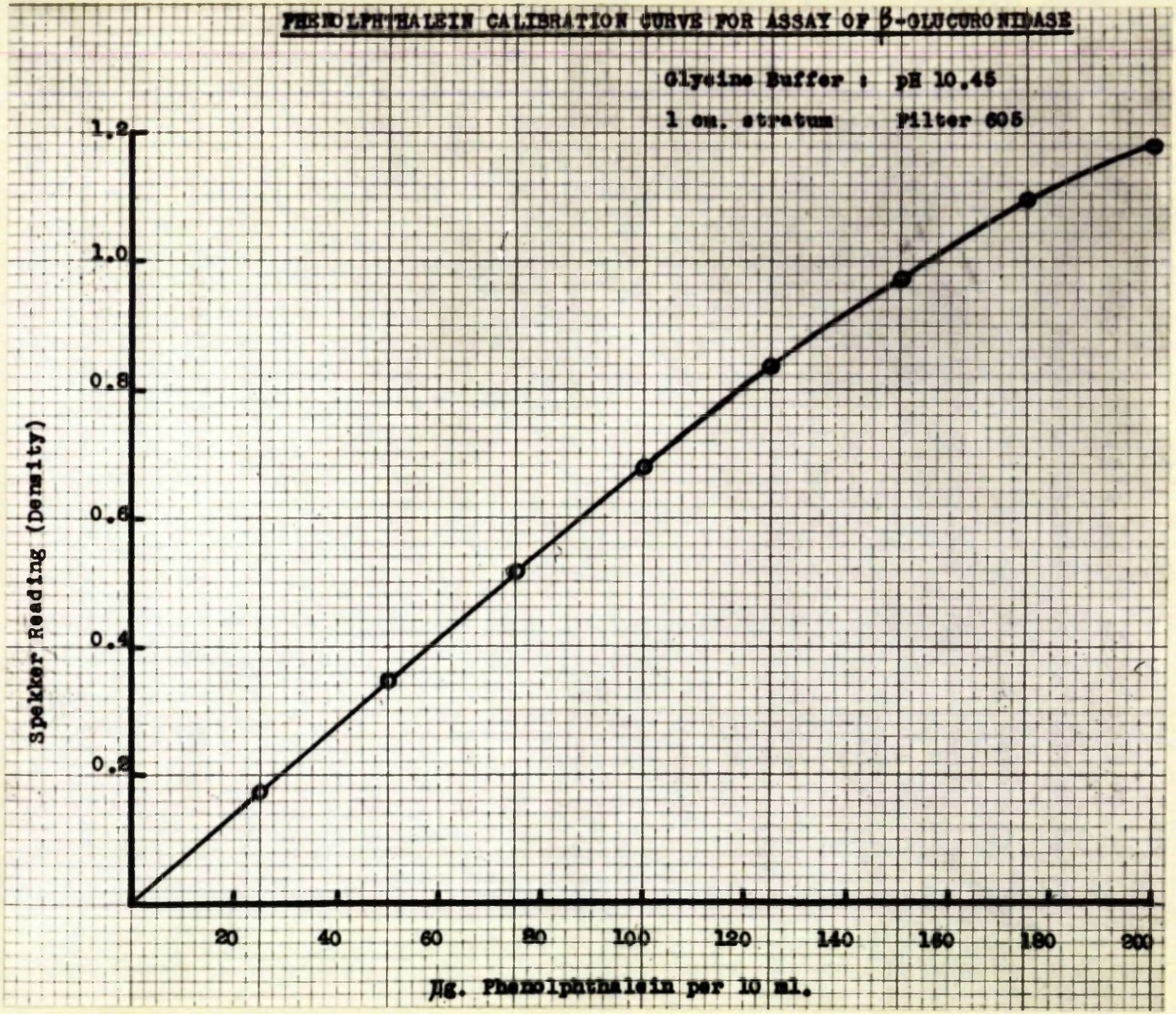
(b) Preparation of Phenolphthalein Monoglucuronide.

(Talalay et al, 1946)

The sodium salt of phenolphthalein phosphate was synthesised from phenolphthalein and phosphorus oxy-:chloride, the latter being prepared by the careful distillation of phosphorus pentoxide and phosphorus pentachloride. The white crystals, which were re-:crystallised from methanol-formamide (80/20, v/v) were used in the biological synthesis of phenolphthalein monoglucuronide, (Talalay, 1945).

Four rabbits were given daily intraperitoneal in-:jections of 0.5 gm. of sodium phenolphthalein phosphate until 24 gm. of the salt had been used, and the pooled urines were collected under toluene, and treated daily with 30% sodium chloride and colloidal iron to yield a clear solution free from suspended phosphates and faecal material. Free phenolphthalein was removed by extrac-:tion with ether and the glucuronide partitioned into ethyl acetate. After decolourisation with charcoal and filtration, the phenolphthalein glucuronide was isolated as the cinchonidine salt and recrystallised from dioxane. Pure glucuronide was regenerated from a weighed amount of/

Fig. 1.


PHENOLPHTHALEIN CALIBRATION CURVE FOR ASSAY OF β-GLUCURONIDASE
Glycine Buffer : pH 10.45
1 cm. stratum Filter 605
Spekker Reading (Density)
1.2
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
120
140
160
180
200
µg. Phenolphthalein per 10 ml.

of the salt by the addition of hydrochloric acid and extraction with ethyl acetate. The solvent was evaporated under reduced pressure and the residue dissolved in the calculated amount of dilute sodium hydroxide to give a 0.01 M solution.

(c) Assay of Activity of Glucuronidase Preparation.

The unit of glucuronidase activity is defined as such an amount of enzyme as will liberate 1 µg. of phenolphthalein in 1 hour at 38°C., from phenol-:phthalein glucuronide at pH 5.0 under standard con-:ditions. Preparations of the order of 200,000 units were obtained from each ox spleen and in the later part of the work this was found to be approximately the amount of enzyme which was required to treat a 24 hour collec-:tion of urine.

A graph for the estimation of this enzyme activity was prepared by dissolving phenolphthalein in 80 per cent ethanol to give a stock solution of 1 mg. per ml. and dilutions were made in 0.4 M. glycine buffer of pH 10.45 to cover the range 0 - 200 µg. phenolphthalein per final 10 ml. of solution. The resultant pink colour was read on a Spekker absorptiometer employing a green Wratten filter 605, and a calibration curve prepared, Fig. 1.

A/

TABLE I

Example of the Assay of an Ox Spleen Glucuronidase Preparation.

Material		Spekker Reading	Mg. Phenol-phthalein
TEST			
Enzyme Preparation 0.05ml. Phenolphthalein Glucuronide Substrate 0.5ml. Acetate Buffer pH 5.0 4.0ml. Water 0.45ml. 1 Hr. at 37°C.	Finally 5.0ml. Glycine Buffer pH 10.45	1.07	165
ENZYME BLANK			
Enzyme Preparation 0.05 ml. Acetate Buffer pH 5.0 4.0 ml. Water 0.95ml. 1 Hr. at 37°C.	-do-	0.031	3
SUBSTRATE BLANK			
Phenolphthalein Glucuronide Substrate 0.5ml. Acetate Buffer pH 5.0 4.0ml. Water 0.5ml. 1 Hr. at 37°C.	-do-	0.076	11
Nett Enzyme Activity			151

Volume of enzyme preparation from two ox spleens=130 ml.

$\therefore$ Total enzyme activity $= \frac{151 \times 130}{0.05}$

$=$ 393,000 Fishman units per 130ml.

A typical assay which indicates the quantities of reagents used is shown in Table I.

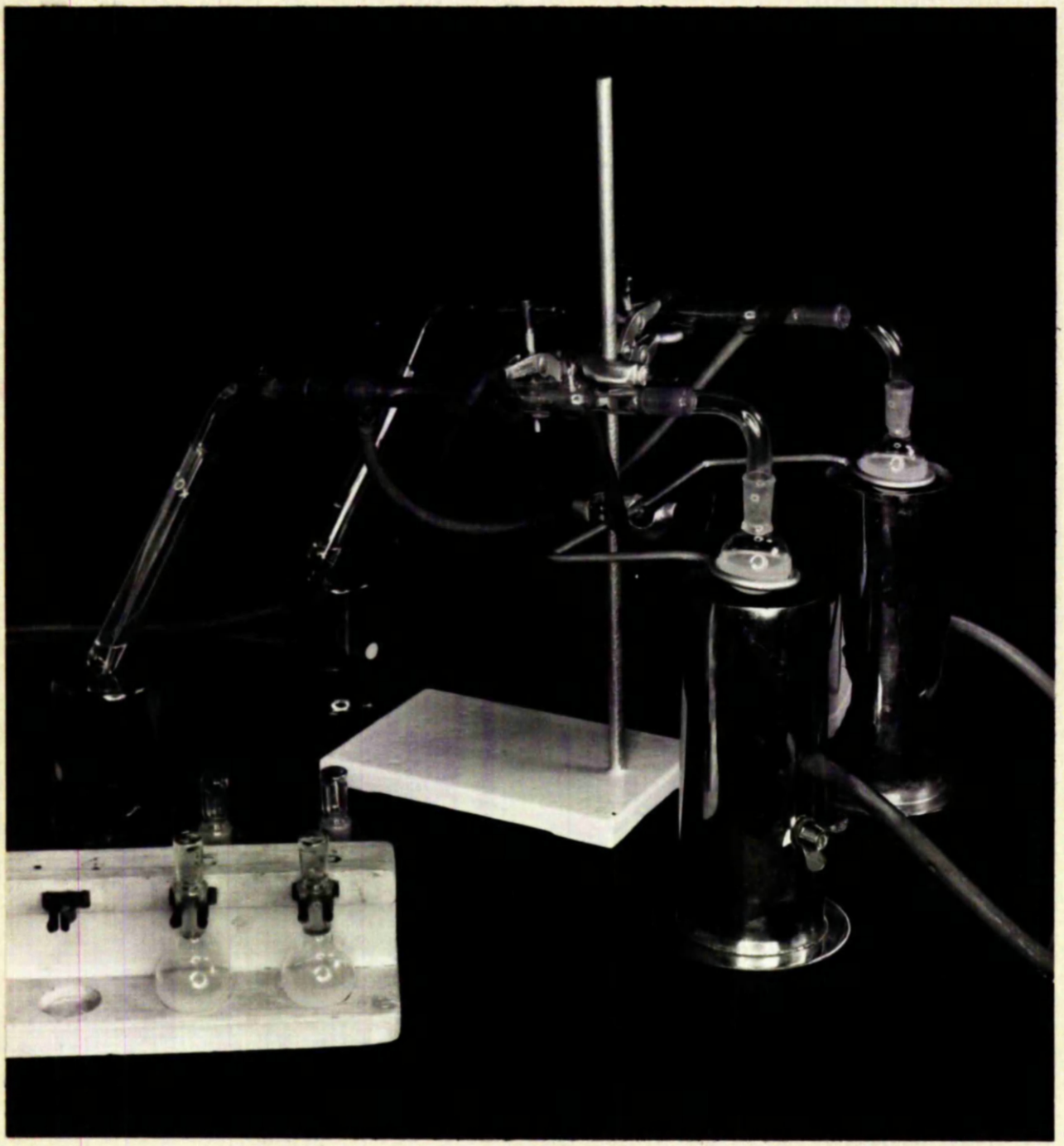
2. CHEMICAL ESTIMATION OF FORMALDEHYDOGENIC STEROIDS.

Unlike the estimation of 17-ketosteroids where the Zimmerman reaction (1935) was early adopted as the accepted method of estimation and an agreed technique (M.R.C. committee on Clinical Endocrinology, 1951) has been recommended, no single method has yet established itself as the most satisfactory and convenient method for the estimation of urinary corticoids and at intervals over the past few years new techniques based on different principles have been reported. It has been difficult therefore to compare results of different groups of investigators since so often they are estimating different combinations of compounds.

As already stated it was decided at the outset of these experiments to use the method of Corcoran and Page (1948), which estimates the so-called "formaldehydogenic steroid substances" and a detailed description of the method is appended. Particular attention was paid to the paper of MacFadyen (1945) where important information regarding acid concentrations and other experimental details were first recorded.

The periodic acid oxidation method has been the subject of/

Fig. 2.



Micro-distillation Apparatus for Distillation of Formaldehyde.

of certain criticism (Hollander, Di Mauro and Pearson, 1951) in connection with the apparent ability of certain urines which had been taken to pH 1.0 to fix formaldehyde and as a consequence give low apparent yields when cort-:icoids are estimated by this method. Paterson and Marrian (1953) were able to show less interference in alkali washed chloroform extracts from urines than in un-:washed extracts, but that this treatment did not wholly eliminate the inhibition and low recoveries could still be expected. Wilson (1953) using this method, but employing isothermal diffusion in Conway units as a means of trans-:ference of the formaldehyde, claimed to eliminate the difficulty by extracting the periodic acid oxidised sample with ether before diffusion. No such precaution has been employed in this work.

Estimation of Urinary Corticoids.

Principle: The Corticosteroids are extracted from urine with chloroform and the extract is oxidised directly by periodic acid without any pre-:liminary benzene/water partitioning. The formaldehyde formed from the oxidation of the α-ketol or glycolene groups attached at the C-17 atom, is distilled and estimated by the colour produced with chromotropic acid re-:agent.

Special Apparatus: Micro distillation apparatus consist-:ing of 25 ml. round bottomed flasks, still head, condenser and delivery tube (all B 14 cones and tapers). See Fig. 2.

Reagents/

Reagents:
(1) Chloroform (chemically pure and redistilled before use.)
(2) 0.1 N-Sodium hydroxide.
(3) Sodium sulphate (anhydrous).
(4) Acetic acid (Analar).
(5) 0.05 M-Periodic acid reagents. Prepared by dissolving 1.15 g. KIO_4 in 60 ml. N-H_2SO_4 and diluting to 100 ml.
(6) 6% Stannous chloride. Prepared daily by dissolving 1.5 g. $SnCl_2.2H_2O$ in 25 ml. cold distilled water to which a few drops of conc. HCl were added. The reagent was centrifuged and the supernatent liquid stored in a glass stoppered tube.
(7) 0.2% Chromotropic acid in 15 M-H_2SO_4. Prepared by dissolving 0.2 gm. 1.8 dihydroxynaphthalene sulphonic acid (Hopkin and Williams - specially purified) in 4 ml. distilled water and making up to 100 ml. with 15 M-H_2SO_4. Purification by treatment with Na_2SO_3 and repeated acetone precipitation is necessary if a pure product is not available.
(8) 9 M-H_2SO_4.

Preparation of Calibration Curve.

Calibration curves were prepared using each of the follow:ing standard substances.

(i) Redistilled formaldehyde, standardised according to an an adaptation of the method of Blank and Zinken-:beiner, 1899.

(ii) Resublimed hexamethylene tetramine, which was hydrolys-:ed for 4 hours with N-H_2SO_4 in a Thunberg tube. (1 mg. $C_6H_{12}N_4$ = 1.285 mg. H.CHO)

(iii) Hydrocortisone (free alcohol). The acetate cannot be used without preliminary hydrolysis as formaldehyde is not formed during oxidation.

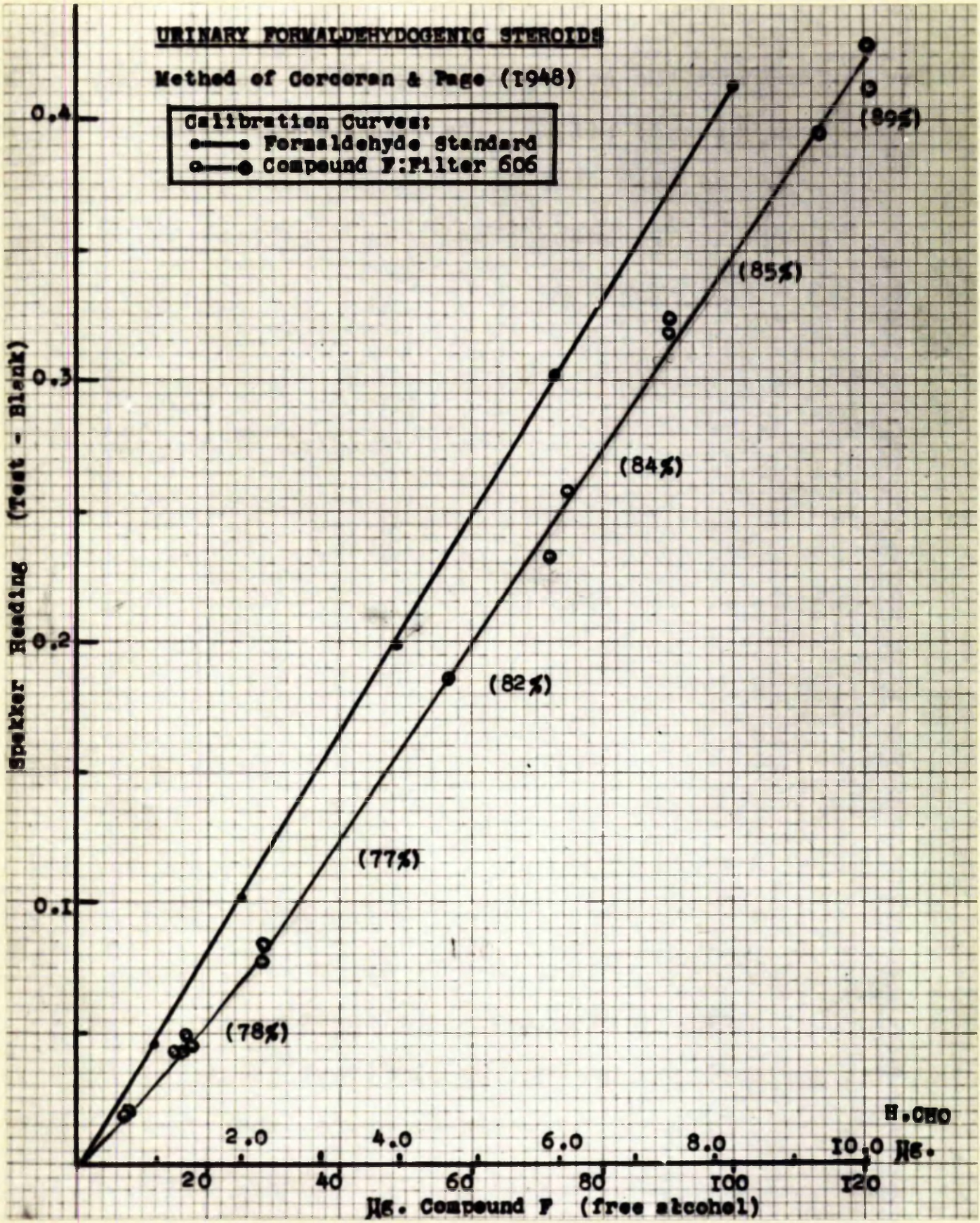
A formaldehyde solution was therefore prepared from (i) and/

and (ii) and diluted to give a range 0 - 10 µg. H.CHO per 3 ml. solution. These aliquots were taken into test tubes graduated at 10 ml. and 5 ml. chromotropic acid reagent were added to each. The contents were mixed, stoppered with glass tears and placed in a boiling water bath for ex-:actly 30 minutes. The tubes were cooled, made up to 10 ml. with 9 M-H_2SO_4 and read on a Spekker absorptometer using a wratten filter No. 606 and the results plotted. Methods (i) and (ii) gave identical results.

It was confirmed that when using hexamethylene tetramine, simultaneous hydrolysis and colour development over a period of 30 minutes, gave only about 46% of the theoretical yield of formaldehyde, and that a preliminary acid hydrolysis was necessary in order to obtain 100 per cent yield.

For method (iii) a few milligrams of compound F were accurately weighed on a microbalance and dissolved in chloroform. Aliquots were distributed for tests and controls in 25 ml. flasks and the chloroform evaporated under reduced pressure at a temperature below 50°C. The contents of the flasks were oxidised with periodic acid and the formaldehyde distilled as described in the method set out below and the distillate diluted to 10 ml. with distilled water. Chromo-:tropic acid reagent was added to a 3 ml. aliquot of the distillate/

Fig. 3.



Calibration Curve for Interpolation of Formaldehydogenic Steroids.

distillate and the colour developed and read as indicated in methods (i) and (ii). The results are shown in Fig. 3.

It can be seen that 80 to 85 per cent of the theoretic-:al amount of formaldehyde was recovered over the greater part of the range shown.

The incomplete recovery could denote either, (1) that the standard substance was not 100 per cent pure, or (2) that oxidation was incomplete over the period of 30 min. allow-:ed or (3) that the distillation failed to allow all the formaldehyde to pass over, or (4) that there was incomplete absorption of the formaldehyde in the water in the receiving tube.

The 85 per cent recovery is probably a combination of some or all of these factors although Doughaday, Jaffe and Williams, (1948) showed progressive increase in formalde-:hyde yield up to 165 min. oxidation. They chose 30 min. as the most convenient time and found 86 per cent recovery compared with the value obtained after 165 min.

It follows, taking into consideration the various fractions employed during the different stages of the method for estimating free corticoid in urine, that each milligram hydrocorticosterone per 24 hour collection should yield 2.94 µg. H-CHO in the final 3 ml. distillate employed.

Method/

Method: Estimation of Free Corticosteroids in Urine.

A fresh 24 hour collection of urine was required and for estimating free corticoids an 8 hour sample usually sufficed. If extraction was being made at pH 1.0 the urine was first brought to that pH with 10% H_2SO_4 using a pH meter with glass electrode.

Extraction: The urine was placed in a separating funnel and extracted with 4 x 100 ml. chloroform. Spillage was avoided and 15 min. was allowed between extractions, the contents of the funnel being shaken several times during that interval. The chloroform extracts were collected in one flask and thoroughly mixed.

Separation, Washing and Evaporation: The chloroform extract was centrifuged and the urine emulsion broken and the urine discarded. About 4 gm. sodium sulphate were added and the chloroform shaken to dehydrate. The extract was chilled in ice and filtered through glass wool into a clean separating funnel. It was washed twice with 40 ml. 0.1 N-NaOH and twice with water. The aqueous extracts were shaken with 40 ml. $CHCl_3$ and 30 ml. of this was added to 300 ml. of the final chloroform extract, so that finally the equivalent of 6 hour urine collection was obtained.

This extract was evaporated in a 500 ml. Quickfit flask and the chloroform removed by vacuum distillation in a thermostatically controlled water bath at a temperature below 50°C.

Blank: A chloroform blank was run through occasionally using water at the appropriate pH. It was found that the blank obtained, even from distilled chloroform often gave a slightly higher reading than the test "unoxidised" sample but this was not so if the chloroform was shaken up with water and washed with alkali and water as for a urine extract.

To the residue in the test flask 10 ml. chloroform were added and the flask shaken to dissolve the material and 5 ml. were pipetted in small 25 ml. Quickfit flasks, marked TOX and the remainder, together with two 1 ml. lots of chloroform used to rinse the large flask, were placed in the second flask marked TU. The "blank" samples were similarly treated and the chloroform once more evaporated at reduced pressure.

Oxidation/

Oxidation: Reagents (5) and (6) were prepared and 0.5 ml. glacial acetic acid was added to all four flasks and rotat-:ed to wet the walls, after which 8.5 ml. water were added to each.

To TOX and BOX were added 0.5 ml. periodic acid re-:agent, the flasks stoppered and the oxidation allowed to continue at room temperature for 30 minutes with occasion-:al shaking. At the end of this period, 0.5 ml. stannous chloride reagent was added to each flask with vigorous mixing.

To TU and BU, 0.5 ml. stannous chloride was first added, to be followed by 0.5 ml. of periodic acid.

Distillation: Using the micro-distillation shown Fig. 2. the contents of the flasks were carefully distilled. The receiving tubes, graduated at 10 ml., contained 1 ml. dis-:tilled water and distillation was continued until a total of 9.5 ml. had been collected. The tip of the delivery tube having been withdrawn and rinsed before the completion of the distillation. Each tube was made up to 10 ml. with distilled water and the contents mixed.

Development of Colour: Into a dry tube duly labelled and graduated at 10 ml. was transferred 3 ml. distillate and 5 ml. chromatropic acid reagent added. The tubes were stoppered with glass tears and placed in boiling water bath for 30 minutes. After cooling, the contents were diluted to 10 ml. with 9 M-H_2SO_4, and read in the Spekker absorptiometer and interpolated on hydrocortisone graph.

Calculation: (TOX - TU) - (BOX - BU) gave µg.

hydrocortisone in sample analysed.

$$\mu g. \times \frac{10}{3} \times 2 \times \frac{400}{300} \times \frac{24 \text{ hour urine vol.}}{\text{vol. of initial sample}}$$

= µg. per 24 hours.

3. RECOVERY EXPERIMENTS.

(a) Recovery of Hydrocortisone Added to Neutral Urine.

A collection of fresh pooled male urine was made and

five/

five 500 ml. aliquots were taken. The first sample was used as a control in which to determine the initial level and the requisite amount of chloroform solution of hydrocortisone was added to the other four to give additions as indicated in Table II. The free corticoid content of the samples was determined by the method previously described and Spekker readings interpolated on graph (iii), Fig. 3, and recoveries calculated.

TABLE II

Recovery of Hydrocortisone Added to Urine.

Sample	Added Hydrocortisone µg.	Found µg.	Nett Recovery µg.	% Recovery
1.	Nil	164	-	-
2.	125	261	97	80
3.	250	324	160	67
4.	500	555	391	81
5.	1000	989	825	86

It can be seen that the recovery averaged 78%, that is only 78% of the recovery obtained by direct oxidation of hydrocortisone/

hydrocortisone. This could be explained as being due to incomplete extraction by the chloroform, destruction of hydrocortisone in solution or fixation of formaldehyde by urine extract as suggested by Paterson and Harrian (1953). It could not be due to impurity of hydrocortisone as this was the same material as was used for the preparation of calibration curve. The loss was further investigated by adding hydrocortisone to urine before extraction and to the dried chloroform residue from a second extract as indicated.

(1) 500 ml. urine extracted with 4 x 100 ml. chloroform. 500 ml. aliquot taken, evaporated and divided into "oxidised" and "unoxidised" samples. Distilled, made up to 10 ml. with water and 3 ml. taken for colour development.

(2) 500 ml. urine + 1000 µg. hydrocortisone in chloroform and treated as (1) above.

(3) 500 ml. urine extracted as in (1), but 1000 µg. hydrocortisone added to chloroform residues and continued as in (1).

Results are shown in Table III.

It can be seen that by eliminating the extraction process that a further 10% recovery was effected but that there is still a loss or fixation of formaldehyde by the urine extract, compared with that obtainable by direct oxidation/of

TABLE III

Recovery of Hydrocortisone Added to Urine & Urine Extract.

Test	Initial Level µg.	Calculated on Final Aliquot.		
		Total Corticoid as Hydrocortisone. µg.	Total Found µg.	% Recovery
1.	13	13	13	-
2.	13	130*	103	79
3.	13	163†	146	89

(1) Initial level = 13 µg. per final aliquot.

i.e. $13 \times \frac{10}{3} \times \frac{2}{1} \times \frac{400}{300}$ = 116 µg. per 500 ml.

* (2) $(116 + 1000) \times \frac{300}{400} \times \frac{1}{2} \times \frac{3}{10}$ = 130 µg. per final aliquot.

† (3) $\left[(116 \times \frac{300}{400}) + 1000\right] \times \frac{1}{2} \times \frac{3}{10}$ = 163 µg. per final aliquot.

of hydrocortisone.

(b) Effect of pH upon Extraction of Free Corticoids.

Aliquots were taken from a 24 hour urine collection and the pH of the samples were adjusted as indicated below, Table IV. The urine or aqueous washings were extracted four times with 100 ml. chloroform and estimated according to the method already described.

TABLE IV

Effect of pH upon Extracting of Corticoid Material from Urine.

Test No.	Sample	mg. Corticoid / 24 Hr.
1.	Urine taken to pH 1.0	1.01
2.	Fresh urine, pH 5.6	0.58
3.	Urine taken to pH 10.0	0.46
4.	Alkali washings from pH 1.0 re-extracted with chloroform	0.05
5.	Alkali washings (4) acidified to pH 1.0 and again re-extracted with chloroform, and oxidised without further washing.	0.35

It can be seen that the greatest yield of corticoid material was/

was obtained at pH 1.0, was lower when the fresh urine was extracted without adjustment of pH and was lower still when taken to pH 10.0.

Further examples, Table V, of the effect of acidification upon the yield of formaldehydogenic material are shown below.

TABLE V

Effect of Acidification upon Yield of Corticoid Material from Urine.

Neutral Urine mg. Corticoid/24 Hr.	Urine taken to pH 1.0 mg. Corticoid/24 Hr.
0.51	0.92
0.31	0.53
0.33	0.70

Lieberman and Dobriner (1948) attributed the increase on acidification to hydrolysis of sulphate conjugates and found that glucuronides were not hydrolysed in this manner. The effect of acidification and the time acidified urines are allowed to stand before extraction has been the subject of much discussion, (Paterson, 1952).

Extraction of the alkali wash liquor with chloroform yielded practically no formaldehydogenic material but if the/

the same liquid was then taken to pH 1.0 and re-extracted, and distilled without an alkali wash, this resulted in a significant yield of formaldehyde. This suggests that alkali soluble steroid or phenolic material is removed at this stage which is capable of yielding formaldehyde on oxidation with periodic acid.

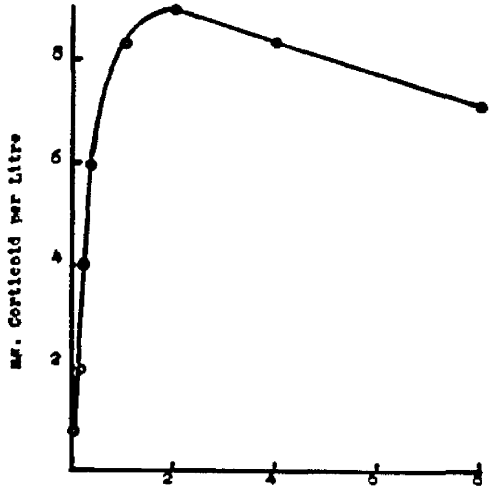
4. DETERMINATION OF MAXIMUM CONDITIONS FOR HYDROLYSIS OF GLUCURONIC ACID CONJUGATES IN URINE.

Employing standardised ox spleen glucuronidase preparation and the formaldehydogenic steroid method already described, experiments were conducted to find the optimal conditions for the hydrolysis of urinary corticoids conjugated with glucuronic acid. Information regarding duration of action, concentration of enzyme and hydrogen ion concentration was being sought.

In each instance 50 ml. of pooled male urine was used and where necessary was buffered with 2 ml. M-sodium acetate buffer of pH 5.0, 2 ml. chloroform were added to inhibit bacterial action and the whole incubated at 37°C. in the dark for the time specified in the different series of the tests.

On completion of the hydrolysis the urine was cooled to/

Fig. 4(a)



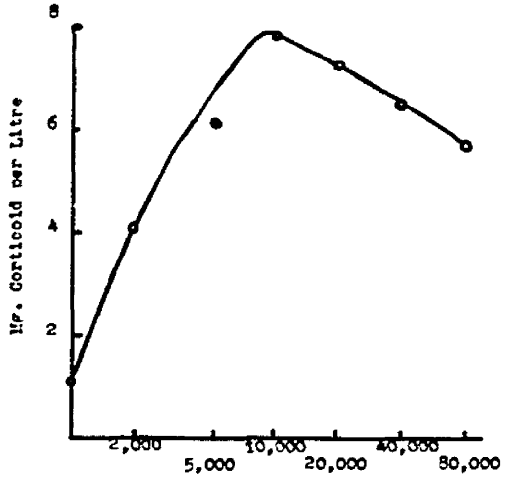
Time in Days of Incubation at 37°C.

50 ml. pooled male urine. Buffered to pH 5.0

5,000 units - Glucuronidase

Fig. 4(b)

DETERMINATION OF MAXIMUM CONDITIONS FOR THE LIBERATION OF GLUCURONIC ACID CONJUGATED CORTICOIDS FROM URINE

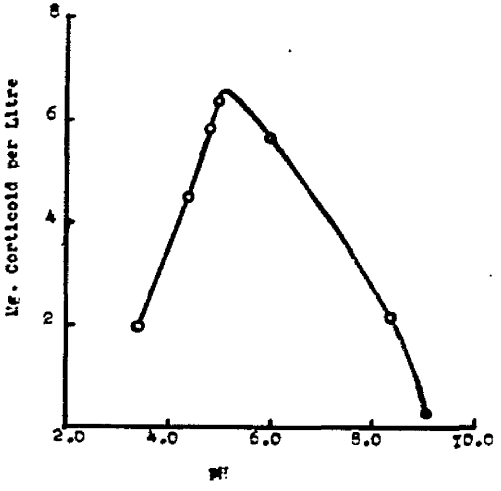


Units -Glucuronidase

50 ml. pooled male urine. Buffered to pH 5.0.

Incubated at 37°C. for 24 hours.

Fig. 4(c)



50 ml. pooled male urine. 5,000 units -Gluc'ase

24 hour incubation at 37°C.

to room temperature and extracted twice with 50 ml. and once with 25 ml. of chloroform, washed twice with 20 ml. 0.1 N-sodium hydroxide and once with 20 ml. of water. The aqueous extracts were re-extracted with 25 ml. of chloro-:form which was added to first extractions. The mixed chloroform extract was dehydrated with anhydrous sodium sulphate and 100 ml. aliquot was taken to dryness and then divided into two parts (see p.18), one of which was oxidised with periodic acid, the other serving as a control, according to the method described on p.14. The final results are charted on Fig. 4. It can be seen from Fig. 4(a) that a 48 hour incubation achieves optimal action at pH 5.0, at what was later shown to be submaximal enzyme concentration, and with only 24 hours incubation it was found, Fig. 4(b), that about 10,000 units of glucuronidase were necessary at pH 5.0 to achieve the greatest effect. The activity over pH range 3.0 to 9.0 was also studied and it was shown, Fig. 4(c) that maximum activity of the enzyme was at or near pH 5.0.

These findings are essentially in agreement with those of Cohen (1951).

It can be concluded therefore from these experiments that for optimal hydrolysis of formaldehydogenic steroids in/

in urine, one should allow 100 units of glucuronidase to act for 48 hours at pH 5.0 per ml. of urine.

In pregnancy urine there is a most striking difference in the yield of chloroform soluble material before and after treatment with glucuronidase preparation consisting mainly of pregnandiol, and the explanation for the need for such excessive quantities of enzyme mentioned above no doubt lies in the fact that the corticosteroid conjugates con-:stitute only a small part of the glucuronic acid glycoside type of compound found even in normal urine and that sub-:strate competition therefore exists, but the main reason no doubt lies in the mass law which necessitates a gross ex-:cess of enzyme in order to force the reaction to any degree of completion.

Fishman (1939) has shown that considerable differences exist in the Michaelis constant for different glucuronides and was able to show that the naturally occuring oestriol glucuronoside was more readily hydrolysed than the borneol or menthol compounds.

5. RECOVERY OF HYDROCORTISONE ADDED TO URINE IN THE PRESENCE OF GLUCURONIDASE PREPARATION.

A further short experiment was performed to test the effect of the presence of glucuronidase, in the quantities found necessary in the previous section, upon the recovery of/

of added hydrocortisone.

In these tests 50 ml. of urine were buffered to pH 5.0 and 5000 units of glucuronidase was added as indicated. In the first sample to which 2 ml. chloroform were added, the action of glucuronidase upon the urine was used to serve as a control. To the second sample was added 1000 µg. hydro-:cortisone in 2 ml. chloroform and a third sample was treat-:ed in a manner similar to the first. All three were then incubated at 37°C. for 48 hours and 1000 µg. hydrocortisone in chloroform was added to the third immediately after ex-:traction with chloroform. The results are shown in Table VI.

TABLE VI

Recovery of Hydrocortisone Added to Urines Treated with Glucuronidase.

Sample No.	Initial µg./50 ml.	Added µg.	Found µg.	Recovered µg.	% Recovery
1.	1390	-	1390	-	-
2.	1390	1000	2160	770	77
3.	1390	1000	2420	1030	103

There appears therefore to be some degree of destruction of/

of hydrocortisone during the time of contact with glucuron-:idase in the urine, (c.f. Fig. 4(a) and 4(b)). The in-:itial concentration of corticoid in the above urine was high (27.8 mg./litre), and much higher concentrations ob-:tained than when the free corticoid recoveries were being studied and no apparent loss was evident in the third specimen.

6. PROPORTION OF FREE TO GLUCURONIC ACID CONJUGATED CORT-:ICOSTEROID IN URINE.

Urines from a number of normal subjects and from a miscellaneous group of patients were studied in order to obtain information which would serve as a guide to the use-:fulness of this scheme of hydrolysis and to the type of result which could be expected later when biological assays were also being conducted. In this series of tests eight hour aliquots were taken from 24 hour urine collections, adjusted to pH 1.0 and extracted, as detailed in descrip-:tion of method in earlier section, and one hour aliquots were taken to pH 5.0, 100 Fishman units of glucuronidase preparation were added for each millilitre of urine and the whole incubated at 37° C. for 48 hours to give "Free + Gluc-:uronic acid conjugates". From Table VII it can be seen that the average excretion of free urinary corticoid material, as/

TABLE VII

EFFECT OF GLUCURONIDASE ON YIELD OF FORMALDEHYDOGENIC CORTICOIDS.

CASE NO.	AGE yrs.	VOLUME ml./ 24 Hr.	PREFORMED CREATININE gm./24 Hr.	CORTICOIDS mg./24 Hr. FRESH URINE Free pH 1.0	After G'ase.	G'ase. Free	BOILED URINE After G'ase.	G'ase. Free
NORMAL MEN								
1.	43	940	1.16	0.73	9.2	12.3	7.5	10.2
2.	26	930	1.61	0.67	10.7	15.9	-	
3.	39	940	0.68	0.62	6.3	10.2	9.2	14.8
4.	47	520	0.45	0.83	5.5	6.6	5.8	7.0
5.	37	1,100	2.01	0.80	8.6	10.7	8.8	11.0
6.	61	1,020	1.61	0.87	10.3	11.8	9.5	10.9
7.	51	776	1.02	0.56	9.7	17.3	10.9	19.5
8.	43	2,120	2.04	1.09	19.1	17.5	19.3	17.7
9.	58	1,800	1.85	0.60	8.8	14.7	8.4	14.0
10.	40	1,570	1.67	1.12	15.4	13.8	-	-
AVERAGE:				0.80	10.4	13.8		
NORMAL WOMEN								
1.	40	870	0.89	0.59	8.3	14.1	8.5	14.4
2.	35	1,220	1.12	0.38	4.6	12.1	-	-
3.	53	940	1.38	0.47	7.7	16.4	8.1	17.2
4.	26	640	0.49	0.31	9.9	31.9	-	-
5.	42	1,010	0.88	0.38	11.9	31.4	-	-
6.	-	1,120	1.32	0.64	11.7	18.3	-	-
7.	43	660	0.94	0.52	17.8	34.2	-	-
8.	57	1,730	1.04	0.31	5.1	16.4	6.2	20.0
9.	32	1,320	1.20	0.71	18.2	25.6	-	-
10.	38	980	1.37	0.54	9.3	17.2	9.5	17.6
AVERAGE:				0.49	10.5	21.8		

as extracted by chloroform at pH 1.0, in the small series studied is 0.80 mg. and 0.49 mg. per 24 hours for normal males and females respectively, and that the total extract-:able corticoid after treatment of the urine with glucur-:onidase is 10.4 mg. and 10.5 mg., in other words one can expect on an average a 14-fold increase in formaldehydogenic material after treatment of male urine with glucuronidase and about a 20-fold increase in female urine. This means that although women excrete lower levels of free corticoid material, the level of total corticoid is almost identical and the results infer that there is almost equal output of adrenal corticosteroids in the two sexes, but that in the female there is more complete utilisation, or reduction to inert metabolic end-products or conjugation. Tables VII and VIII show that by boiling the urine and cooling prior to the addition of glucuronidase, a further moderate increase is usually apparent.

Notes on Cases Reported on Table VIII.

Case 1 A patient who suffered from Cushing's disease of very rapid onset who had submitted to a subtotal adrenalectomy and who still showed a high 17-ketosteroid excretion. Cortisone resulted in a marked increase in total corticoids with attendant clinical changes. Prednisolone caused a distinct fall in total corticoids and also succeeded in lowering the 17-ketosteroids to within normal limits.

Case 2/

Table VIII

EFFECT OF GLUCURONIDASE IN URINARY CORTICOSTEROIDS IN A CELLAR BOIS GROUP OF CASES.

CASE NO.	CONDITION	TREATMENT	SEX	AGE yrs.	VOLUME ml./24Hr.	TOTAL CORTICOSTEROIDS mg./24Hr.	CORTICOIDS mg./24 Hr. FRESH URINE Free pH 1.0	FRESH URINE After G'ase.	FRESH URINE G'ase./Free	BOILED URINE After G'ase.	BOILED URINE G'ase./Free
1.	Cushing's Disease (After sub total adrenalectomy)	(a) Nil	F	28	1760	1.34	1.20	17.7	14.7		
		(b) Cortisone			1320	0.75	-	51.1	-	-	-
		(c) Prednisolone			1540	0.68	-	5.4	-		
2.	Rheumatoid Arthritis	ACTH	F	34	770	0.95	0.17	6.1	35.9	-	-
3.	Rheumatoid Arthritis	ACTH	F	50	740	0.88	3.16	50.5	16.0	-	-
4.	Rheumatoid Arthritis	Cortisone	F	38	1530	1.22	1.74	16.6	9.5	-	-
5.	Osteogenesis Imperfecta	Methyl androstene-diol	F	7	628	0.25	0.26	3.7	14.2	-	-
6.	Idiopathic Oedema	NIL	F	50	3080	0.89	0.45	7.1	15.8	-	-
7.	Simmonds' Disease	Untreated	F	49	500*	0.40	0.19	2.9	15.4	-	-
8.	Simmonds' Disease	Thyroid	M	60	850	0.82	0.34	7.1	20.9	-	-
9.	Simmonds' Disease	(a) Untreated	F	63	1480	0.75	NIL	10.0		10.2	
		(b) Cortisone			930	0.94	1.70	6.5	3.8	6.5	3.8
		(c) Cortisone			1540	0.97	2.06	9.3	4.5	4.5	6.7
10.	Pemphigus	(a) NIL	F	52	1500	1.25	0.72	5.1	7.1	-	-
		(b) Cortisone			2330	1.39	1.36	28.1	20.7	31.6	23.2
		(c) ACTH			510*	0.64	6.4	19.2	3.0	24.9	3.9
11.	Pemphigus	Cortisone & ACTH	F	67	2400	1.40	5.0	76.5	15.3	-	-
12.	Uncomplicated Pregnancy	NIL	6 months		1060	0.87	0.93	5.5	5.1	-	-
13.	"	NIL	7 months		1390	1.28	1.57	14.7	9.4	-	-
14.	"	NIL	8 months		1760	1.03	0.95	20.6	21.7	-	-

* Heavily infected urine.

Case 2 Rheumatoid arthritic patient who failed to respond to ACTH.

Case 3 In contrast to above patient, this case showed a marked response to ACTH which was evident in both free and conjugated fractions.

Case 4 Long standing case of rheumatoid arthritis who had received cortisone, with alternate boosting doses of ACTH, over a period of several years.

Case 5 Young child with osteogenesis imperfecta receiving methyl androstenediol as a low androgenic anabolic steroid for her bone condition.

Case 6 Patient with idiopathic oedema who showed normal corticoid excretion levels.

Case 7 A case of untreated Simmonds' disease in which there existed a urinary infection. Part of the free corticoid may be accounted for by the action of bacterial glucuronidase acting upon conjugated material.

Case 8 An example of Simmonds' disease in the male. The patient at this point was receiving only a small dose of thyroid and showed subnormal levels of urinary corticoid. He has subsequently been maintained in excellent health on 12.5 - 25 mg. cortisone per day.

Case 9 No free corticoid could be demonstrated in this patient's urine although a normal level was found after treating the urine with glucuronidase. This has been reported previously in cases of Simmonds' disease (see also Cases 9 and 10 in Table XII). The patient showed a normal response to cortisone on two later occasions.

Case 10 Resistant and unstable case of pemphigus who has been the subject of a separate report (Neill, 1955). During the third assay, the patient had an active infection of the urinary tract and the very high free fraction was undoubtedly due to hydrolysis of conjugated material by bacterial enzymes. The third collection appears to be incomplete as judged by/

by the creatinine excretion.

Case 11 Case of pemphigus on intensive therapy with ACTH and cortisone simultaneously. Gross increase in both free and conjugated fractions.

Cases 12, 13 and 14. All three cases show moderate increase in free corticoids as compared with average of series of normal females. In cases 13 and 14, the conjugated fractions are also increased.

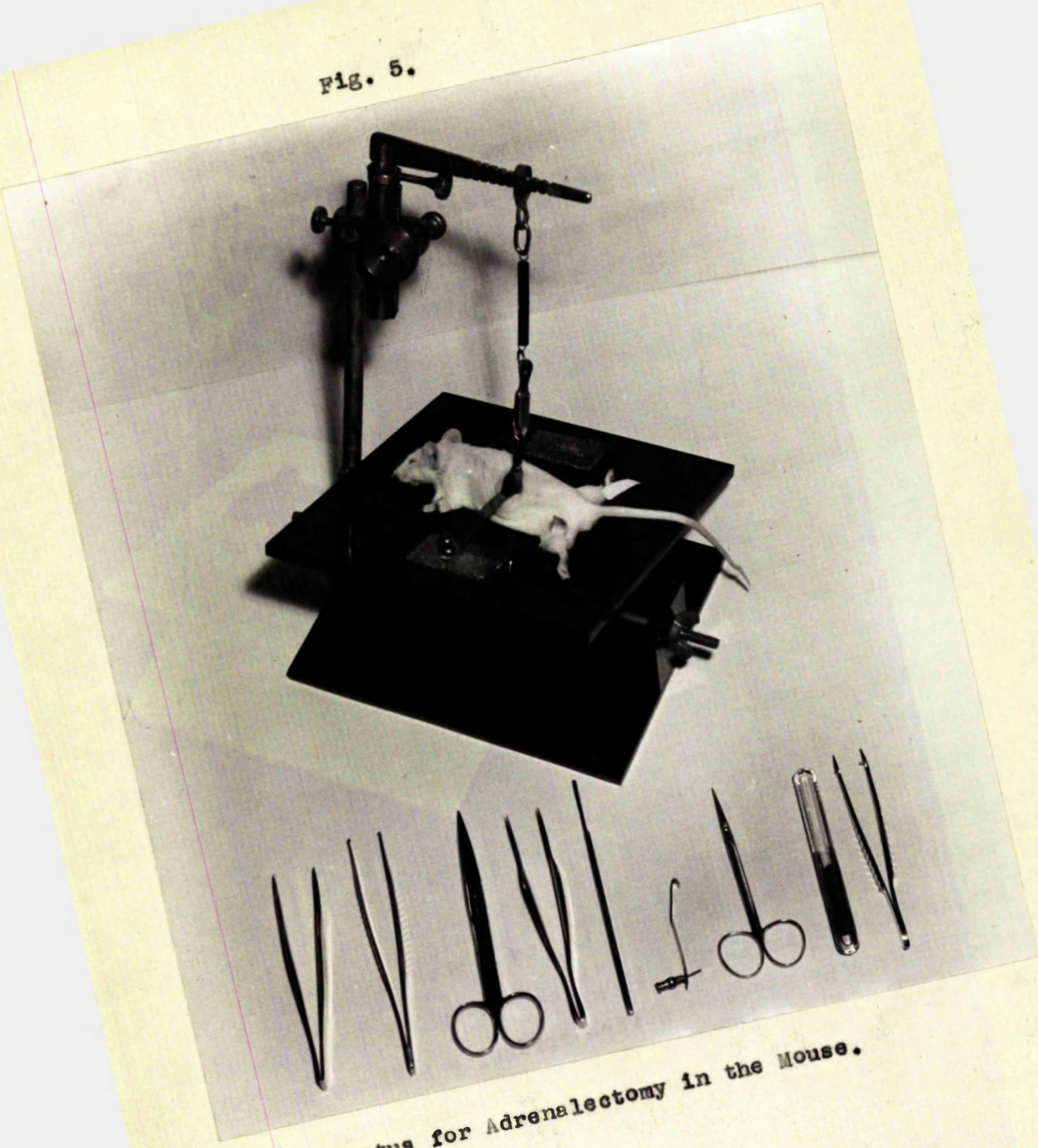
Chapter III

BIOLOGICAL ASSAY OF URINARY CORTICOIDS.

It is recognised that the effect of the glucocortic-:oid hormones in fed animals is to suppress the oxidation of glucose and thus cause an excessive deposition of glyco-:gen in the liver, whereas in fasting animals neoglucogenes-:is results from the accelerated breakdown of protein sources under the action of the hormones, (Evans, 1936; Long, Katzim and Fry, 1940; and Wells and Kendall, 1940.) These processes are advanced in the livers of animals well supplied with amino acids, (Guest, 1941; Mirski, Rosenbaum, Stein & Wertheimer, 1938).

The two principal methods for the assay of glucocorti-:coids by means of liver glycogen in the adrenalectomised mouse are those of Eggleston, Johnston, and Dobriner (1946) and Venning, Kazmin and Bell (1946). In the former an effort is made to maintain existing liver glycogen stores by injection of the hormone and thus combat depletion which would normally take place during the fasting period of the test. In the latter method the glycogen stores are first intentionally reduced by a prolonged fast and the degree of replenishment gives a measure of the activity of the adreno-:cortical preparation. 'Chemical maturity', both from the point of view of the age of the animal and previous dietary history/

Fig. 5.



Apparatus for Adrenalectomy in the Mouse.

history seemed necessary if satisfactory results were to be obtained. Irrespective of which method is used adequate post-operative care and feeding are essential.

1. ADRENALECTOMY OF THE MOUSE.

The programme entailed the development of a satisfactory method of adrenalectomy of the mouse. Several descriptions appear in the literature of methods which have been devised for adrenalectomy of the rat. (Richter, 1941; Grollman, 1941), but it was found that the same procedures when applied to the mouse presented considerably more difficulty in virtue of the fact that the animals were so much smaller and also because the adrenal gland in the mouse, particularly on the right side does not "shell out" as easily as in the rat.

The following scheme has been evolved which allows one person to adrenalectomise mice, with a considerable degree of success, at the rate of about six per hour. The apparatus used is illustrated in Fig. 5. It consisted of a plastic board, 6" x 5" with two cork inserts. To one side was a rod, which could be raised or lowered by a rack and pinion, and carried a horizontal beam projecting over the board to which was attached by means of a fine spring, a Diffenbach's "bull dog" artery clamp. The board was so arranged that it could be tilted and held at an angle of 45" and when in use the board/

board was illuminated with a Horstman "Pulsite" lamp, the magnifying lens of which assisted during dissection.

The mouse was anaesthetised by a subcutaneous injection of 0.2 ml. of a freshly prepared one per cent nembutal sol-:ution and left until it was under the effect of the drug.

It was then stretched on the board, its legs being held by string elastic threaded through suitably placed holes. The hair of the back was removed over a suitable area with a set of electric clippers and the skin swabbed with ether. A midline incision about 1 cm. in length was made at the level where the kidney could be palpated, almost from the middle of the back upward. The skin on the left side was retracted and held in position by a small retractor which could be pinned into the cork insert. The fascia of the muscle was next incised with a pair of fine eye scissors, up-:wards and inwards towards the upper pole of the kidney. The central portion of the muscle was grasped in the Diffenbach clamp and the outer portion collected and held by the re-:tractor. By raising the rack and pinion a good exposure of the upper pole of the kidney could be obtained and using a fine pair of forceps and a probe the kidney capsule and the adrenal gland were freed from fat and connective tissue. The adrenal vessels were occluded by another pair of forceps for/

for about thirty seconds after which the adrenal was easily removed without haemorrhage. The retractor and clamp were withdrawn, the facia pulled well over and the process repeated on the right side. This was technically more difficult and greater care was necessary if haemorrhage was to be avoided. When the second adrenal had been successfully removed the peritoneum was closed and the skin sutured with two stitches of fine gut. Casualty rate was low and with experience 90 to 95 per cent adrenalectomies were proved to be complete.

Adrenalectomised mice could be maintained in good condition on the special diet stated below and using 0.9% NaCl as drinking water. Mice were kept on occasions for periods of up to six weeks, but all died within two days if tap water was substituted for saline solution.

2. ESTIMATION OF LIVER GLYCOGEN.

Glycogen was estimated by the method of Good, Kramer and Somogyi (1933). Other methods have recently become available, (Kemp and Van Heijningen, 1954), but where large numbers of estimations have to be done in rapid succession it was found that the method of Good et al. was to be preferred.

The animals were killed by snapping their necks and their/

their livers removed within 40 seconds and immediately immersed in 5 ml. hot 30 per cent potassium hydroxide solu-:tion contained in strong 4" x $\frac{3}{4}$" round bottomed test tubes which had been graduated at 10 ml. Each tube was supplied with a numbered glass rod to assist in the homogenisation and ultimate solution of the liver tissue.

The tubes were immersed for 20 minutes in a boiling water bath by which time digestion was complete. At this point the tubes were removed and cooled, after which 5.7 ml. 95 per cent ethanol was added with constant stirring. This maintains the 1.1 : 1. alcohol ratio advised in the original technique. The contents were once again brought to boiling point in the water bath when it was found that the glycogen was thoroughly coalesced into a precipitate which could be easily centrifuged. The glycogen deposit, after being drained by inversion was dissolved in 4.0 ml. of N-sulphuric acid, stoppered with a glass tear and hydrolys-:ed in a boiling water bath for two and a quarter hours. The contents of the tubes were then neutralised by the addition of 0.7 ml. 5 N-sodium hydroxide and diluted to 10 ml. from which 1 ml. samples were taken and the glucose estimat-:ed by the colorimetric method of Nelson (1944).

3. RECOVERY OF ADDED GLYCOGEN./

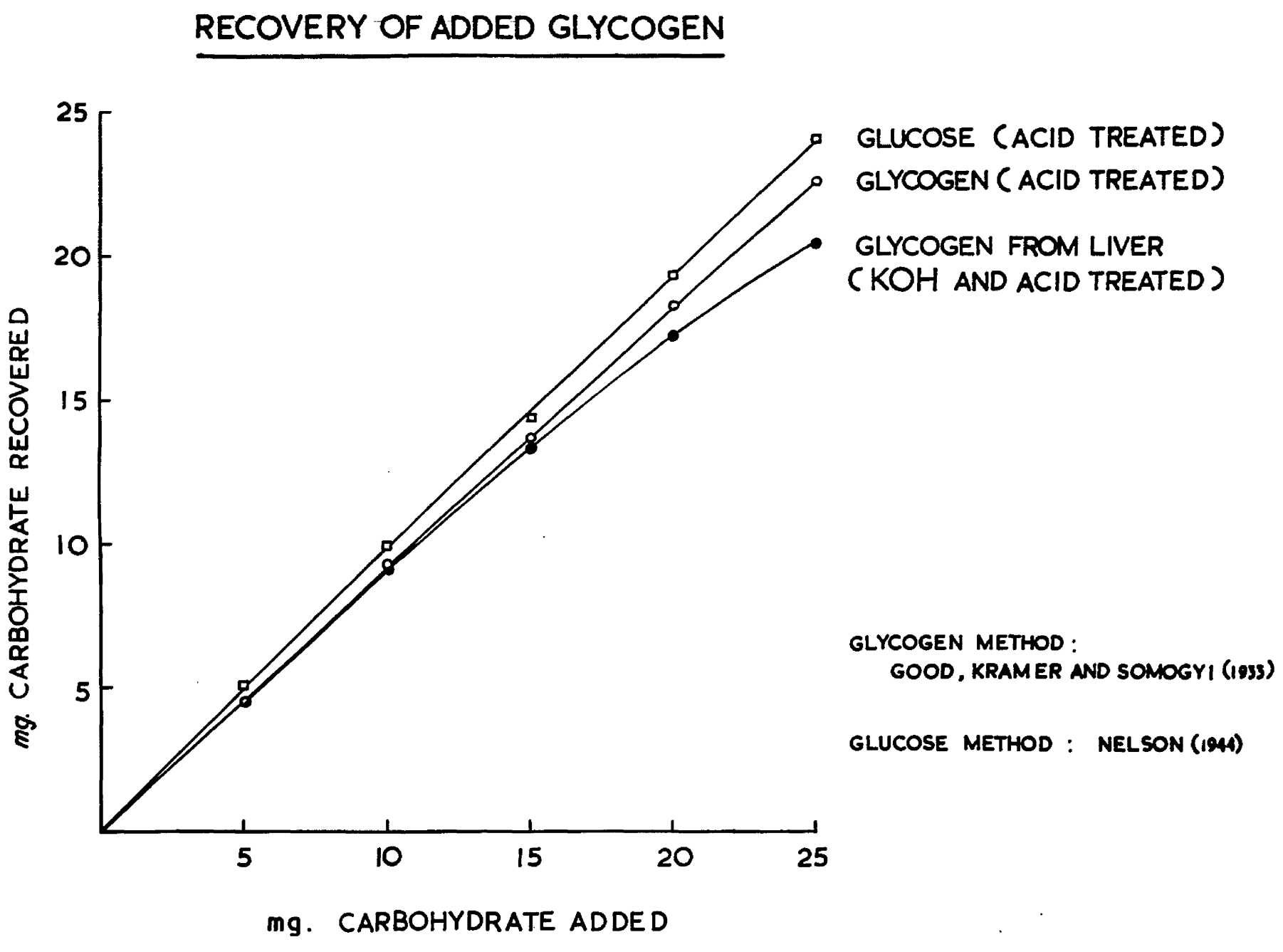
3. RECOVERY OF ADDED GLYCOGEN.

About 25 gm. guinea pig liver was homogenised in 0.9% sodium chloride with the aid of an "Atomixer" and allowed to stand for two days in the refrigerator by which time the glycogen content had fallen almost to Zero. This homogenate was used in the recovery experiments described below.

200 mg. glycogen (B.D.H.) were dissolved in exactly 8 ml. water. Tubes graduated at 10 ml. were prepared and to each was added 2.5 ml. of liver homogenate and 0.5 ml. 60% KOH and 1.0, 0.8, 0.6 ml. etc. glycogen were added, the volume in each case being made up to 6 ml. with water. These open tubes were heated in a boiling water bath for 20 minutes, cooled and treated with 5.7 ml. alcohol as in Good's method. The glycogen was precipitated by boiling, after which the tubes were cooled, centrifuged, and then decanted and drained. The contents were hydrolysed by boiling with 4 ml. N-sulphuric acid for 2½ hours, the solu: :tions were then treated with 0.7 ml. 5 N-NaOH, diluted to 10 ml. with water and 1 ml. taken for the estimation of glucose by Nelson's method.

The same volumes of glycogen solution were taken in separate tubes, made up to 1 ml. with water and treated directly/

Fig. 6.


RECOVERY OF ADDED GLYCOGEN
25
20
15
10
5
mg. CARBOHYDRATE RECOVERED
5
10
15
20
25
mg. CARBOHYDRATE ADDED
GLUCOSE (ACID TREATED)
GLYCOGEN (ACID TREATED)
GLYCOGEN FROM LIVER
(KOH AND ACID TREATED)
GLYCOGEN METHOD :
GOOD, KRAMER AND SOMOGYI (1933)
GLUCOSE METHOD : NELSON (1944)

directly with sulphuric acid alone for 2½ hours, after which the glucose was estimated as indicated above.

It can be seen from Fig.6 that there is significant loss as a result of the alkali treatment, especially at the higher levels, when compared with "acid alone" specimens, but even with direct acid treatment there is a recovery of only 91%. This suggested that part of the explanation for the low glycogen recovery lay in the degree of purity of the glycogen and that 90 to 95 per cent of glycogen could be recovered and estimated from liver tissue. When samples of glucose ranging from 5 to 25 mg. were treated with the acid, exactly as for hydrolysis of glycogen, 96 per cent recovery was obtained.

4. CONTROL EXPERIMENTS.

In all these experiments the adrenalectomised mice were maintained in a thermostatically controlled room at a temperature of 78 ± 2°C. They were fed on the following modification of McCollum's Lactation Diet as recommended by Forbes, Griswold and Albright (1950).

High Protein Diet for Adrenalectomised Mice.

1215	g.	Whole meal flour
270	g.	Casein.
180	g.	National dried milk.
27	g.	Calcium carbonate.
18	g.	Sodium chloride.
90	g.	Margarine.

1% Cod liver oil.

For experiments by the method of Eggleston the mice were given, on the evening prior to the tests, the same diet except that bran was substituted for the whole meal flour. This latter modification was suggested by Forbes et al. (1950) since they found that there was a tendency for a diet lacking in bulk to remain too long in the intestine during the fasting period with the result that absorption con-:tinued from the gut which affected the fasting glycogen level. This latter complication was not found to be sig-:nificant in the method of Venning, according to Nissim (1953), where a more prolonged period of fast takes place.

Eggleston et al. (1946) showed that adrenalectomised mice could have their livers depleted of glycogen as a result of a seven hour fast. It was decided therefore to estimate the average liver glycogen content in the following four groups of mice.

Group (1) Normal Mice.

These animals were given the high protein diet for four days and were killed at 10 a.m. on the morning of the fifth day, under the conditions described for estimation of liver glycogen p. 38. The body weight of the animal was noted and the liver removed within 40 sec. and placed in hot 30% KOH. It/

It can be seen that there is little difference between standard deviation as assessed on mg. glycogen per 10 g. body weight and mg. glycogen per 100 g. liver. The latter involved additional weighing of the liver and is time consuming, so that it was decided to express results exclusively as glycogen per 10 g. body weight.

Group (11) Normal Mice (Fasted for 7 hours)

These animals were given the high protein diet for four days and on the morning of the fifth day they were placed in separate cages and kept without food or water for seven hours, after which they were killed and their livers removed quickly and placed in hot 30% potassium hydroxide solution. As was to be expected, there was a considerable fall in the liver glycogen content and a wide scatter was also evident in the individual results.

Group (111) Sham Operated Mice (Fasted for 7 hours)

The mice of this group were subjected to the same operative procedure as described for adrenalectomy, but the adrenal glands were left intact. They were given the same dietary regime as animals in the previous group.

It can be seen that the surgical manipulation had caused a fall in the average glycogen level.

Group/

Group (IV) Adrenalectomised Mice (Fasted for 7 hours)

The mice in this group were adrenalectomised by the procedure outlined on p. 36. They were maintained on the high protein diet with 0.9% sodium chloride solution as drinking water for four days, and transferred on the morn-:ing of the fifth day to individual cages, because of the risk of cannibalism, the cages containing only cotton wool. They were fasted from 8 a.m. on the morning of the fifth day and sacrificed at 3 p.m.

A summary of the results of the above tests is shown on Table IX.

TABLE IX

Liver Glycogen Content of Mice Maintained under Different Conditions.

Group No.	Condition	No. of Mice.	Liver Glycogen mg./10g. body weight.	Glycogen mg./ 100 g. liver
I.	Normal Mice	20	12.4 ± S.D. 3.02	1.99 ± S.D. 0.55
II.	Normal Mice 7 hour Fast	20	3.51 ± 2.24	
III.	Sham Operat-:ed Mice 7 hour Fast.	20	1.18 ± 0.88	
IV.	Adrenalecto-:mised Mice 7 hour Fast.	20	0.17	

5. ASSAY OF COMPOUND F.
(Method of Venning, Kazmin and Bell, 1946)

The mice were adrenalectomised, usually on a Sunday or Monday and placed on the high protein diet till Thursday evening at 5 p.m. when they were transferred to individual cages, without food and given only 0.9% saline as drinking water. The mice during the earlier experiments were given a subcutaneous injection of 0.25 ml. adreno-cortical extract to tide them over the immediate post operative period, but it was later found that if adequate care was taken in respect of food and temperature conditions this was unnecessary. On Friday morning the saline was removed and the tests started. Injections of 0.2 ml. solution were given at 9.00, 9.45, 10.30, 11.15 a.m., 12.00 noon, 1.00 and 2.00 p.m. and the animals were killed at 3.00 p.m.

Olsen, Jacobs, Richert, Thayer, Kopp and Wade (1944) showed that when oil was used as the vehicle for corticoid material in biological assays using rats, only 60% of the response was obtained as compared with the same amount of material injected in 10% ethanol. Propylene glycol has been used as the solvent in the eosinophile method but was unsatisfactory in the glycogen method as it resulted in glycogen deposition of itself. Ten per cent ethanol was therefore/

therefore used as solvent in the bioassays.

Estimation of Basal Glucose Requirement.

Adrenalectomised mice were injected with 1.4 ml. of 10 per cent ethanol containing the quantities of glucose listed in Table X, in seven injections of 0.2 ml.

TABLE X

Basal Glucose Requirement for Six Hours after 16 Hour Fast.

No. of Mice	Glucose mg.	Liver Glycogen mg./ 10 g. body weight
8	50	Nil
6	75	0.14
6	100	2.14
6	200	7.4

It can be seen that 75 mg. can be given to adrenalectomised mice, which have been fasted for 16 hours, over the period of the injections without significantly increasing liver glycogen. The quantity of glucose met the basal requirements of the animals and rendered them more sensitive to any simultaneously injected corticoid material.

Log dose - Response Regression Line.

Samples of pure cortisone acetate and hydrocortisone (free alcohol)/

(free alcohol) were gifted by Messrs. Upjohn (England). Since the hydrocortisone was suitable as a standard for the chemical estimation of formaldehydogenic corticoids and it had a slightly higher potency in glycogenic units than cortisone (Olson, Thayer and Kopp, 1944), it was decided to use it also as the standard for the biological assay.

Several experiments were conducted in order to prepare a log dose-response regression curve. In these the hydro-:cortisone was weighed out on a semi-micro balance accurate-:ly to 10 µg. The material was dissolved in ethanol, 50% sterile glucose solution was added and the volume made up with distilled water, such that the final solution contain-:ed 100 µg. Compound F and 75 mg. glucose in a final 1.4 ml., the ethanol concentration being 10 per cent.

The mice had been adrenalectomised and placed on the high protein diet as previously described, p. 41.

Example

2.15 mg. Compound F in 3.01 ml. ethanol.

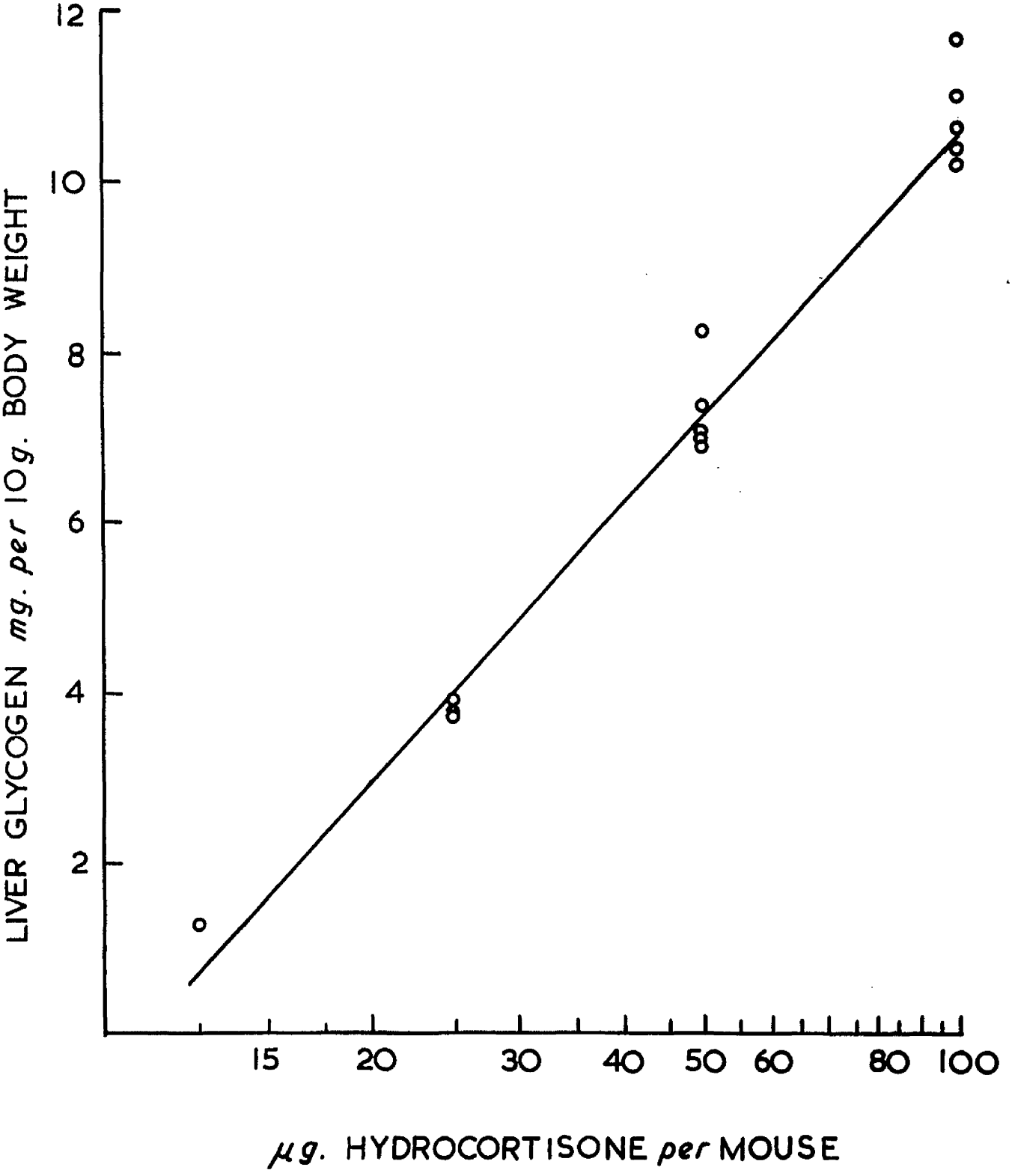
2.8 ml.	taken	)
3.0 ml.	50% glucose solution	) Solution A
22.2 ml.	sterile water	)
28.0 ml.		

1.4 ml. per mouse = 100 µg. Compound F, 75 mg. glucose, in 10% ethanol.

As a diluent/

Fig. 7.

LOG DOSE-RESPONSE CURVE FOR HYDROCORTISONE



As a diluent:

5.6 ml. ethanol	)
6.0 ml. 50% glucose	) Solution B
44.4 ml. sterile water	)

By serial dilutions of Solution A in Solution B a range 100 µg., 50 µg., and 25 µg. Compound F were prepared. Detailed results of the control experiments, using hydrocortisone as standard substance, are collected on p. 73 et seq. and the regression equation has been calculated by the method of least squares and evaluated as :

$$Y = 11.04\ x - 11.42,$$

where Y is the response and x the logarithm of the dose. The log dose-response curve upon which results have been interpolated is shown in Fig. 7.

The mean square of errors, s, calculated on these controls was computed to be 2.648 and the index of precision, λ, was therefore 0.2398. This latter value is about twice that of the authors of the method ($\lambda = 0.158$) and shows a less satisfactory assay, but is about half of that of Nissim ($\lambda = 0.505$), who has discussed the statistical aspect of this assay, the significance of sensitivity and accuracy, and has concluded that the main factor in the co-efficient of variation was the strain of animal used. Venning et al. worked with mice from a closely inbred colony, while Nissim purchased/

purchased his stock directly from dealers. The mice used in these experiments had been inbred for about one year prior to the start of the tests and continuously thereafter.

Chapter IV

CHEMICAL AND BIOLOGICAL ASSAY OF FREE AND CONJUGATED CORTICOIDS IN HUMAN URINE.

With the experience gained in the earlier part of the work and recognising the limitations of the methods already studied, attention was directed to the main purpose of the investigation, the comperison of the results obtained from the estimation of free and conjugated urinary corticoids, by chemical and biological methods, in normal urines and in urines from certain abnormal conditions.

It was decided to confine the estimations to that fraction of corticoid material in fresh urine which could be directly extracted with chloroform and to a second fraction which could be further liberated from the same urine specimen by glucuronidase enzyme acting at pH 5.0 and extract-:able with chloroform.

A few preliminary trials were run in order to find what volume of normal male urine in terms of day collections it would be necessary to process and inject in order to ob-:tain a satisfactory response by the biological method. It was soon found that by taking the whole of a 24 hour collection that one tenth of the final extract was usually satisfactory for "test" and "blank" estimation of free corticoid by the chemical method and that if the remaining

eight-tenths were dissolved in 10% ethanol to which 750 mg. of glucose were added to give a final volume of 14 ml., seven injections of 0.2 ml. per mouse, that is the equival-:ent of eight hundredths of a 24 hour collection, gave a satisfactory response by the biological method.

With the conjugated material one fiftieth was adequate for the "test" and "control" estimations by the chemical method and the remaining 48/50ths were again taken up in 10% ethanol, with 750 mg. glucose, to give a final volume of 14 ml. The final preparation for injection in the case of the free material had a pink fluorescent appearance, while the conjugated extract yielded a brown opalescent solu-:tion.

Time Table for the Conduct of the Experiments.

DAY

1. Twelve to twenty mice were adrenalectomised according to method already described (p.36). The animals were placed in thermostatically controlled room at a temp-:erature of 78 ± 2°C. and placed on high protein diet and given 0.9% sodium chloride solution as drinking water.

   24 hour urine collection started.

   Glucuronidase enzyme was reconstituted and activity assayed as already described (p.12).

2./

DAY

2. Fresh 24 hour urine was extracted with chloroform and the extract washed with 0.1 N-NaOH and water and taken to dryness at reduced pressure at a temperature below 50°C. and the dry extract stored in cool dark cupboard.

The urine was adjusted to pH 5.0 and M-sodium acetate buffer at the same pH, added to give a final 0.05 M. concentration. Glucuronidase was then added, 100 units per ml. of urine and the flask and its contents in-:cubated at 37°C. for 48 hours.

3. -

4. After 48 hours incubation the urine was cooled and ex-:tracted with chloroform. The extract was washed with 0.1 N-NaOH and water and taken to dryness under reduc-:ed pressure.

5. The "free" and "conjugated" residues were normally dissolved in 10 ml. and 50 ml. chloroform respectively and two 1 ml. aliquots removed from each flask for "test" and "blank" assays by the chemical method, (p.18). The remainder of the chloroform was once more taken to dryness under reduced pressure and re-:tained for the biological assay.

On the evening of the fifth day the adrenalectomised mice/

DAY

mice were placed in separate cages containing only cotton wool and were given saline as drinking water, but no food.

6. The remainder of the "free" and "conjugated" extracts were taken up in 1.4 ml. ethanol, 1.5 ml. 50% sterile glucose and 12.1 ml. water added to give a final volume of 14 ml. Injections were given at the times indicated (p.45) and at 3.00 p.m. the animals were weighed, then killed and their livers digested in hot 30% KOH (p.38) and the glycogen precipitated with ethanol and allow to drain.

7. The collected glycogen was hydrolysed by dilute sulphuric acid in a boiling water bath and glucose estimated, (p.39).

These combined estimations were made on five normal males and three normal females and on seven patients whose condition are indicated in Table XII. 24 hour urines were usually employed although in certain of the normals and the two cases of hypopituitrism 48 hour urine collections were employed and in the case of patients with nephrosis undergoing treatment with ACTH, only a fraction of a 24 hour collection was required.

Observations on Results Tabulated in Table XII.

Cases 1 - 5/

TABLE XIX

Comparison of the Chemical (formaldehydogenic) and Biological (Mouse Liver Glycogen) Methods of Assaying Urinary Corticoids. Results expressed as mg. hydrocortisone per 24 hour.

Case No.	Condition	FREE (NEUTRAL) Chem.	Biol.	Biol. / Chem.	GLUCURONIC ACID CONJUGATES Chem.	Biol.	Biol. / Chem.
1.	Normal Male	1.34	0.26	0.19	21.4	1.04	0.049
2.	"	0.83	0.56	0.68	15.7	0.64	0.040
3.	"	0.61	0.27	0.44	13.5	0.32	0.024
4.	"	0.60	0.28	0.47	13.5	0.42	0.031
5.	"	0.54	0.26	0.48	15.4	0.42	0.027
	Average	0.78	0.33	0.45	15.9	0.57	0.034
6.	Normal Female	0.38	0.18	0.47	9.3	0.22	0.024
7.	"	0.49	0.33	0.67	16.1	0.26	0.016
8.	"	0.33	0.27	0.82	11.7	0.22	0.019
	Average	0.40	0.26	0.65	12.7	0.23	0.016
9.	Simmonds' Disease (F)	0.09	Nil	-	0.0	0.08	0.009
10.	" (F)	0.26*	Nil	-	6.0	0.08	0.013
11.	Cushing's Disease (M)	1.63	0.54	0.33	37.4	1.35	0.036
12.	Pemphigus (M) (On cortisone)	1.98	0.57	0.29	35.4	0.96	0.029
13.	Nephrosis (M) (On ACTH)	2.69*	0.77	0.29	65.6	3.84	0.059
14.	Nephrosis (F) (On ACTH)	6.79	1.43	0.21	48.0	1.87	0.039
15.	Adrenal Tumour (M)	0.92	0.31	0.34	29.1	0.49	0.017
		17-Ketosteroids: 209 mg. per 24 hours.					

* Heavily infected urines.

Cases 1 - 5 (Normal Males):

It can be seen that in normal male subjects about one half of the free fomaldehydogenic material as estimated by the method of Corcoran and Page appeared to be biologically active, whilst of the corticoid material thereafter liberated by glucuronidase about one thirtieth was biologically active.

Cases 6 - 8 (Normal Females):

The females again showed a lower free fraction than the males as estimated by the chemical method and lower biological activity in both fractions.

Cases 9 and 10: In these cases of Simmonds' disease it was significant that there was no biological activity in either the free or conjugated fraction, although the latter showed appreciable amounts when estimated by the chemical method. The free corticoid was negligible in the first case and would probably have been lower in the second, but for the bacterial action.

Case 11: This patient was suffering from Cushing's disease and showed a high free fraction, bearing in mind that urine had not been taken to pH 1.0, and the extract appeared to contain relatively less active material, since the ratio of biological to chemical corticoid was lower/

lower than found in the normal subjects. The conjugat-:ed material was definitely raised by both chemical and biological estimates. The patient subsequently submitt-:ed to subtotal adrenalectomy with considerable ameliora-:tion of his symptoms.

Case 12: This male patient was receiving a course of cortisone therapy as part of his treatment for pemphigus. The findings are surprisingly similar to those in Case 11 and show relatively less biological activity in the free fraction. The difference between his total excretion and a normal male level would amount to about 20 mg. per day, the fraction of corticoid material ex-:pected to be excreted on a dosage of 100 mg. cortisone per day.

Cases 13 and 14: These were cases which showed all the manifestations of the nephrotic syndrome. A course of ACTH therapy was given which resulted in a temporary relief in both cases. The biological assay demonstrated that only about a quarter of the free material estimated chemically was biologically active. The conjugated values were the highest of the series and in Case 13 it was possible to obtain a satisfactory response with the equivalent of 25 ml. urine per mouse. The question

can/

can be raised of the possibility of increased permeabil-:ity of the renal tubules to steroid material in such cases.

Case 15: This was a patient suffering from an adrenal tumour. The 17-ketosteroids in the sample studied were estimated to be 209 mg. per diem but although the cort-:icoids were elevated by chemical method they were normal by biological assay and gives evidence of the dissociation of adrenal function. In such a case one would expect to find no significant change in 17-ketosteroid excretion under stimulation with ACTH and no depression on treat-:ment with cortisone in contradistinction to cases of simple adrenal hyperplasia.

It would appear from Table XII, that when a high cort-:icoid concentration is presented to the kidney, the free fraction consists of a higher proportion of inert metabolites and in addition a relatively higher proportion of biologically active material is conjugated without reduction, the overall effect being a more rapid elimination of what is potentially toxic material.

Detailed data on the chemical and biological assays in all these cases is collected together on p.78 et seq.

Chapter V

CONCLUSIONS AND SUMMARY

A study of the Corcoran and Page (1948) method for the estimation of urinary corticoids has been made in which formaldehyde, hexamethylenetetramine and hydrocortisone (free alcohol) have been used as standard substances.

The first two compounds gave identical results provided the hexamethylenetetramine was first given a preliminary acid hydrolysis. Hydrocortisone proved to be a more satis-:factory standard, even though it was found under the con-:ditions of the test to yield only 80 - 85 per cent of the theoretical amount of formaldehyde, because it was subjected to the same oxidation and distillation procedure as the urine extracts.

Recovery of hydrocortisone added to neutral urine was found to be between 70 and 80 per cent when calculated from the hydrocortisone calibration curve, that is, only 60 per cent of the theoretical yield. Part of this loss was known to be due to incomplete oxidation and part was shown to be due to incomplete extraction by chloroform since the addition of hydrocortisone to a dried urine extract improved recovery al-:though there was still a loss or fixation of formaldehyde.

Preparations/

Preparations of ox spleen β-glucuronidase were prepared and standardised and used for the liberation of urinary conjugates. It was found that in order to obtain optimal yields 100 Fishman units of the enzyme must be added per ml. of urine and allowed to act at pH 5.0 for 48 hours at 37°C.

It was apparent that excessive amounts of enzyme and prolonged incubations were to be avoided as both these conditions resulted in reduced yield and it was also evident that when urines were heavily infected unexpectedly high yields of free corticoid material could be anticipated, supporting the earlier evidence (Cohen and Marrian, 1935), that bacterial action could result in liberation of conjugated steroid material, in fact preparations of Escherichia coli have been used for the liberation of urinary conjugates, (Patterson, 1937). If urine was taken to pH 1.0 before extraction, a higher yield of corticoid material was obtained than if it were extracted without adjustment, whereas taking the urine to pH 10.0 resulted in lower yields. The alkali wash solution which is normally discarded contained material capable of yielding formaldehyde and strict conditions of extraction and washing are necessary if reproducible results are to be obtained. Recoveries of hydrocortisone added to urines which were subjected to the action/

action of glucuronidase were of the same order as that listed for the free material but if the hydrocortisone was added to a urine extract complete recovery was obtained confirming loss during extraction, but due to the much higher concentra-:tions existing loss due to fixation of formaldehyde was not apparent.

In a series of estimations performed on urines from ten male and ten female patients who were considered normal in relation to adrenal function, the yield of free formaldehydo-:genic steroid from urine taken to pH 1.0 averaged 0.85 and 0.49 mg. per 24 hours respectively, expressed as hydrocortis-:one, and is somewhat less than the normals quoted by the authors of the method who found 1.04 mg. and 0.56 mg. per 24 hours for 23 cases from each sex. The yield of total cortic-:oid material after incubation with glucuronidase was found to be 10.4 and 10.5 mg. per 24 hours in the present series.

Urines from a miscellaneous group of fourteen cases receiv-:ing various forms of treatment were examined and the ratio of free to conjugated, even where high levels were experienced, was not greatly different from that found in the normal groups and supports the contention that the kidney clears free and conjugated material at different rates (Bongiovanni and Eberlein/

Eberlein, 1955b).

Cortisone and ACTH were found to produce their usual effects and with the exception of one or two resistance cases resulted in increased yields of both free and conjugated material. Two out of three cases of late pregnancy showed moderately raised corticoid levels.

Preparatory to the study of the biological methods for the assay of urinary corticoids, a technique for the adrenal-:ectomy in the mouse was evolved, the main advantage of the method being that no assistance was required during the opera-:tion.

The recovery of glycogen added to liver homogenates was examined and it was found that the main source of loss in the method used (Good et al., 1933) occurred during the digest-:ion process while the tissue is being dissolved in hot pot-:assium hydroxide, but that 90 - 95 per cent recovery could be effected.

Glycogen was estimated in the livers of groups of normal mice, fasted mice, adrenalectomised and sham adrenalectomised mice. It was found that a seven hour fast reduced the liver glycogen content of mice to 20 per cent of their average morn-:ing level. Sham operated mice fell to about half that value when/

when fasted for the same period four days after their opera-:tion, and adrenalectomised mice were almost completely de-:pleted of liver glycogen during a seven hour fast period.

The method of Venning, Kazmin and Bell (1946) was follow-:ed for the assay of hydrocortisone and it was found that after an overnight fast, 75 mg. glucose could be injected over a period of six hours, without significantly raising the liver glycogen level. Such injections met the basal calorie requirements of the animals and rendered them more sensitive to simultaneously injected corticoid material.

A log dose-response curve, which allowed the assay of glucocorticoid material over the range 12.5 - 100 µg. express-:ed as hydrocortisone, was prepared. The sensitivity was of the same order as in the original work, but the coefficient of variation was greater, the latter being dependent to a very large extent upon the purity of the strain of mouse used in the tests.

Comparison was made of the results obtained by chemical and biological method for the estimation of corticoids in urine, the fresh urine being extracted directly without prior acidification and a further extract was obtained from the same urine specimen after being submitted to the action of glucuron-:idase enzyme.

The/

The difficulty of adrenalectomising a sufficient number of animals at one time and co-ordination of times of injection, etc. precluded the use of four point assays and as a consequence unknowns have been compared against independent controls, and calculation of fiducial limits is therefore not strictly applicable. A wide range of results has however been encountered within the series and the following general conclusions have been drawn.

The average output of free corticoid by five normal men by the chemical method was found to be 0.78 mg. and by the biological assay, 0.32 mg. per 24 hours and three women averaged 0.40 mg. and 0.26 mg. respectively per 24 hours.

For the conjugated material the two methods gave 15.9 mg. and 0.57 mg. per 24 hours for men and 12.7 and 0.23 mg. for women, on the same basis.

Using the eosinophile response in adrenalectomised mice Rosenbergh et al, (1954), found that biologically active material so estimated, ranged in normal urines from 0.34 - 0.76 mg. per 24 hours, from urines extracted at pH 7.0. Aggleston et al, (1946), obtained a value lying between 0.19 - 0.38 mg. per 24 hours for a normal urine which had been directly extracted when examined by their own mouse liver glycogen method/

method.

Cope and Hurlock (1952) estimated normal urine to con-:tain 0 - 0.36 mg. corticoid material in the free state and 0 - 0.64 mg. in urines subjected to Baylis's triple extrac-:tion (1952) as estimated by the mouse eosinophile response, but only 0.07 - 0.25 mg. cortisone plus compound F out of a possible 1.14 - 3.58 mg. on a semi-quantitative basis (1953) after eluting the different steroids from paper chromatograms and applying the Porter and Silber reaction.

It is important therefore when interpreting urinary corticoid results obtained by chemical method to recognise that only a very small part of the total material is biolog-:ically active.

Two cases of Simmonds' disease showed no bilogical activity in either fraction although a significant amount of conjugated material could be detected by the chemical method, indeed this is one of the few types of case in which Forbes et al, (1950), considered the laborious method of bioassay justif-:ied.

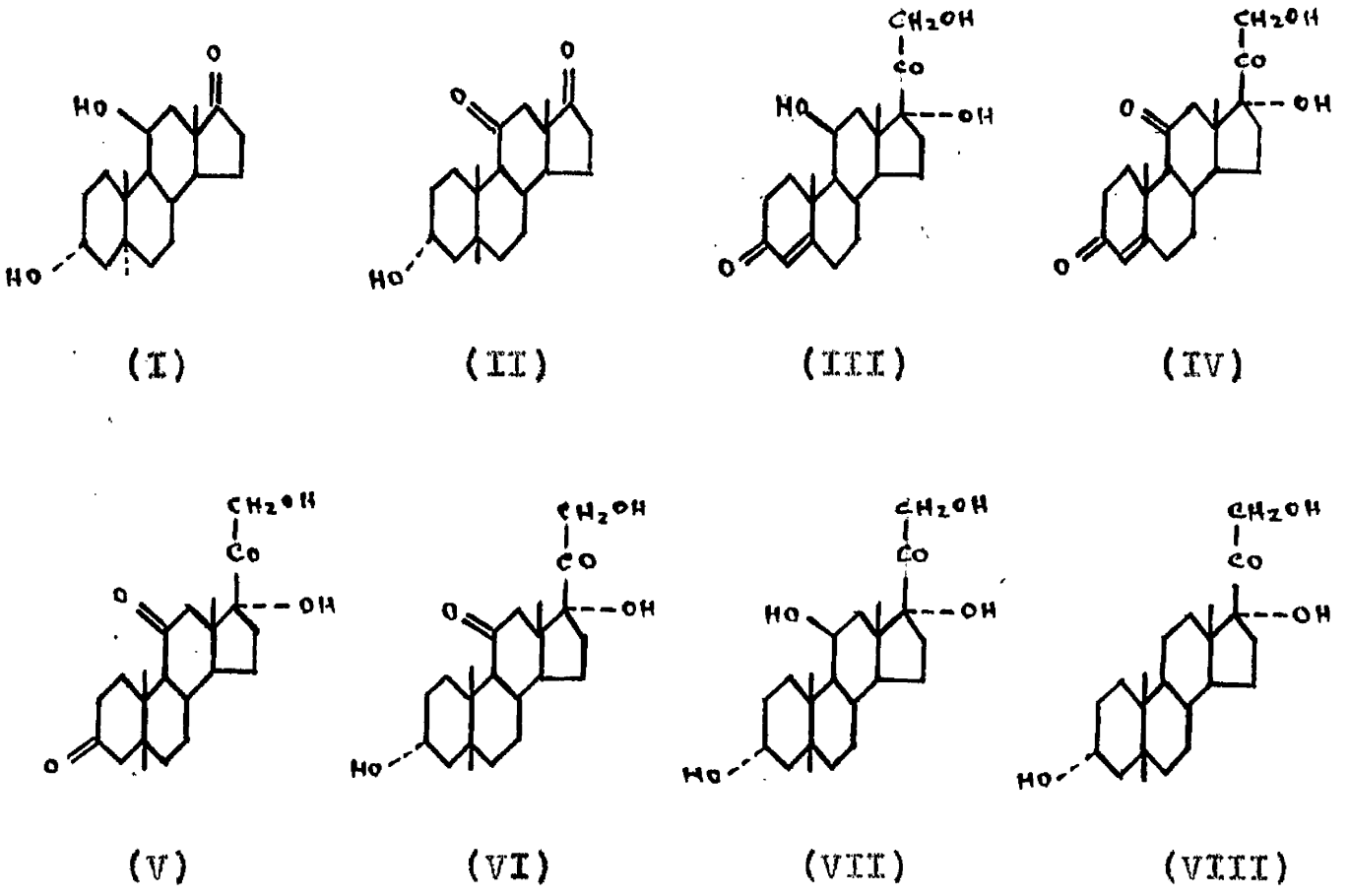
The effect of cortisone and ACTH on the excretion pattern was to cause a significant rise in the free material, though less of it appeared biologically active relative to the total as/

as compared with the normals. The main bulk of the corticoid was still conjugated and inert. An active case of Cushing's disease showed a similar pattern to the above.

A patient suffering from an adrenal tumour was studied in which a very high 17-ketosteroid level was met, and although the chemical assays were elevated the corticoid excretion fell within normal values when estimated by the biological method.

It has been shown that the greater part of the urinary corticoid as estimated chemically is biologically inert with respect to glycogen deposition. This could be anticipated from work done on metabolic end-products of testosterone and progesterone. The normal metabolic path for active steroids is reduction followed by conjugations either with glucuronic acid or sulphuric acid prior to excretion by the kidney. For adrenal steroids Dobriner and Lieberman (1952) have postulated first reduction of the Δ^4-bond in ring A of the molecule, to be followed by reduction of the C_3-ketone group to the alcohol. Further steps may be reduction of the C_{20}-ketone, and even reduction of C_{21}-carbinol group to CH_3. Any metabolite which retains a C_{11}-O or -OH group or C_{17} -OH is considered "labelled" and steroids such as 11-hydroxyandrosterone (1) and/

and 11-ketoaetiocholanolone (11) known to occur in urine are therefore taken as being derived from adrenal sources, the C_{17}-side chain of the steroid having been oxidised.



(I) (II) (III) (IV)

(V) (VI) (VII) (VIII)

Compound F (III) was first identified with certainty in human urine in a case of Cushing's disease, and E (IV) and F were later found in post-operative urines and from cases after stimulation with corticotrophin, (Mason & Sprague, 1948; Mason, 1950). These same compounds were found in normal male urine (Schneider, 1950; 1952) in small amounts, together with/

with dihydrocortisone (pregnane 17α, 21-diol-3,11,20 trione), (V) and tetrahydrocortisone (pregnane 3α, 17α, 21 triol-11, 20 dione), (VI), the latter proving to be the main adrenal steroid metabolite but terahydro-compound F (VII) (pregnane 3α, 11β, 17α, 21-tetraol-20-one) also appears to be present in normal urine (Cope and Hurlock, 1953). Tetrahydro-compound S, (VIII) (pregnane 3α, 17α, 21-triol-20-one) has been found to be principal constituent in the urine from a case of congenital adrenal hyperplasia with hypertension, a condition in which it has been suggested that there is ab-:sence of an enzyme necessary for the introduction of a hydroxyl group at C_{11}-atom, (Eberlein and Bongiovanni,1955).

Nelson and Samuels (1952) using a chromatographic separa-:tion and the Porter and Silber reaction were able to deter-:mine the free plasma corticoids and found a range of 4-10 µg. per 100 ml. in normal subjects. Baylis and Steinbeck(1953) showed by means of paper chromatograms that compound F was the main active constituent and amounted to 3-5 µg. per 100 ml., that no compound S could be detected, and that the balance was more polar under the conditions and consisted of tetrahydro-compound E and tetrahydro-compound F. It has been further demonstrated that the total corticoid material ex-:tractable from plasma after treatment with glucuronidase is usually/

usually about three times the level of free material, even after ACTH stimulation, when both fractions may rise to six times the normal level, (Reddy et al, 1956). The plasma clearance values for free and conjugated corticoid are widely different, 11.2 ml. and 71.0 ml. per minute respectively, when computed in relation to creatinine clearance values, (Bongiovanni & Eberlein, 1955b), showing a preferential re-:absorption of the free material.

It must be accepted therefore that although compound F appears to be the main active adrenal steroid present in plasma, interconversion takes place possibly in the kidney, in such a way that a higher level of reduced compound E is also present and it is this compound in the form of its glucur-:onic acid derivative which is normally presented in largest quantity to the kidney for excretion.

Cope & Hurlock (1953) were able to show that these re-:duction products were biologically inactive, and the overall picture agrees with the findings of this investigation.

There is little doubt that the majority of the chemical methods at present in use are capable of giving a satisfactory estimate of urinary corticoids especially where the total corticoid material is being estimated. The fact that the greater/

greater part of this material is biologically inactive does not detract from the value of such estimations since these reduction products appear to reflect the overall change as is evident when serial estimations are being performed during adrenal stimulation with adrenocorticotrophic hormone. There are indications however, that greater emphasis will be laid upon both qualitative and quantitative identification of par-:ticular metabolites and it is here that chromatographic sep-:aration will play an increasingly important part.

The biological methods of assay of glucocorticoids are laborious, time consuming and sensitive only in the hands of workers specialising in this particular field and have little or no place in routine biochemistry although they must still remain as the final reference, when physiological activity is being investigated.

SUMMARY

1. A study has been made of the formaldehydogenic method for the determination of urinary corticoids and of the factors influencing the liberation of glucuronic acid conjugates by ox spleen β-glucuronidase.

2. The estimation of urinary corticoids by the mouse liver glycogen deposition method has also been investigated and a method is offered whereby one operator can adrenalectomise mice at the rate of about six animals per hour.

3. A comparison has been made of the above chemical and biological methods for the assay of corticoids in extracts from human urine. It has been found that in urine from normal individuals, of the free corticoid material directly extractable with chloroform, slightly more than half is biologically active and of the material conjugated with glucuronic acid and liberated by β-glucuronidase about one thirtieth is biologically active.

4. In the two cases of Simmonds' disease studied, a very low free corticoid excretion was found by the chemical method/

method and no biologically active material could be detected. The conjugated fractions were only slightly reduced compared with the average normal value when estimated chemically but once more only minimal amounts of glycogenic material were demonstrable.

5. Where high total corticoid levels were met, as in urines from a case of Cushing's disease, from a patient suffering from pemphigus on treatment with cortisone, and from two cases of nephrosis during a course of ACTH therapy, the free fraction contained a smaller proportion of glycogenic material than was found in normal individuals, suggesting that excess adrenocortical steroid was being treated as potentially toxic material and its excretion expedited. Urine from both nephrotic patients also showed a higher proportion of active steroid in their conjugated fractions than did the normals.

6. The significance of these findings is discussed in relation to recent work on blood corticosteroid levels.

CHAPTER VI

ADDITIONAL EXPERIMENTAL DATA

CONTROL EXPERIMENTS WITH HYDROCORTISONE

Calculation of Regression Equation by Method of Least Squares.

Dose Mg.	Log dose (x)	f	fx	Response y	fy	fx.y	fx^2	fy^2
12.5	1.0969	6	6.5814	1.28	7.69	8.4352	7.2191	$2.39^2 + 0.35^2$
25	1.3979	22	30.7538	3.80	83.61	116.8784	42.9907	etc. for all
50	1.6990	28	47.5720	7.30	204.53	347.4965	80.8248	of 86 terms
100	2.0000	30	60.0000	10.74	322.32	644.6400	120.0000	
S =		86	144.9072		618.15	1,117.4501	251.0347	5870.3243

$$S(fx'.y) = S(fx.y) - \frac{(Sfx \,.\, Sy)}{f}$$

$$= 1,117.4501 - \frac{144.9072 \times 618.15}{86}$$

$$= 75.8875$$

$$S(x'^2) = S(fx^2) - \frac{(Sfx^2)}{f}$$

$$= 251.0347 - \frac{(144.9072)^2}{86}$$

$$= 6.870902.$$

$$S(y'^2) = S(fy)^2 - (Sfy)^2$$

$$= 5870.3243 - \frac{(618.15)^2}{86}$$

$$= 1427.1923$$

If v is the variation in y,

then $v^2 = \frac{1427.1915}{85} = 16.7905$

$v = 4.097$

Regression coefficient, $b = \frac{S\,(fx' \,.\, y)}{S(x'^2)}$

$= \frac{75.8875}{6.870902} = 11.04475.$

Regression Equation:

$Y = y' + b\,(x - x')$

$= 7.18779 + 11.04475\,(x - 1.684947)$

$= 11.04x - 11.42.$

$S(y'^2) = S(y^2) - (Sy)^2 = 5870.3243 - \frac{(618.15)^2}{86}$

$= 1427.1923$

s^2 (Mean square of errors) $= \frac{1}{n-2}\, S(y'^2) - \frac{S(fx'.y')^2}{s(fx'^2)} = \frac{1}{84}\, 1427.1923 - \frac{(75.8875)^2}{6.8709}$

$= 7.01229$

$s = 2.648.$ Index of precision $= \frac{s}{} = \frac{2.648}{11.0449} = 0.2398$

Average coefficient of variation $= \frac{4.097}{7.188} \times 100 = 55.8$

Experimental Results Obtained During Preparation of log Dose-Response Curve.

Adrenalectomised Mice Injected with Standard Doses of Hydrocortisone.

(See page 40)

Level	Body Weight	Spekker Reading	Glycogen per Liver	Glycogen per 10 gm. Body Weight	Av.	S.D.
12.5 Mg.	20	0.088	4.79	2.39		
	22	0.014	0.77	0.35		
	22	0.096	5.23	2.38	1.28 ±	0.91
(n=6)	21	0.005	0.27	0.13		
	21	0.030	1.63	0.79		
	28	0.085	4.62	1.65		
25 Mg.	20	0.250	13.72	6.87		
	23	0.024	1.32	0.57		
	22	0.156	8.56	3.89	3.73 ±	1.80
(n=8)	19	0.056	3.06	1.61		
	25	0.219	12.00	4.79		
	22	0.176	9.66	4.39		
	22	0.148	8.12	3.69		
	21	0.154	8.46	4.03		
25 Mg.	25	0.240	13.02	5.22		
	23	0.100	5.45	2.37		
	21	0.149	8.15	3.88		
(n=8)	20	0.028	1.52	0.76	3.91 ±	1.69
	25	0.220	11.97	4.78		
	21	0.250	13.61	6.48		
	24	0.210	11.39	4.75		
	23	0.128	6.94	3.02		
25 Mg.	32	0.272	13.40	4.20		
	30	0.257	12.65	4.24		
	28	0.155	7.62	2.75		
(n=6)	26	0.014	0.79	0.27	3.75 ±	2.21
	25	0.392	19.29	7.71		
	27	0.182	8.94	3.34		

Preparation of log Dose-Response Curve.

(Continued)

Level	Body Weight	Spekker Reading	Glycogen per Liver	Glycogen per 10 gm. Body Weight	Av.		S.D.
50 Mg.	22	0.203	11.2	5.12			
	23	0.310	17.1	7.40			
	22	0.223	12.3	5.58	7.40	±	2.26
(n=7)	20	0.182	10.0	5.00			
	23	0.462	25.3	11.02			
	22	0.411	22.7	10.33			
	22	0.295	16.1	7.37			
50 Mg.	22	0.261	14.4	6.54			
	22	0.442	23.3	10.59			
(n=4)	22	0.372	20.5	9.33	8.29	±	1.72
	22	0.269	14.8	6.72			
50 Mg.	23	0.260	14.2	6.18			
	24	0.178	9.7	4.04			
	19	0.234	12.8	6.73	7.10	±	3.61
(n=6)	22	0.094	5.1	2.23			
	21	0.480	26.3	12.51			
	25	0.500	27.4	10.94			
50 Mg.	23	0.210	11.5	5.00			
	24	0.323	17.7	7.38			
	27	0.538	29.5	10.92	7.02	±	2.70
(n=7)	21	0.082	4.4	2.09			
	24	0.283	15.5	6.44			
	20	0.283	15.5	7.75			
	20	0.348	19.1	9.55			
50 Mg.	24	0.203	11.2	4.67			
	26	0.462	25.3	9.73			
	20	0.295	16.2	8.10	6.94	±	2.06
(n=4)	19	0.182	10.0	5.27			

Preparation of log Dose-Response Curve.

(Continued)

Level	Body Weight	Spekker Reading	Glycogen per Liver	Glycogen per 10 gm. Body Weight	Av.		S.D.
100 µg. (n=5)	17	0.462	25.4	14.92	11.68	±	2.20
	23	0.338	18.6	8.09			
	21	0.433	23.9	11.38			
	29	0.602	33.1	11.42			
	21	0.478	26.4	12.58			
100 µg. (n=6)	20	0.246	13.4	6.70	10.65	±	3.40
	25	0.500	27.4	10.98			
	21	0.208	11.4	5.43			
	18	0.460	25.2	14.00			
	23	0.556	30.5	13.26			
	20	0.496	27.1	13.52			
100 µg. (n=6)	22	0.462	22.9	10.42	10.21	±	4.02
	19	0.710	35.2	18.52			
	16	0.192	9.5	5.93			
	23	0.462	22.9	9.95			
	17	0.250	12.4	7.30			
	17	0.313	15.5	9.12			
100 µg. (n=7)	23	0.312	17.0	7.39	10.39	±	3.02
	20	0.376	20.5	10.25			
	21	0.428	23.5	11.09			
	22	0.298	16.2	6.37			
	23	0.698	37.9	16.46			
	22	0.396	21.6	9.82			
	22	0.460	25.0	11.38			
100 µg. (n=6)	26	0.427	21.1	8.11	11.01	±	2.61
	21	0.655	32.3	15.39			
	29	0.574	28.3	9.76			
	32	0.600	29.6	9.26			
	30	0.835	41.2	13.71			
	22	0.438	21.6	9.81			

DETAILED EXPERIMENTAL DATA

on

CASES 1 - 15,

from

TABLE XII

CASE No.1. Condition: Normal Male

Urine Volume :4540 ml. per 48 hours.
Preformed Creatinine :3.62 gm. per 48 hours.
pH : 6.18

FREE CORTICOIDS

4500 ml. urine taken. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 880 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 16 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.286	0.108

Interpolation Difference: 44.0 µg. Comp. F.

$$\therefore \text{ Free Corticoid} = \frac{44.0 \times 10}{1000} \times \frac{16}{3} \times \frac{1000}{2} \times \frac{4540}{880} \times \frac{1}{4500}$$

$$= \underline{1.34\text{mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

2270 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
120 ml. M-sodium acetate buffer pH 5.0 added.
250,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 830 ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.478	0.110

Interpolation Difference: 106.6 µg.

$$\therefore \text{ Conjugated Corticoid} = \frac{106.6 \times 10}{1000} \times \frac{50}{3} \times \frac{1000}{1} \times \frac{2270}{830} \times \frac{1}{2270}$$

$$= \underline{21.4\text{mg. Compound F per 24 hours.}}$$

Serial No. 467.

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		28	0.273	15.24	5.44	
2.		20	0.077	4.26	2.13	
3.	8 th	28	0.228	12.60	4.50	3.47
4.	160	25	0.090	4.98	1.99	+
5.		28	0.107	5.91	2.11	-
6.	per	27	0.228	12.60	4.67	1.43
7.	mouse	-	-	-	-	
8.		-	-	-	-	

$$\therefore \quad \frac{23}{1000} \times \frac{160}{8} \times \frac{1000}{880} \times \frac{4540}{4500} \times \frac{1}{2}$$

$$= \underline{0.26 \text{ mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		29	0.640	35.38	12.20	
2.		25	0.370	20.42	8.18	
3.	48 th	29	0.451	24.88	8.58	9.87
4.	500	28	0.422	23.26	8.29	+
5.	per	29	0.820	45.34	15.42	-
6.	mouse	24	0.284	15.70	6.53	3.01
7.		-	-	-		
8.		-	-	-		

$$\therefore \quad \frac{83}{1000} \times \frac{500}{48} \times \frac{1000}{830} \times \frac{2270}{2270}$$

$$= \underline{1.04 \text{ mg. Compound F per 24 hours.}}$$

CASE No.2. Condition: Normal Male

Urine Volume : 3900 ml. per 48 hours.
Preformed Creatinine : 2.32 gm. per 48 hours.
pH : 7.44

FREE CORTICOIDS

3800 ml. urine taken. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 860 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 16 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.150	0.075

Interpolation Difference: 25.9 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{25.9 \times 10}{1000} \times \frac{16}{3} \times \frac{1000}{2} \times \frac{3900}{860\ 3800}$$

= 0.82mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

1950 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
40 ml. M-sodium acetate buffer pH 5.0 added.
179,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 830ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.360	0.093

Interpolation Difference: 78.4 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{78.4 \times 10}{1000} \times \frac{50}{3} \times \frac{1000}{1} \times \frac{1950}{830\ 1950}$$

= 15.7mg. Compound F per 24 hours.

Serial No.471.

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		27	0.339	16.75	6.20	
2.		25	0.245	12.12	4.84	
3.	7 th	27	0.499	24.68	9.14	6.39
4.	160	22	0.283	14.00	5.83	+
5.		24	0.191	9.44	3.93	-
6.	per	24	0.409	20.22	8.43	1.85
7.	mouse	-				
8.		-				

$$\therefore \quad \frac{41}{1000} \times \frac{160}{7} \times \frac{1000}{860} \times \frac{3900}{3800} \times \frac{1}{2}$$

= 0.56 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		20	0.296	14.62	7.31	
2.		20	0.275	13.60	6.80	
3.	48 th	24	0.464	22.96	9.56	7.48
4.	500	24	0.207	10.22	4.27	+
5.	per	26	0.494	24.40	9.38	-
6.	mouse	23	0.352	17.40	7.57	1.80
7.		-	-			
8.		-	-			

$$\therefore \quad \frac{51}{1000} \times \frac{500}{48} \times \frac{1000}{830} \times \frac{1950}{1950}$$

= 0.64 mg. Compound F per 24 hours.

CASE No.3. Condition: Normal Male

Urine Volume : 1560 ml. per 24 hours.
Preformed Creatinine : 1.72 gm. per 24 hours.
pH : 7.02

FREE CORTICOIDS

1500 ml. urine taken. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 870ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.112	0.072

Interpolation Difference: 15.2 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{15.2 \times 10}{1000} \times \frac{10}{3} \times \frac{1000}{1} \times \frac{1560}{870} \times \frac{1}{1500}$$

$$= \underline{0.61 \text{mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

1500 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
30 ml. M-sodium acetate buffer pH 5.0 added.
150,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 800 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 25 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading: (1:2 dil. of distillate).	0.277	0.070

Interpolation Difference: 2 x 62.3µg.

$$\therefore \text{Conjugated Corticoid} = \frac{124.6 \times 10}{1000} \times \frac{25}{3} \times \frac{1000}{1} \times \frac{1560}{800} \times \frac{1}{1500}$$

$$= \underline{13.5 \text{ mg. Compound F per 24 hours.}}$$

Serial No. 475

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		24	0.093	4.60	1.92	
2.		28	0.110	5.43	1.94	
3.	8 th	22	0.168	8.31	3.78	2.54
4.	100	20	0.148	7.36	3.68	+
5.		24	0.050	2.49	1.04	-
6.	per	22	0.043	2.12	0.96	1.17
7.	mouse	28	0.236	11.64	4.16	
8.		25	0.142	7.01	2.81	

$$\therefore \quad \frac{18}{1000} \times \frac{100}{8} \times \frac{1000}{870} \times \frac{1560}{1500}$$

$$= \underline{0.27 \text{ mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		25	0.313	15.45	6.18	
2.		19	0.010	0.50	(0.26)	
3.	23 th	27	0.114	5.72	2.12	3.54
4.	250	26	0.271	13.38	5.23	+
5.	per	23	0.112	5.55	2.41	-
6.	mouse	24	0.182	9.00	3.75	1.47
7.		28	0.152	7.50	2.68	
8.		22	0.107	5.29	2.40	

$$\therefore \quad \frac{23}{1000} \times \frac{250}{23} \times \frac{1000}{800} \times \frac{1560}{1500}$$

$$= \underline{0.32 \text{ mg. Compound F per 24 hours.}}$$

CASE No.4. Condition: Normal Male

Urine Volume : 1650 ml. per 24 hours.
Preformed Creatinine : 2.13 gm. per 24 hours.
pH : 6.55

FREE CORTICOIDS

1600 ml. urine taken. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 885ml. $CHCl_3$ taken.
Evaporated. Dissolved in 8 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.131	0.078

Interpolation Difference: 19.5 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{19.5 \times 10}{1000} \times \frac{8}{3} \times \frac{1000}{1} \times \frac{1650}{885 \quad 1600}$$

= 0.60 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

1600 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
32 ml. M-sodium acetate buffer pH 5.0 added.
160,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 870 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.293	0.061

Interpolation Difference: 68.4 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{68.4}{1000} \times \frac{10}{3} \times \frac{50}{1} \times \frac{1000}{870} \times \frac{1650}{1600}$$

= 13.5mg. Compound F per 24 hours.

Serial No. 479.

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		26	0.038	1.89	0.73	
2.		23	0.183	9.08	3.59	
3.	6 th	24	0.102	5.05	2.10	2.58
4.	80	27	0.121	6.00	2.22	±
5.		25	0.020	0.21	(0.08)	
6.	per	24	0.171	8.48	3.54	1.02
7.	mouse	24	0.159	7.88	3.28	
8.		-	-			

$$\therefore \quad \frac{18}{1000} \times \frac{80}{6} \times \frac{1000}{885} \times \frac{1650}{1600}$$

= 0.28 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		20	0.100	4.95	2.48	
2.		25	0.323	15.98	6.39	
3.	48 th	23	0.258	12.74	5.54	5.52
4.	500	23	0.334	16.50	7.18	+
5.	per	26	0.412	20.40	7.85	-
6.	mouse	22	0.337	16.69	7.57	1.96
7.		18	0.110	5.44	3.02	
8.		18	0.136	7.39	4.10	

$$\therefore \quad \frac{34}{1000} \times \frac{500}{48} \times \frac{1000}{870} \times \frac{1650}{1600}$$

= 0.42 mg. Compound F per 24 hours.

CASE No.5. Condition: Normal Male

Urine Volume : 2280 ml. per 24 hours.
Preformed Creatinine : 2.55 gm. per 24 hours.
pH : 6.65

FREE CORTICOIDS

2000 ml. urine taken. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 880 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.122	0.091

Interpolation Difference: 12.0 µg. Comp. F.

$$\therefore \text{ Free Corticoid} = \frac{12.0 \times 10}{1000} \times \frac{10}{3} \times \frac{1000}{1} \times \frac{2380}{880} \times \frac{2380}{2000}$$

$$= \underline{\underline{0.54 \text{mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

2000 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
100 ml. M-sodium acetate buffer pH 5.0 added.
176,500 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled, Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 820 ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.313	0.099

Interpolation Difference: 63.8 µg.

$$\therefore \text{ Conjugated Corticoid} = \frac{63.8}{1000} \times \frac{10}{3} \times \frac{50}{1} \times \frac{1000}{820} \times \frac{2380}{2000}$$

$$= \underline{\underline{15.4 \text{mg. Compound F per 24 hours.}}}$$

Serial No. 496.

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		29	0.065	3.42	1.18	
2.		24	0.124	6.51	2.71	
3.	8 th	28	0.080	4.21	1.50	2.31
4.	100	28	0.149	7.82	2.79	+
5.		30	0.086	4.52	1.51	-
6.	per	24	0.114	5.99	2.50	0.74
7.	mouse	30	0.191	10.02	3.34	
8.		24	0.134	7.04	2.93	

$$\therefore \quad \frac{17}{1000} \times \frac{100}{8} \times \frac{1000}{880} \times \frac{2140}{2000}$$

= 0.26 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		26	0.618	32.48	(12.50)	
2.		30	0.139	7.30	2.43	
3.	48 th	23	0.252	13.24	5.76	
4.	500	30	0.253	13.30	4.44	4.42
5.	per	26	0.252	13.24	5.09	+
6.	mouse	29	0.154	8.08	2.79	-
7.		24	0.284	14.92	6.22	1.32
8.		24	0.193	10.15	4.22	

$$\therefore \quad \frac{28}{1000} \times \frac{500}{48} \times \frac{1000}{820} \times \frac{2380}{2000}$$

= 0.42 mg. Compound F per 24 hours.

CASE No.6. Condition: Normal Female

Urine Volume : 1610 ml. per 48 hours.
Preformed Creatinine : 1.21 gm. per 48 hours.
pH : 6.62

FREE CORTICOIDS

1600 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 530 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.130	0.069

Interpolation Difference: 21.8 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{21.8 \times 10 \times 10 \times 600 \times 1610}{1000 \quad 3 \quad 2 \quad 530 \quad 1600}$$

$$= \underline{\underline{0.38 \text{ mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

1600 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
80 ml. M-sodium acetate buffer pH 5.0 added.
176,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 520 ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.406	0.073

Interpolation Difference: 96.7 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{96.7 \times 10 \times 50 \times 600 \times 1610}{1000 \quad 3 \quad 2 \quad 520 \quad 1600}$$

$$= \underline{\underline{9.3 \text{ mg. Compound F per 24 hours.}}}$$

Serial No. 547

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		33	0.334	16.50	5.00	
2.		40	0.425	21.00	5.25	
3.	8 th	34	0.163	8.05	2.37	4.13
4.	100	38	0.213	10.52	2.77	+
5.		38	0.441	21.80	5.74	-
6.	per	38	0.283	13.99	3.68	1.27
7.	mouse	-				
8.		-				

$$\therefore \quad \frac{25}{1000} \times \frac{100}{8} \times \frac{600}{530} \times \frac{1610}{1600} \times \frac{1}{2}$$

= 0.18 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		40	0.346	17.05	4.26	
2.		36	0.423	20.82	5.79	
3.	48 th	33	0.537	26.42	8.00	5.89
4.	500	32	0.238	11.72	3.66	+
5.	per	29	0.397	19.56	6.74	-
6.	mouse	35	0.488	24.10	6.89	1.52
7.		-				
8.		-				

$$\therefore \quad \frac{37}{1000} \times \frac{500}{48} \times \frac{600}{520} \times \frac{1610}{1600} \times \frac{1}{2}$$

= 0.22 mg. Compound F per 24 hours.

CASE No. 7. Condition: Normal Female

Urine Volume : 1020 ml. per 24 hours.
Preformed Creatinine : 1.01 gm. per 24 hours.
pH : 7.62

FREE CORTICOIDS

1000 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 510ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml., then 3ml. taken	1 ml. Control
Spekker Reading:	0.141	0.105

Interpolation Difference: 12.0 Mg. Comp. F.

$$\therefore \text{ Free Corticoid} = \frac{12.0 \times 10}{1000} \times \frac{10}{3} \times \frac{600 \times 1020}{1} \times \frac{}{500\ 1000}$$

= 0.49mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

1000 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
50 ml. M-sodium acetate buffer pH 5.0 added.
100,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 500 ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml., then 3 ml. taken	1 ml. Control
Spekker Reading:	0.375	0.105

Interpolation Difference: 79.2 Mg.

$$\therefore \text{ Conjugated Corticoid} = \frac{79.2 \times 10}{1000} \times \frac{50}{3} \times \frac{600 \times 1020}{1} \times \frac{}{500\ 1000}$$

= 16.1 mg. Compound F per 24 hours.

Serial No. 538

FREE CORTICOIDS

Biological Method

All → 10 ml. at Nelson stage.

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		30	0.453	10.60	3.53	
2.		28	0.241	5.63	2.01	
3.	8 th	28	0.468	10.93	3.90	3.43
4.	100	33	0.621	14.51	4.39	+
5.		31	0.175	4.09	1.32	-
6.	per	28	0.650	15.20	5.43	1.23
7.	mouse	-				
8.		-				

$$\therefore \quad \frac{22}{1000} \times \frac{100}{8} \times \frac{600}{510} \times \frac{1020}{1000}$$

= 0.33 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

All → 10 ml. at Nelson stage.

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		30	0.543	12.68	4.23	
2.		34	0.347	8.11	2.39	2.84
3.	48 th	29	0.261	6.10	2.11	+
4.	500	30	0.288	6.73	2.24	-
5.	per	33	0.429	10.02	3.04	0.70
6.	mouse	27	0.351	8.21	3.04	
7.		-				
8.		-				

$$\therefore \quad \frac{20}{1000} \times \frac{500}{48} \times \frac{600}{500} \times \frac{1020}{1000}$$

= 0.26 mg. Compound F per 24 hours.

CASE No. 8. Condition: Normal Female.

Urine Volume : 870 ml. per 24 hours.
Preformed Creatinine : 0.83 gm. per 24 hours.
pH : 7.41

FREE CORTICOIDS

850 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 510 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.111	0.090

Interpolation Difference: 8.2 Mg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{8.2}{1000} \times \frac{10}{3} \times \frac{10}{1} \times \frac{600}{510} \times \frac{870}{850}$$

$$= \underline{\underline{0.33 \text{mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

850 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
43 ml. M-sodium acetate buffer pH 5.0 added.
100,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 150ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 470ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.269	0.093

Interpolation Difference: 53.8 Mg.

$$\therefore \text{Conjugated Corticoid} = \frac{53.8}{1000} \times \frac{10}{3} \times \frac{50}{1} \times \frac{600}{470} \times \frac{870}{850}$$

$$= \underline{\underline{11.7 \text{mg. Compound F per 24 hours.}}}$$

Serial No. 511.

FREE CORTICOIDS

Biological Method

All → 10 ml. at Nelson stage.

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		29	0.255	6.01	2.07	
2.		32	0.376	8.85	2.77	
3.	8 th	28	0.494	11.62	4.16	2.56
4.	100	33	0.416	9.80	2.97	+
5.		34	0.205	4.82	1.42	-
6.	per	29	0.244	5.75	1.98	0.88
7.	mouse	-				
8.		-				

$$\therefore \quad \frac{18}{1000} \times \frac{100}{8} \times \frac{600}{510} \times \frac{870}{850}$$

$$= 0.27 \text{ mg. Compound F per 24 hours.}$$

CONJUGATED CORTICOIDS

All → 10 ml. at Nelson stage.

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		30	0.226	5.32	1.77	
2.		28	0.317	7.46	2.67	
3.	48 th	28	0.045	1.06	0.38	1.67
4.	100	36	0.277	6.52	1.81	+
5.	per	31	0.113	2.66	0.86	-
6.	mouse	28	0.297	7.00	2.50	0.72
7.		-				
8.		-				

$$\therefore \quad \frac{15}{1000} \times \frac{500}{48} \times \frac{600}{470} \times \frac{870}{800}$$

$$= 0.22 \text{ mg. Compound F per 24 hours.}$$

CASE No.9. Condition: Simmonds' Disease (Untreated)

Urine Volume : 1280 ml. per 48 hours.
Preformed Creatinine : 1.47 gm. per 48 hours.
pH : 6.14

FREE CORTICOIDS

1270 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 500 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.090	0.079

Interpolation Difference: 4.3 µg. Comp. F.

∴ Free Corticoid = $\frac{4.3 \times 10 \times 10 \times 600 \times 1280}{1000 \quad 3 \quad 2 \quad 500 \quad 1270}$

= 0.09mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

1270 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
64 ml. M-sodium acetate buffer pH 5.0 added.
125,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 150ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 470ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.377	0.091

Interpolation Difference: 83.9 µg.

∴ Conjugated Corticoid = $\frac{83.9 \times 10 \times 50 \times 600 \times 1280}{1000 \quad 3 \quad 2 \quad 470 \quad 1270}$

= 9.0mg. Compound F per 24 hours.

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FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		21	0.005	0.11	0.05	
2.		22	0.010	0.21	0.09	
3.	8 th	23	0.008	0.16	0.07	0.17
4.	100	25	0.005	0.11	0.05	+
5.		22	0.355	16.99	(7.68)	-
6.	per	23	0.062	1.31	0.57	0.20
7.	mouse	-				
8.		-				

$$\therefore \quad \frac{Nil \times 100 \times 600 \times 1280 \times 1}{1000 \quad 8 \quad 500 \quad 1270 \quad 2}$$

= Nil mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		24	0.146	3.06	1.27	
2.		27	0.006	0.12	0.04	
3.	48 th	25	0.013	0.27	0.11	0.47
4.	500	22	0.048	1.01	0.46	±
5.	per	27	0.058	1.22	0.45	0.44
6.	mouse	Died				
7.						
8.						

$$\therefore \quad \frac{12 \times 500 \times 600 \times 1280}{1000 \quad 48 \quad 470 \quad 1270} \times \frac{1}{2}$$

= 0.08 mg. Compound F per 24 hours.

CASE No. 10. Condition: Simmonds' Disease (Untreated)

Urine Volume : 640 ml. per 48 hours.
Preformed Creatinine : 1.24 gm. per 48 hours.
pH : 6.71

FREE CORTICOIDS

600 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 500 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.083	0.051

Interpolation Difference: 12.2 µg. Comp. F.

$$\therefore \text{ Free Corticoid} = \frac{12.2}{1000} \times \frac{10}{3} \times \frac{10}{2} \times \frac{600}{500} \times \frac{640}{600}$$

$$= \underline{\underline{0.26 \text{mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

600 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
30 ml. M-sodium acetate buffer pH 5.0 added.
70,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 500 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading:	0.276	0.090

Interpolation Difference: 56.3 µg.

$$\therefore \text{ Conjugated Corticoid} = \frac{56.3}{1000} \times \frac{10}{3} \times \frac{50}{2} \times \frac{600}{500} \times \frac{640}{600}$$

$$= \underline{\underline{6.0 \text{ mg. Compound F per 24 hours.}}}$$

Serial No. 484

FREE CORTICOIDS

Biological Method

All → 10 ml. at Nelson stage.

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		30	Nil	Nil	Nil	
2.		23	Nil	Nil	Nil	
3.	8 th	23	0.010	0.22	0.10	0.05
4.	100	22	0.003	0.06	0.03	
5.		23	0.007	0.16	0.01	
6.	per	30	Nil	Nil	Nil	
7.	mouse	28	0.020	0.46	0.16	
8.		28	0.011	0.25	0.08	

$$\therefore \quad \frac{\text{Nil}}{1000} \times \frac{100}{8} \times \frac{600}{500} \times \frac{640}{600} \times \frac{1}{2}$$

= Nil mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

All → 10 ml. at Nelson stage.

Mouse No.	Material.	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		24	0.149	3.39	1.42	
2.		31	0.036	0.82	0.26	
3.	48 th	25	0.100	2.29	0.91	0.71
4.	500	26	0.081	1.83	0.70	±
5.	per	26	0.070	1.60	0.61	0.41
6.	mouse	26	Nil	Nil	Nil	
7.		29	0.098	2.24	0.77	
8.		25	0.111	2.52	1.00	

$$\therefore \quad \frac{12.5}{1000} \times \frac{500}{48} \times \frac{600}{500} \times \frac{640}{600} \times \frac{1}{2}$$

= 0.08 mg. Compound F per 24 hours.

CASE No.11. Condition: Cushing's Disease.

Urine Volume : 2090 ml. per 24 hours.
Preformed Creatinine : 1.46 gm. per 24 hours.
pH : 6.58

FREE CORTICOIDS

2000 ml. urine taken. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 895ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml., then 3ml. taken	1 ml. Control
Spekker Reading:	0.210	0.079

Interpolation Difference: 41.8 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{41.8 \times 10}{1000} \times \frac{10}{3} \times \frac{1000}{1} \times \frac{2090}{895} \times \frac{}{2000}$$

= 1.63mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

2000 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
40 ml. M-sodium acetate buffer pH 5.0 added.
266,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 850 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 100ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml., then 3 ml. taken	1 ml. Control
Spekker Reading:	0.389	0.076

Interpolation Difference: 91.2 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{91.2 \times 10}{1000} \times \frac{100}{3} \times \frac{1000}{1} \times \frac{2090}{850} \times \frac{}{2000}$$

= 37.4mg. Compound F per 24 hours.

Serial No.485

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		23	0.137	7.87	3.42	
2.		21	0.259	14.90	7.09	
3.	8 th	25	0.371	21.36	8.53	5.96
4.	100	21	0.306	17.60	8.38	+
5.		21	0.131	7.53	3.58	-
6.	per	26	0.234	13.45	5.18	1.97
7.	mouse	20	0.192	11.03	5.52	
8.		-	-	-	-	

$$\therefore \quad \frac{37 \times 100 \times 1000 \times 2090}{1000 \quad 8 \quad 895 \quad 2000}$$

$$= \underline{0.54 \text{ mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		22	0.326	13.75	6.25	
2.		20	0.183	10.52	5.26	
3.	49 th	24	0.500	28.79	11.98	7.71
4.	1000	22	0.259	14.90	6.78	+
5.	per	26	0.447	25.64	9.85	-
6.	mouse	23	0.215	12.37	5.38	2.17
7.		20	0.292	16.78	8.39	
8.		26	0.351	20.20	7.77	

$$\therefore \quad \frac{54 \times 1000 \times 1000 \times 2090}{1000 \quad 49 \quad 850 \quad 2000}$$

$$= \underline{1.35 \text{ mg. Compound F per 24 hours.}}$$

CASE No.12. Condition: Pemphigus (100 mg. cortisone acetate orally per diem)

Urine Volume : 1870 ml. per 24 hours.
Preformed Creatinine : 0.92 gm. per 24 hours.
pH : 6.19

FREE CORTICOIDS

1640 ml. urine taken. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 870ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml., then 3ml. taken	1 ml. Control
Spekker Reading:	0.272	0.127

Interpolation Difference: 43.3 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{43.3 \times 10}{1000} \times \frac{10}{3} \times \frac{1000}{1} \times \frac{1870}{870} \times \frac{1}{1640}$$

$$= \underline{\underline{1.98 \text{mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

1640 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
80 ml. M-sodium acetate buffer pH 5.0 added.
160,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 250ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 880ml. $CHCl_3$ taken.
Evaporated. Dissolved in100ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml., then 3 ml. taken	1 ml. Control
Spekker Reading:	0.383	0.120

Interpolation Difference: 77.3 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{77.3 \times 10}{1000} \times \frac{100}{3} \times \frac{1000}{1} \times \frac{1870}{880} \times \frac{1}{1640}$$

$$= \underline{\underline{33.4 \text{mg. Compound F per 24 hours.}}}$$

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		22	0.147	7.28	3.31	
2.		28	0.307	15.20	5.43	
3.	8 th	26	0.499	24.65	9.47	5.57
4.	100	31	0.272	13.48	4.36	+
5.		20	0.145	7.17	3.59	-
6.	per	24	0.141	6.97	2.91	2.44
7.	mouse	24	0.297	14.70	6.13	
8.		21	0.399	19.72	9.39	

$$\therefore \quad \frac{35}{1000} \times \frac{100}{8} \times \frac{1000}{870} \times \frac{1870}{1640}$$

$$= \underline{0.57 \text{ mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		23	0.551	27.25	11.85	
2.		26	0.627	31.00	11.92	
3.	97 th	24	0.354	17.50	7.29	9.10
4.	1000	22	0.262	12.97	5.90	+
5.	per	26	0.528	26.17	10.06	-
6.	mouse	27	0.414	20.53	7.60	2.32
7.		-	-	-	-	
8.		-	-	-	-	

$$\therefore \quad \frac{72}{1000} \times \frac{1000}{97} \times \frac{1000}{880} \times \frac{1870}{1640}$$

$$= \underline{0.96 \text{ mg. Compound F per 24 hours.}}$$

CASE No.13. Condition: Nephrosis on ACTH

Urine Volume : 2140 ml. per 24 hours.
Preformed Creatinine : 1.39 gm. per 24 hours.
pH : 8.45

FREE CORTICOIDS

2000 ml. urine taken. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 900ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading:	0.312	0.083

Interpolation Difference: 68.0 γg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{68.0 \times 10}{1000} \times \frac{10}{3} \times \frac{1000}{1} \times \frac{2140}{900} \times \frac{}{2000}$$

$$= \underline{\underline{2.69 \text{ mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

2000ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
100ml. M-sodium acetate buffer pH 5.0 added.
218,000 units glucuronidase added and incubated for 48 hours at 37°C.
Cooled. Extracted x4 with 250 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 800 ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading: (1:3 dil.)	0.420	0.079

Interpolation Difference: 3 x 98.1 γg.

$$\therefore \text{Conjugated Corticoid} = \frac{294.3 \times 10}{1000} \times \frac{50}{3} \times \frac{1000}{1} \times \frac{2140}{800} \times \frac{}{2000}$$

$$= \underline{\underline{65.6 \text{ mg. Compound F per 24 hours.}}}$$

Serial No. 499

FREE CORTICOIDS

Biological Method

KOH digest → 10 ml. as usual.

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		25	0.388	20.31	8.13	
2.		27	0.589	30.83	11.42	
3.	8 th	23	0.460	24.08	10.48	7.56
4.	100	20	0.223	11.66	5.83	+
5.		25	0.200	10.47	4.18	-
6.	per	22	1.027	53.61	(24.37)	2.76
7.	mouse					
8.						

$$\therefore \quad \frac{52}{1000} \times \frac{100}{8} \times \frac{1000}{900} \times \frac{2140}{2000}$$

= 0.77 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

KOH digest → 20 ml.

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		28	0.595	62.27	22.23	
2.		26	0.469	49.06	18.89	
3.	38 th	26	0.404	42.26	16.32	15.62
4.	500	29	0.431	45.08	15.52	+
5.	per	21	0.140	14.62	6.97	-
6.	mouse	21	0.277	28.96	13.79	4.71
7.		22	0.069	(Toxic)		
8.		-				

$$\therefore \quad \frac{281}{1000} \times \frac{500}{38} \times \frac{1000}{800} \times \frac{2140}{2000}$$

= 4.94 mg. Compound F per 24 hours.

(Approx.) repeated with $\frac{8}{500}$ th

Case No.13 continued

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		29	0.453	23.28	8.03	
2.		22	0.319	16.40	7.45	
3.	8 th	25	0.290	14.91	5.97	6.89
4.	500	25	0.149	7.66	3.06	+
5.	per	30	0.340	17.49	5.93	2.42
6.	mouse	25	0.533	27.41	10.98	
7.		-				
8.		-				

$$\therefore \quad \frac{46}{1000} \times \frac{500}{8} \times \frac{1000}{800} \times \frac{2140}{2000}$$

$$= \underline{3.84 \text{ mg. Compound F per 24 hours.}}$$

CASE No.14. Condition: Nephrosis on ACTH

Urine Volume : 1280 ml. per 24 hours.
Preformed Creatinine : 0.98 gm. per 24 hours.
pH : -

FREE CORTICOIDS

1260 ml. urine taken. Extracted x4 with 150 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 480ml. $CHCl_3$ taken.
Evaporated. Dissolved in 10 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10ml.,	1 ml. Control then 3ml. taken
Spekker Reading: (1:2 dil.)	0.386	0.112

Interpolation Difference: 2 x 80.3 µg. Comp. F.

$$\therefore \text{ Free Corticoid} = \frac{160.6 \times 10}{1000} \times \frac{10}{3} \times \frac{600}{1} \times \frac{1280}{480} \times \frac{1}{1260}$$

$$= \underline{\underline{6.79 \text{ mg. Compound F per 24 hours.}}}$$

CONJUGATED CORTICOIDS

1260 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
63 ml. M-sodium acetate buffer pH 5.0 added.
125,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 150ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 520ml. $CHCl_3$ taken.
Evaporated, Dissolved in 50 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised Distilled → 10 ml.,	1 ml. Control then 3 ml. taken
Spekker Reading: (1:3 dil.)	0.368	0.088

Interpolation Difference: 3 x 82.0 µg.

$$\therefore \text{ Conjugated Corticoid} = \frac{246.0 \times 10}{1000} \times \frac{50}{3} \times \frac{600}{1} \times \frac{1280}{520} \times \frac{1}{1260}$$

$$= \underline{\underline{48.0 \text{ mg. Compound F per 24 hours.}}}$$

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FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		28	0.583	28.52	10.18	
2.		26	0.478	23.38	8.98	
3.	4/100 th	26	0.248	12.12	4.66	6.78
4.		26	0.480	23.47	9.04	+
5.		27	0.178	10.42	3.87	-
6.	per	28	0.308	15.06	5.37	2.22
7.	mouse	22	0.227	11.10	5.05	
8.		28	0.405	19.81	7.08	

$$\therefore \quad \frac{45}{1000} \times \frac{100}{4} \times \frac{600}{480} \times \frac{1280}{1260}$$

= 1.43 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		27	0.160	7.82	2.90	
2.		25	0.290	14.19	5.67	
3.	10/500 th	27	0.210	10.28	3.81	5.17
4.		27	0.302	14.77	5.47	+
5.	per	30	0.620	30.31	10.10	-
6.	mouse	26	0.184	9.00	3.46	2.08
7.		28	0.288	14.09	5.03	
8.		27	0.272	13.35	4.95	

$$\therefore \quad \frac{32}{1000} \times \frac{500}{10} \times \frac{600}{520} \times \frac{1280}{1260}$$

= 1.87 mg. Compound F per 24 hours.

CASE No.15 Condition: Adrenal Tumour

(17 ketosteroids : 209 mg./24 hr.)

Urine Volume : 508 ml. per 24 hours.
Preformed Creatinine : - gm. per hours.
pH : 6.35

FREE CORTICOIDS

460 ml. urine taken. Extracted x4 with 100 ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 320 ml. $CHCl_3$ taken.
Evaporated. Dissolved in 20 ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised → Distilled → 10ml., then 3ml. taken	1 ml. Control → Distilled → 10ml., then 3ml. taken
Spekker Reading:	0.102	0.077

Interpolation Difference: 10.0 µg. Comp. F.

$$\therefore \text{Free Corticoid} = \frac{10.0 \times 10 \times 20 \times 400 \times 508}{1000 \quad 3 \quad 1 \quad 320 \quad 460}$$

$$= \underline{0.92 \text{mg. Compound F per 24 hours.}}$$

CONJUGATED CORTICOIDS

460 ml. urine taken after extraction of 'Free'.
Taken to pH 5.0 using glass electrode.
23 ml. M-sodium acetate buffer pH 5.0 added.
50,000 units glucuronidase added and incubated for 48 hours at 37°C.

Cooled. Extracted x4 with 100ml. $CHCl_3$.
Washed with 0.1 N-NaOH, water and 340ml. $CHCl_3$ taken.
Evaporated. Dissolved in100ml. $CHCl_3$.

Chemical Method

	1 ml. Oxidised → Distilled → 10 ml., then 3 ml. taken	1 ml. Control → Distilled → 10 ml., then 3 ml. taken
Spekker Reading:	0.299	0.073

Interpolation Difference: 67.1 µg.

$$\therefore \text{Conjugated Corticoid} = \frac{67.1 \times 10 \times 100 \times 400 \times 508}{1000 \quad 3 \quad 1 \quad 340 \quad 460}$$

$$= \underline{29.1 \text{mg. Compound F per 24 hours.}}$$

Serial No. 526

FREE CORTICOIDS

Biological Method

Mouse No.	Material	Body Weight gm.	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		33	0.232	11.30	3.42	
2.		30	0.233	11.35	3.78	
3.	18 th	31	0.100	4.88	1.57	2.84
4.	200	29	0.208	10.15	3.50	+
5.		28	0.034	1.66	0.41	1.24
6.	per	30	0.251	12.20	4.07	
7.	mouse	28	0.182	8.85	3.16	
8.		-				

$$\therefore \quad \frac{20}{1000} \times \frac{200}{18} \times \frac{400}{320} \times \frac{508}{460}$$

= 0.31 mg. Compound F per 24 hours.

CONJUGATED CORTICOIDS

Mouse No.	Material	Body Weight	Spekker Reading (Density)	Liver Glycogen mg/Liver	Liver Glycogen mg/10 gm. Body Weight	Av. S.D.
1.		33	0.030	1.46	0.44	
2.		25	0.122	5.95	2.38	
3.	48 th	28	0.220	10.71	3.83	2.14
4.	1000	26	0.128	6.24	2.40	+
5.	per	32	0.211	10.25	3.21	-
6.	mouse	30	0.103	5.01	1.67	1.10
7.		27	0.057	2.78	1.03	
8.		-				

$$\therefore \quad \frac{17}{1000} \times \frac{1000}{48} \times \frac{400}{320} \times \frac{508}{460}$$

= 0.49 mg. Compound F per 24 hours.

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