ASSAYS OF URINARY CORTICOIDS.

A COMPARISON BETWEEN RESULTS OF CHEMICAL AND BIOLOGICAL METHODS.

by

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THESIS

Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the Degree of Master of Science.

McGill University,
Montreal, Canada.

May 3rd., 1947.

A C K N O W L E D G M E N T S.

The writer is particularly grateful to Dr. J. S. L. Browne and Dr. E. H. Venning for their general supervision, encouragement, and criticism throughout this undertaking. Appreciation is also due Dr. H. Sobel and Dr. R. D. Heard for demonstrating their chemical method of urinary assay.

Thanks are also due Miss M. Ripstein for helping in preliminary extractions on about five occasions. Mr. V. Kazmin and his assistants performed the glycogenic bioassays under the general supervision of Dr. E. H. Venning, and other assistants performed the 17-ketosteroids and creatinine determinations.

The patients studied at the Royal Victoria Hospital were largely drawn to the author's attention by Dr. J. S. L. Browne and also by Dr. F. L. Selye. Those at the Montreal General Hospital were under the care of Dr. E. S. Mills and Dr. R. Powell. The patients at the Notre Dame Hospital were under the care of Dr. C. E. Grignon. Specimens from the Cleveland Clinic were forwarded by Dr. A. C. Corcoran from patients of Dr. E. P. McCullagh. Those from the Johns Hopkins Hospital were in charge of Dr. J. E. Howard and Dr. J. E. Luetscher, Jr. The patient at the Royal Edward Sanitarium was under the care of Dr. F. Learn Phelps. The author is accordingly grateful to all these cliniciams for their cooperation.

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OUTLINE OF THE PURPOSE OF THIS THESIS.

The experimental data which will be presented consist of estimations on urine samples of normal humans and of patients suffering from a variety of clinical conditions. The urines have been assayed by a biological and by a chemical method, both of which purport to measure substances similar to those isolated from the adrenal cortex. The chief purpose of this undertaking was to define the correlation between the results of the bioassay and chemical assay on each sample of urine. A secondary purpose was to determine the suitability of these methods (particularly the chemical method) for use in hospital laboratories as tools to aid clinicians in their assessment of adrenal cortical function for the diagnosis and management of the individual patients.

The introductory review includes a discussion of the biological and chemical properties of extracts of the adrenal cortex, and of chemical substances derived therefrom. Evidence of adrenal corticoid activity in blood by bioassay is reviewed. Then the history of investigations of urine samples by various biological assays is given, and is followed by a detailed discussion of several chemical methods for assaying urine. The effect of specific hyperfunction and hypofunction of the adrenals, and of various non-specific states of stress on urinary corticoids as determined by both types of assay is outlined. An attempt is made to correlate the previously reported results of urinary levels by biological assays and by chemical assays. The relative advantages of biological and chemical assays are outlined, both from the viewpoint of the scientific accuracy and of the value to the hospital clinician, where economy of time and of costly

materials may be additional important considerations. Finally, an attempt is made to clarify the scientific basis for the interpretation of urinary corticoid assay as an index of significant clinical variation in adrenal function.

The second part of the thesis presents the methods and the experimental results with certain pure substances and with urinary extracts from normal individuals and from various patients. Interpretative comments on the assays follow, with particular reference to the correlation between the results of the bioassay and of the chemical assay. Further comments are given on the reflection of clinical states by the urinary assays. Suggestions are made for certain future research in this field. Finally, the conclusions from the thesis are presented.

BIOLOGICAL AND CHEMICAL PROPERTIES OF EXTRACTS OF THE ADRENAL CORTEX AND OF PURE SUBSTANCES DERIVED THEREFROM.

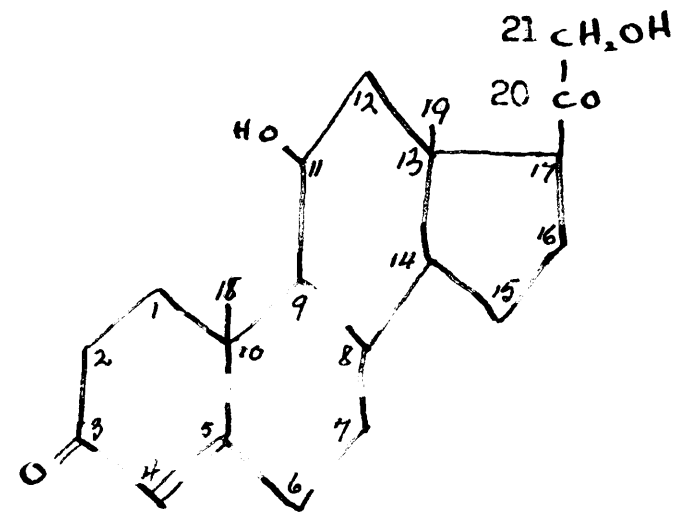
Almost simultaneously in 1927 three groups of workers (41, 91, 92) succeeded in preparing from adrenal cortical tissue extracts capable of prolonging the lives of adrenalectomized animals. This was followed by a determined search by many investigators for the isolation of the active hormone as a pure chemical substance. Wintersteiner and Pfiffner (42), Kendall, Mason and coworkers (93, 94, 56, 66, 52), and Reichstein and co-workers (20), have been prominent in this work. Reichstein (20) (see page 352), in his review published in 1943, lists twenty-eight steroids isolated in pure form from the adrenal cortex up to that date. Not all these are specific to the adrenal (for example progesterone and oestrone are found). Many may be natural metabolites of each other and others may be artefacts produced by the rather strenuous chemical procedures required for their

separation and for the regeneration of the free substances from the isolated acetyl derivatives. All twenty-eight compounds have the four ring steroid nucleus, and in all save four (oestrone and three members of androstane series) there is the pregnane type of two carbon atom side chain. These C-21 compounds fall into groups depending on their oxygen content. Thus there are 8 members of the O-5 series, 9 of the O-4 series, 5 of the O-3 series and 2 of the O-2 series. The relatively few compounds of absolutely certain biological activity are scattered between three of the above series as follows:

O-5 series - (17-hydroxycorticosterone (Kendall's cpd. F)
(17-hydroxy, 11-dehydrocorticosterone (Kendall's cpd. E)

O-4 series - (corticosterone (Kendall's cpd. B)
(11-dehydrocorticosterone (Kendall's cpd. A)
(17-hydroxy, 11-desoxycorticosterone

O-3 series - 11-desoxycorticosterone



The empirical diagrammatic structure of corticosterone is given for reference.

The complete chemical name for corticosterone is Δ^4-pregnene-11-21 diol, 3-20 dione. It is worthy of note that Reichstein's isolations

of 11-desoxycorticosterone and 17-hydroxy, 11-desoxycorticosterone have not been confirmed by other workers, and there is thus the possibility that they are artefacts. One must also emphasize that the non-crystallized amorphous solid material obtained from various extracts is in certain respects more potent biologically per mg. than the pure crystalline compounds, which raises the possibility of the existence of new chemical substances or of more active complex compounds of the known substances.

Biological Properties.

Adreno-cortical extracts have been shown to have a remarkably wide variety of effects in experimental animals. Examples on adrenalectomized animals follow:

(a) Life maintenance and (usually) normal growth: Hartman et al on cat (44); Kuizenga and Cartland on rat (60, 75); Pfiffner et al (54); and also Kendall et al on dogs (95, 96).

(b) Maintenance of normal blood urea: Pfiffner et al on dogs (54).

(c) Maintenance of normal carbohydrate metabolism: Britton and Silvette (72); Long et al (30); Grattan and Jensen (67).

(d) Restoration of muscle-work performance (probably related to carbohydrate metabolism): Ingle (65), and Everse and de Fremery (73).

(e) Increase in resistance to various forms of stress: Selye and Schenker (cold) (32); Marmorston-Gottesman and Perla (histamine)(43); and other traumata and drugs.

The following effects are evident in both adrenalectomized and normal animals:

(f) Alterations in sodium and potassium metabolism: Harrop (74), and Thorn et al (68, 69) on urinary excretion.

(g) Decreased size of the thymus and lymph glands, Wells and Kendall (97); decreased number of circulating lymphocytes, Dougherty and White (89); and increased circulating antibodies, Chase, White and Dougherty (98).

The final effect is seen in normal animals:

(h) Decreased weight of the adrenal glands, Wells and Kendall (97).

TESTS FOR BIOLOGICAL ACTIVITY OF CORTICAL EXTRACTS AND PURE STEROIDS.

On the basis of the above biological effects several methods of bioassay have been developed. Noteworthy is the fact that different preparations or batches of cortical extract do not give parallel values in the various types of assays. This lack of parallelism is even more obvious when different pure steroids are tested and is evidence for qualitative (as well as quantitative) differences in their effects. In Table 1, attention is particularly directed to the negative or insignificant effects of 11-desoxycorticosterone in Ingle's work test (65), in Long's data on carbohydrate metabolism (30), and also in Dougherty and White's data (89) on the effect on lymphoid tissue and release of antibodies. 11-dehydro, 17-hydroxy corticosterone is anomalous in causing an increased excretion of sodium in normal dogs (Thorn, 68, 69).

TABLE I.

RELATIVE ACTIVITIES OF CRYSTALLINE COMPOUNDS IN DIFFERENT METHODS OF ASSAY.

Name	Series	Survival of rat or dog (75, 95, 96)	Cold Resistance (9, 76)	CHO Metabolism (Long 30)	Liver Glycogen (Venning 7)	Ingle's Work Test (65)	Lymphocyte Effect (89)	Sodium Retention (Thorn 69)
17-hydroxy, 11-dehydro-corticosterone	$C_{21}O_5$	+	++	++	+++	+++	+++	0(negative)
Corticosterone	$C_{21}O_4$	++	+++	++	++	++	+++	++
11-desoxycor-ticosterone	$C_{21}O_3$	+++	+	0	0	±	0	+++
Progesterone	$C_{21}O_2$	±	0	0	0	0	?	+

NOTES: for Table 1.

(a) 17-hydroxy corticosterone gives results in different assays rather similar to those of 17-hydroxy-11-dehydrocorticosterone. Also 11-dehydrocorticosterone has activity similar to that of corticosterone. Thirdly, 17-hydroxy, 11-desoxycorticosterone appears slightly less active than desoxycorticosterone in survival and in sodium effect, and is also ineffective in the carbohydrate group of assays.

(b) Whole adrenal cortical extract cannot be compared directly on a weight basis with the above pure compounds, but is, of course, active by all the above tests including the sodium retention test of Thorn. "Amorphous" fractions differ in their modes of preparation and probably in their composition from laboratory to laboratory. Thus, it is difficult to compare such with pure substances, but they appear to be relatively more powerful in the survival tests than in Long's carbohydrate test, Ingle's work test or Thorn's sodium retention test.

SPECIFITY OF BIOLOGICAL ASSAYS FOR ADRENAL CORTICAL SUBSTANCES.

None of the biological methods listed on the previous page appears certainly specific for substances derived from the adrenal cortex. The question as to the exact source of the active substances in potent urinary extracts is of prime importance but will be considered later. It is noted that progesterone in large doses is effective in the survival tests in rats (Kuizenga quoted by 20). Possibly this explains the finding of several workers, e.g. Firor and Grollman (47), that adrenalectomized

pregnant rats survive till labour. Selye (61), using survival in rats as the criterion, found considerable activity in two steroid compounds devoid of oxygen at C-11 and of the conjugated 3-keto group. Neither has been found in adrenal cortical materials. Furthermore, in regard to tests for carbohydrate activity, both stilbestrol and also certain cardiac glycosides show some activity in certain tests (see Reichstein and Shoppee, (20, p. 407). The deposition of liver glycogen in adrenalectomized rats (Reinecke and Kendall 25) or mice (Venning 7) is probably specific for corticosteroids, but as yet, published evidence is not available for the full range of steroids such as the cardiac glycosides.

FRACTIONATION OF EXTRACTS: SOLUBILITY CHARACTERISTICS.

The active substances in the adrenal cortex are soluble in such organic solvents as benzene, ethyl acetate, chloroform, ethylene dichloride and ether. Such solvents have the property of being volatile under reduced pressures at low temperatures; thus considerable concentration of the active material is obtainable. Partition between benzene and water has been frequently used to remove non-specific lipid soluble substances, and also to separate the various pure adrenal steroids. The conditions of extraction of benzene-water partition differ from worker to worker and it is thus difficult to lay down definite laws. The relative volumes of benzene and water and the number of washings of the one with the other are important considerations. A few illustrations are given. Reichstein (20, p. 406) gives a table showing relative solubilities

in water on distribution between benzene and water (no conditions defined). Desoxycorticosterone is only slightly soluble, corticosterone and 11-dehydro-corticosterone moderately soluble, and the two 17-hydroxy-11-oxy compounds are very soluble in water. More detailed information is given by Talbot (6). He added the steroid to 30 cc. benzene which was then extracted ten times with 30 cc. lots of water. The combined water extract was subsequently extracted four times with 45 cc. lots of chloroform. The respective steroid contents of the benzene and the chloroform residues were then determined (See Table II).

TABLE II.

DISTRIBUTION OF CRYSTALLINE CORTICOSTEROIDS BETWEEN BENZENE AND WATER

Steroid Used	Amount of Steroid Added	Steroid Recovered	
		Benzene Phase	Water Phase
	mg.	mg.	mg.
Desoxycorticosterone	0.48	0.40	0.01
11-dehydrocorticosterone	0.21	0.15	0.05
Corticosterone	0.21	0.12	0.08
17-hydroxy-11-dehydro-corticosterone	0.23	0.00	0.23
17-hydroxycorticosterone	0.25	0.00	0.25

Kuizenga and Cartland (59) in fractionating adrenal cortex extract used an ethyl acetate solution which was evaporated and the residue taken up in 200 cc. of water. This was washed by equal volumes of benzene ten times. Then the benzene was concentrated to 200 cc. which was washed by equal volumes of water ten times. The process was continued by three more steps, ending in a benzene solution. There were thus obtained two "benzene residues" consisting of solid material nearly insoluble in water, and in these residues 11-dehydro corticosterone was identified. The final benzene solution yielded crystals of the two 17-hydroxy corticosterones (Compound E and F of Kendall). These latter had evidently passed readily back and forth between benzene and water and probably would have been nearly equally divided between the two phases after one equal volume washing rather than ten such washings. Using their rat survival method the final benzene solution contained 89% of the activity of the original ethyl acetate solution, demonstrating remarkably little loss in fractionation, and further that the greater part of the active material is characterized by ready solubility in both water and benzene. As Kuizenga (75) showed that desoxycorticosterone is highly active in his test and as Talbot's table (see Table II above) shows that desoxycorticosterone is even less soluble in water than is dehydrocorticosterone, the inferences are that the extract contained no appreciable amount of desoxycorticosterone, or that "carrier substances" had altered the usual solubility characteristics of free desoxycorticosterone.

CHEMICAL PROPERTIES OF ADRENAL STEROIDS.

In this paragraph two properties will be specially considered: (1) The sensitivity of the compounds to acids and alkalis, and (2) their reducing properties. 21-acetyl derivatives are relatively stable to alkali but it is stated that all free α-ketol groups are sensitive to alkali (20, p. 348 and 366). The normality, volumes, temperature and time of exposure are obviously important variables which determine the relative effect. Mason (56) on p. 465 states: "in the presence of 0.01 N alkali compounds of the C_{21} O_5 series undergo a rearrangement of the side chain with the quantitative formation of acid in the presence of air. Compounds of the C_{21} O_4 series are not affected by cold 0.1 N sodium hydroxide". Mason was dealing with the pure substances. Kuizenga (59) washed his ethyl acetate solutions of gland extractives with weak acids and weak alkali, and between the eventual benzene solution and benzene residues he accounted for approximately 94% of the original rat survival activity (mostly as C_{21} O_5 compounds), thus implying insignificant destruction. Talbot (6) purifies 100 cc. chloroform residues with three successive 10 cc. volumes of 0.1 N sodium hydroxide. He gives no data for pure steroids in water, but his Table III (p. 542) shows approximately quantitative recovery of two samples of 17-hydroxy corticosterone and of two samples of 17-hydroxy-11-dehydrocorticosterone when added to known urinary extracts and tested by a chemical method. This indicates no significant loss or destruction by alkali washings. (N.B. The poorer recoveries of corticosterone and 11-dehydrocorticosterone may be assumed to be due to incomplete partition into water from benzene rather than to loss in the alkali washings). Talbot (6) (on page 540) states that 0.1 N

NaOH washings and water washings combined result in less than 4% loss of added steroid. Heard and Sobel's (2) Table II, shows that free desoxycorticosterone (dissolved in 0.1 cc. acetic acid) was recoverable from water on extraction with ether-chloroform when 150 cc. solvent was washed four times with 10 cc. 0.1 n NaOH. Recoveries from urine were also good in this method, the steroid being assayed by a chemical procedure. The general conclusion may be reached that the important α-ketol side chain of all the known active corticosteroids is sensitive to moderately strong alkali, but that washings with small volumes of cold 0.1 N NaOH have no significant destructive effect.

Sensitivity to acids appears to be less clearly defined. Reichstein (20) on p. 348, states that all substances with a hydroxyl group at C_{11} or C_{17} are sensitive to acids, and on p. 366, that desoxycorticosterone is relatively stable to acids. Again the volume and normality of acids used and the eventual effective pH acting on the steroid are presumably the important considerations. Kuizenga (59), quoted above, obtained large recoveries of 17-hydroxy (i.e. C_{21} O_5) compounds after washing ethyl acetate extracts with weak acids. I have not found detailed evidence of sensitivity to acids. It might be assumed that exposure to HCl at pH 1.0 for 30 - 60 minutes in the cold would be sufficient to destroy the "sensitive" hydroxyl groups at C_{17} and C_{11}. Evidence regarding acid hydrolysis of urinary extracts will be discussed later. The impression that all the active corticosteroids withstand weak and even moderately strong acid exposure (only being destroyed by vigorous acid treatment) is advanced but does not appear to be founded on proven direct evidence.

Reducing power is common to many steroids isolated from the adrenal cortex. The principle reducing group is the α-ketol side chain, which resembles the active group of reducing sugars such as fructose. Thus alkaline silver solutions (Reichstein, 20), the cupric ion (Nelson 22, Talbot 6), and the molybdic reagent (Folin-Wu 21, Heard and Sobel 1, 34) are reduced. These reactions, however, are not specific and other groups in steroids isolated from the adrenal cortex or miscellaneous steroids also have reducing power. In addition, certain combinations of oxygen atoms at positions 17, 20, 21, allow oxidation with periodic acid with the release of one or two carbon radicles (Reichstein 20, Lowenstein et al 70). These various reactions are not completely correlated with each other or with biological activity. They will be discussed in detail with reference to the accompanying Table III. The Roman numerals in the table correspond to those of Reichstein (20), see page 352. The compounds at the foot of the table are taken from the table of Heard and Sobel (1), see page 690.

REACTION WITH PERIODIC ACID.

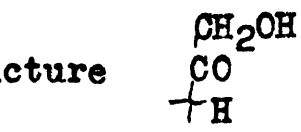
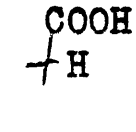
Data is based on that of Reichstein (20), see pages 375, 381, and 385. (N. B. misprint on page 385, in which the -OH groups at position 17 in compounds XVIII and XIX is incorrectly shown as -H). Substances with the glycerol structure $\begin{matrix}CH_2OH\\CHOH\\OH\end{matrix}$ are oxidized by excess periodic acid to =O containing two atoms less of carbon. Ketones are similarly formed from the group $\begin{matrix}CH_3\\CHOH\\OH\end{matrix}$ (p. 385). Substances with the α-ketol side chain give quite a different reaction. Those of the structure $\begin{matrix}CH_2OH\\CO\\OH\end{matrix}$ yield acids $\begin{matrix}COOH\\OH\end{matrix}$ containing one atom less of carbon (p. 375), and those of the

TABLE III

CORTICOSTEROIDS AND RELATED COMPOUNDS - REACTIONS WITH PERIODIC ACID AND REDUCING REAGENTS

Name of Steroid	Periodic Acid Action	Reducing Reagents			Biological	
		Alk.Silver	Cupric	Molybdic	Survival	Glycogenic
A. BIOLOGICALLY ACTIVE CORTICOSTEROIDS						
VII. 17-hydroxycorticosterone	acid + one C	+	+	(+)	+	+++
VIII. 17-hydroxy, 11-dehydrocorticosterone	acid + one C	+	+	+	+	+++
XV. corticosterone	acid + one C	+	+	(+)	++	++
XVI. 17-dehydrocorticosterone	acid + one C	+	+	+	++	++
XI. 17-hydroxy, 11-desoxycorticosterone	acid + one C	+	(+)	(+)	+++	No
XXII. 11-desoxycorticosterone	(acid + one C)	+	+	+	+++	No
B. BIOLOGICALLY INACTIVE COMPOUNDS ISOLATED FROM ADRENAL CORTEX						
I. allopregnane 3-11-17-20-21-pentol	keto + 2 C	No	(No)	(No)	No	
II:III allopregnane 3-11-17-21-tetrol, 20-one	acid + 1 C	+	(+)	(+)	No (III doubtful (75)	
IV. allopregnane 3-17-21-triol, 11-20-dione	acid + 1 C	+	(+)	(+)	(No)	
IX. allopregnane 3-17-20-21-tetrol	keto + 2 C	No	(No)	(No)	No	
X. allopregnane 3-17-21-triol, 20-one	acid + 1 C	+	(+)	+	(No)	
XII. allopregnane 3-11-21-triol, 20-one	(acid + 1 C)	+	(+)	(+)	(No)	
XIII. allopregnane 3-21-diol, 11-20-dione	acid + 1 C	+	(+)	(+)	(No)	
XIX:XVIII. allopregnane 3-17-20-triol	keto + 2 C	(No)	(No)	(No)	(No)	
XX. allopregnane 3-17-diol, 20-one	?	(No)	(No)	(No)	(No)	
XXIII. allopregnane 3-01, 20-one	No reaction	(No)	(No)	(No)	No	
V. Δ4-pregnene 11-17-20-21-tetrol, 3-one	keto + 2 C	No	(No)	(+)	No	
VI. Δ4-pregnene 17-20-21-triol, 3-11-dione	keto + 2 C	No	(No)	(+)	(No)	
XIV. Δ4-pregnene 20-21-diol, 3-11-dione	?	(No)	(No)	(+)	(No)	
XXI. Δ4-pregnene 17-01, 3-20-dione	?	(No)	(No)	(+)	(No)	
XXIV. Progesterone	(No reaction)	No	No	+	+ slightly	
C. MISCELLANEOUS STEROIDS						
H.6 Δ5-pregnene 3-21-diol, 20 one	(acid + 1 C)	(+)	+	+	+(Selye, 61)	
H.19 Δ4-Androstene, 3-17-dione	(No reaction)	(No)	(No)	+	(No)	
H.13 Cholestane, 2-01, 3-one, acetate	(No reaction)	?	+	+	(No)	
H.22 Testosterone	(No reaction)	(No)	(No)	+	(No)	
H.25 Δ5-pregnene 3-01, 20-one	(No reaction)	(No)	(No)	No	+ (Selye, 61)	
H.29 Estrone	(No reaction)	(No).	(No)	No	(No)	

Data obtained from Reichstein (20), Heard and Sobel (1,34) and Talbot (6). Data entered in brackets is for compounds not specifically tested by the sources quoted, but is inferred from the general rules governing

structure  yield acids  (p. 381).

In these last examples the one atom of carbon removed is in the form of formaldehyde and is detectable by the reagent of Lowenstein et al (70). The accompanying table shows that all the known biologically active natural cortico-steroids give the typical reaction, but that an identical reaction is given by several inactive compounds isolated from the adrenal cortex, and also by such miscellaneous steroids as 21-hydroxy-pregnenolone (Δ 5 - pregnene - 3 - 21- diol, 20-one). A number of other inactive compounds from the adrenal cortex give the keto compounds plus a two-carbon compound. The behaviour of these with Lowenstein's reagent would presumably be anomalous.

REACTION WITH ALKALINE SILVER SOLUTION'S AT 20° C.

Data is based on that of Reichstein (20), see p. 375, supported in most instances by direct reference under the individual compounds in his paper. The reaction is apparently quite specific for the α-ketol group. It is therefore given by all the active corticosteroids but also by a number of inactive corticosteroids and would also be given by such miscellaneous steroids as Δ5-pregnene, 3-21-diol, 20-one. It is not relevant to discuss this reducing reagent in any further detail.

REACTION WITH CUPRIC ION ON HEATING.

Talbot (5, 6) examined certain biologically active corticosteroids according to the procedure of Nelson (22). This reagent was designed for the determination of glucose. Talbot found reduction with the biological active 11-oxycorticosteroids. The reagent was further examined (with a slight modification) by Heard and Sobel (1, see page 696). 11-desoxycorticosterone was also found to react.

By inference several of the biologically inactive corticosteroids listed in the accompanying table would react. Heard and Sobel also found reduction with the α-ketol group of Δ 5-pregnene, 3-21-diol, 20-one, and with the cyclic secondary α-ketol of cholestane, 2-ol, 3-one acetate. It should be noted that the conjugated 3-keto group of progesterone failed to react with this reagent. The conclusion may be drawn that Nelson's reagent is not specific, and that among steroid compounds it cannot be correlated solely with biologically active corticosteroids, nor even with compounds at present known to be exclusively derived from adrenal cortical compounds.

REACTION WITH PHOSPHOMOLYBDIC REAGENT ON HEATING.

The data in the accompanying tables (Tables III, IV, V.) are derived from Heard and Sobel (1, 3), and from the thesis of Sobel (34). The procedure for reducing sugars of Folin and Wu (21) was modified by using a reagent consisting of equal parts of the phosphomolybdic solution (Folin-Wu reagent A) with glacial acetic acid. The resulting colour was determined by a photoelectric colorimeter after heating steroids with the reagent for varying times from 15 minutes to 3 hours, cooling, and diluting to constant volume with the reagent. Steroids gave variable curves of intensity increasing with the duration of heating. The colour intensity of different steroids was not found to be consistent for equimolar quantities. On analysis, reducing power was found to be conferred by the primary α-ketol group, but also by the $\alpha\beta$-unsaturated 3-keto group. Compounds having both these groups (as do all the known biologically active corticosteroids) showed reducing power of a high degree of suggesting a summation effect. The presence of either a hydroxyl group at position 17 or an oxygen atom at position 11 apparently decreased the reducing power.

In addition, steroid compounds with cyclic secondary α-ketols in ring A or in ring C showed reducing power. Certain compounds, such as methyl testosterone, behaved anomalously, showing an unexpectedly high degree of reducing power, which was greater than that given by other compounds containing unsaturated 3-keto group without an α-ketol group. The conclusion may be reached that reducing power with this reagent is characteristic of biologically active corticosteroids, but is also inherent in rather a wide range of other steroids, which possess primary or secondary α-ketol groups or $\alpha\beta$-unsaturated 3-keto groups.

A digression into the use of thephosphomolybdic reagent is relevant to the present thesis. Sobel (34) noted some difficulty in preparing identical successive batches of the reagent, although made up from the same chemicals. Thus two out of ten batches of reagents had to be rejected, because standard curves with the same solution of desoxycorticosterone gave optical densities greater than 3% from the usual standard. The cause of this variability was not known, but may be due to incomplete removal of ammonia from the molybdate salt during boiling when preparing the reagent. This difficulty can be overcome by the expedient of performing a new standard curve with each fresh batch of reducing reagent.

The gradual and prolonged increase in colour density with the steroids tested may be related to the manner of developing the colour. Heard and Sobel (1, 34) place an aliquot of steroid dissolved in 0.1 ml. of glacial acid into a glass test-tube (75 mm. by 8 mm.). After adding 2.0 ml. of the phosphomolybdic glacial acetic acid reagent and mixing, the tube is suspended in a steam-heated water bath for the desired length of time. On removal it is cooled and transferred with 8.0 ml. of the reagent to a standard "macro" Evelyn colorimeter tube and read at 650-660 mu. The use of the reagent mixture as the diluent was found to give greater and more

consistent intensity than diluents consisting of water, acetic acid or of the phosphomolybdic solution without acetic acid. Possibly 2.1 ml. of solution in a 3.0 ml. micro test-tube is not very rapidly heated to the temperature necessary for complete reaction with the reagent used. However, it is noted that the author's achieved consistent results at standard times in numerous control estimations.

Various steroids give different shaped curves for the completeness of reduction in relation to time of heating. Tables 4 and 5 are constructed from data given in Sobel's Table V and the text of Sobel's Thesis (34). For each steroid the figures are calculated on the basis of 0.303 millimols (equivalent of 0.1 mg. of desoxycorticosterone).

TABLE IV.

OPTICAL DENSITY OF MOLYBENUM BLUE BY CERTAIN STEROIDS AFTER VARIABLE PERIODS OF HEATING.

AME	A 30 min.	B 60 min.	C 180 min.	Ratio $\frac{A}{C} \times 100$	Ratio $\frac{B}{C} \times 100$
A) α -ketol & unsat. 3 ketone					
l-desoxycorticosterone	.56	.69	.90	62	77
l-desoxycorticosterone acetate	.24	.45	.88	27	51
l-dehydrocorticosterone	.47	.60	.78	60	77
7-hydroxy, 11-dehydro-orticosterone	.40	.51	.65	62	79
B) α-Ketol only					
5-Pregnene, 3-21-diol, 20-one	.48	.57	.68	71	84
itto - 21-acetate	.15	.33	.69	22	48
C) $\alpha\beta$-unsat. 3-ketone only					
ethyl testosterone	.17	.30	.60	28	50
rogesterone	.11	.21	.38	28	55
estosterone	.10	.16	.26	38	62
D) Others					
strone, cholesterol, etc.		.00	$<$.04		

N.B. The quantities of steroids shown were equimolar with 100 gamma of 11-desoxycorticosterone.

TABLE V.

OPTICAL DENSITIES FROM TABLE IV, COMPUTED AS PERCENTILES OF THOSE OF CERTAIN STEROIDS.

	Compared to Desoxycorticosterone 60 min.	180 min.	Compared to Dehydrocorticosterone 60 min.
11-Desoxycorticosterone	100%	100%	115%
ditto 21-acetate	65	98	75
11-Dehydrocorticosterone	87	87	100
17-Hydroxy, 11-dehydrocorticosterone	74	72	85
Δ5-Pregnene, 3-21 diol, 20-one	83	76	95
ditto, 21-acetate	48	77	55
Methyl Testosterone	44	66	50
Progesterone	30	42	35
Testosterone	23	29	27
Estrone, cholesterol, etc.	0	0	0

The percentiles A/C and B/C at the right of Table IV, show that the rates of development of colour density are similar for desoxy-corticosterone, dehydrocorticosterone and for 17-hydroxy, 11-dehydrocorticosteron However, the rate of colour development of the pregnendiolone is slightly more rapid, while the rates for the two acetate compounds listed and for methyl testosterone, progesterone and testosterone are distinctly slower.

The percentiles of Table V show that equimolar amounts of dehydrocorticosterone give 87% of the colour intensity developed by desoxycorticosterone, and that the other compounds give varying but lesser degrees of colour intensity. The slower colour development of the acetates at 60 minutes is again apparent. In the right hand column, where dehydrocorticosterone at 60 minutes heating is taken as 100%, it is seen that the other α-ketols are all more than 75% as intensely reducing, and that the unsaturated 3 - ketones are about 50% or less as reducing.

In comparing unidentified urinary steroids with the pure steroids Heard and Sobel (2) chose the 60 minute heating period arbitrarily. If the unknowns consisted of mixtures of salts such as acetates the colours developed might not be comparably fully developed, and different extracts of unknowns might contain different proportions of such interfering acetates. Secondly, the colour developed by desoxycorticosterone was chosen as a basis of comparison becuase it gave the most intense reaction and is readily available. Yet extracts obtained from urines excreted under different conditions from individuals with various hormonal imbalances might contain varied and unpredictable proportions of different compounds, some of low and others of higher reducing power.

This last consideration might have limited applicability if the method of extraction is such as to finally include only steroids of structure and reducing power closely similar to the corticosteroids. This has not yet been proven by direct isolation. No data is available in the papers of Heard (2) or Sobel (34) to show that the rates of development of colour intensity by urinary extracts correspond to that of desoxycorticosterone. Similar comments may be made on the applicability of Talbot's (6) data to the unknown steroids of urinary extracts, but the data available suggests that his reagent gives maximal colour intensity at 20 minutes. Also, the extraction procedure includes further fractionation and the use of Girard's reagent, thus presumably decreasing the likelihood of interference in the colour by other reducing steroids, and furthermore his reducing reagent has been shown to be more specific in that it does not react with unsaturated 3-keto compounds such as progesterone (1).

SPECIFICITY OF CHEMICAL METHODS FOR ASSAYING CORTICOSTEROIDS - COMPARABILITY.

It is apparent that neither the reducing power nor the periodic acid oxidation are specific for biologically active corticosteroids or even for substances indubitably formed in the adrenal cortex. △ 5-pregnene 3-21-diol, 20-one, for example, reacts with all those listed on ~~page 3~~ Table III. Furthermore, the range of compounds reacting with the cupric and the molybdic reducing reagents are different, so that completely comparable results on mixtures of unknown steroids are not to be expected. Nor can it be assumed that methods based on the periodic acid reaction would give results comparable with any of those using reducing reagents.

Because of the lack of specificity of such chemical reactions methods for assaying unknown mixtures of steroids must depend on the completeness of extraction procedures and on the purification steps such as washings, differential solubility, etc. If such procedures satisfactorily remove the non-specific reducing reactors from unknown mixtures, specifity might be attained despite the non-specifity of the reagent.

In conclusion, chemical methods for assaying corticosteroids from unknown mixtures of steroids and other non-specific reactors must depend on the accuracy of the extraction-purification procedures because the reagents available for quantitative estimation are not themselves specific. The comparability of different methods might well depend more on the relative purification attained by the extraction procedures than on the relative specificity of the final reagents employed.

CLINICAL CONDITIONS. RELATIONSHIP TO BIOLOGICAL PROPERTIES OF CORTICOSTEROIDS.

It is the intention of the writer to illustrate only briefly the close similarity between certain signs and symptoms in human clinical states and the proven physiological observations on the adrenal cortex and corticosteroids in experimental animals. This similarity has stimulated considerable research into methods to estimate the relative lack or excess of corticosteroids in human tissue fluids. The search has naturally been particularly directed to those conditions with clinical evidence of abnormal adrenal function, whether of chronic or acute and transient nature.

Addison's disease (adrenal insufficiency) and panhypopituitarism have both been shown at post-mortem examination to be associated with diseased or aplastic adrenal cortical glands. The clinical signs of asthenia, weight loss, excessive sodium excretion, and low blood sugar with tendency to acute hypoglycaemia closely resemble those of adrenalectomized animals and likewise respond to adequate treatment with active cortical extracts. Cushing's Syndrome has been shown to be associated with tumors or hyperplasias of the adrenal cortex. Here, obesity, some tendency to sodium retention, elevated blood sugar values and evidence of catabolism of protein tissues (thin skin, weak muscles, osteoporosis, etc.) are the reverse of the signs of insufficiency and indeed resemble the effects of excessive adrenal cortical therapy in experimental animals. Albright (19) has brought together considerable evidence on Cushing's Syndrome.

The work of Selye and coworkers (77, 78) has drawn attention to the role of the adrenal cortex in adaptation to non-specific damage, such as trauma, poisons, drugs, infection, fatigue and changes in environmental temperature. This naturally has stimulated search for evidence of alterations of cortical function in similar clinical conditions, e. g. wounds, burns, operative conditions. Finally hypertension is a feature of Cushing's syndrome and elevated blood pressure has followed excessive therapy with synthetic desoxycorticosterone acetate, so attention has been directed to the possible role of the adrenal cortex in clinical hypertension of unknown etiology (i.e. essential hypertension).

ESTIMATIONS OF CORTICOSTEROIDS IN BLOOD.

Of the tissue fluids available for examination in the living human the circulating blood might be expected to yield the most relevant information as to the secretory function of the adrenal cortex. Very little work appears to have been done on the hormone content of the blood of humans, or of experimental animals. This is probably largely due to the evidence that large volumes of blood are required for positive results in the biological assays, and that withdrawl of such large volumes would be hazardous in humans and exsanguinating in the usual laboratory animals.

Anderson et al (10, 23), assayed a number of samples of human blood by the effect on survival of adrenalectomized rats. One hundred cc. of blood was required, and this was extracted by benzene according to the method of Grollman and Firor (46). An equivalent of 1 cc. of blood of the extract in oil was injected subcutaneously once daily from the fifth post-operative day until death to each of a group of adrenalectomized rats. The mean survival period of control untreated rats was 8.1 days; treatment with the blood of a normal adult caused an insignificant prolongation to 9.6 days; while the use of blood from three patients with Cushing's Syndrome was associated with definitely prolonged survival periods of 14.5, 16.0 and 14.2 days respectively. Treatment with Upjohn's adrenal cortical extract resulted in a survival period of 11.8 days when 0.1 cc (equivalent to 4 grams fresh gland) was used daily, and there was apparently indefinite survival when 0.2 cc. cortical extract was used. Therefore, the authors concluded that the Cushing's Syndrome patients had in 1 cc. of circulating blood more adrenal cortical hormone than that extractable from 4 grams fresh gland, and that the

circulating blood of normal humans contained too little for detection by the assay method used. From their results, on the basis of a circulating blood volume of 5000 cc., each patient's circulating cortical hormone was greater than the extractable content of 20 kilograms of beef adrenal (i. e. 500 cc. Upjohns cortical extract). This huge equivalent may be due to relatively greater losses in the commercial preparation of adrenal hormone than in the extraction of the blood. Otherwise it is suggested that the adrenal cortex resembles the ovary, in which various investigators have found extremely minute quantities of oestrogens in comparison with those present in the urine of pregnant women and animals.

The last conclusion is supported by the evidence of Vogt (49). She used the survival to cold exposure method of Selye and Schenker (32) in assaying blood obtained from various large experimental animals such as the dog and the cat. Thirty cc. blood samples of arterial blood failed to show positive responses for cortical hormone by her method. However, after traumatizing-eviscerating operations under anaesthesia, blood obtained from the suprarenal veins showed definite evidence of cortical activity. Comparing this to the activity of Allen and Hanbury's commercial cortical extract, she calculated that one adrenal gland secreted per minute per kilogram body weight, the equivalent of between 0.26 and 2.0 grams of fresh packing-house adrenal gland, or, in other terms, between 0.0034 ml. and 0.0266 ml. of cortical extract. On the basis of her figures the total daily hormone excretion of a 70 kg. human under traumatic conditions, would be the equivalent of over 120 kilograms of adrenal gland or over 1600 ml. of commercial cortical extract. It is,

of course, very doubtful if such a high rate of secretion would be maintained throughout a 24 - hour period, and methods are not available for draining the suprarenal veins without considerable trauma and without sacrificing the animal after one drainage. It should be noted that Vogt found no significant hormone activity in heart blood in cats even if the kidneys were out of the circulation or if the liver was similarly shortcircuited. She concluded that cortical hormone was rapidly inactivated by the blood stream and general body tissues, and that neither renal excretion nor specific mechanisms in the liver (conjugation, metabolism, etc.), played major roles in this inactivation. If Vogt's last conclusions are true, the actual hormone secretion may indeed be very large. Thus Anderson's findings on the circulating blood of Cushing's Syndrome patients would imply an exceedingly high actual hormonal secretion. Another corollary from Vogt's belief in the minor role of renal excretion in the disposal of secreted hormone would be that quantitative estimations of the hormone in urine would give only a very incomplete picture of the actual daily cortical secretion. It seems wise to await confirmation and further extension of Vogt's work under other conditions before placing too much weight on her conclusions and their implications.

In summary of this section of the thesis one may state (1) that satisfactory methods for the estimation of cortical hormone in the circulating blood have not been developed though large amounts of hormone may enter the circulation under certain circumstances, and (2) from the work of Vogt, there would be some doubt as to significance of the urinary content of cortical hormone as a reflection of its secretion.

URINE STUDIES. ISOLATION OF CORTICOSTEROIDS.

If renal excretion is the ultimate channel of corticosteroids and if the steroid structure remains intact during excretion, then the chemical isolation of steroids in urine might prove of great importance in understanding the metabolism and even the rate of secretion of cortical hormones. Unfortunately no steroids with the characteristic α-ketol side chain have been identified in urine (Dobriner, 1945 (26)). Neither have any steroids with oxygen at position 11 been recognized. The compound so far identified which most clearly resembles corticosteroids, has a side chain of the $\begin{matrix} CH_3 \\ CO \\ \vdash OH \end{matrix}$ type, pregnandiol-3 (α), 17-one-20. This was found by Lieberman and Dobriner (57) in the urine of a woman with adrenal hyperplasia, and of another with an adrenal tumor, but not in urine of normal people or pregnant women. Some doubt is thrown on their contention that this compound is solely of adrenal origin because they found it also in urine from a eunuchoid male given testosterone injections, and in urine from a cryptorchid male (in whom islet cell hyperplasia may be suggested as a contributing source). Other compounds of the pregnane series which have been found in the urine of patients with adrenal hyperplasia or carcinoma, are pregnane - 3 (α), 17, 20 triol by Butler and Marrian (79) and pregnane - 3 (α), 20 (α) diol by Venning et al (80).

After the administration of desoxycorticosterone acetate to a male, Cuyler et al (81) isolated large amounts of pregnane - 3 (α), 20 (α) diol and this has been confirmed in the rabbit by Hoffman et al (33) and both in the ovariectomized chimpanzee and in humans with Addison's disease by Horwitt et al (37). However, as this compound only accounted for a maximum of 3% of administered material and also as the same pregnanediol

is a known normal metabolite of progesterone in humans, this finding appears of little practical value in quantitatively determining variations in cortical function.

It would be of considerable interest if one knew the exact chemical structure of the crystalline substance (which reduces alkaline silver at room temperature, melts at 235°C. and is active in the cold exposure biological assay method), which Venning et al (9) found in the acetylated ketonic residue of an extract of pooled post-operative urine. A recent personal communication has given further details on this crystalline substance. Hoffman (71), states that the ultra-violet absorption spectra shows that the λ maximum 238 millimicrons extinction coefficient (assuming a mol. wt. of 404) is 13,800). These are the characteristics of an α-β- unsaturated ketone. By preliminary biological assay in the method of Venning (7) it is approximately one eighth as active as free 17-hydroxy, 11-dehydrocorticosterone (Cpd. E). The acetate of Compound E melts at 239 - 241°C and the acetate of no other known biologically active compound melts at this range. Thus, there is very suggestive evidence that Compound E is excreted in urine and may account for a major portion of its biological activity.

It may be stated that excessive or diminished quantities of 17-ketosteroids of the androstane series (especially the 3 (β) hydroxy group) have been associated with adrenal disorders in humans. In some cases excessive amounts have been chemically isolated, and frequently they have been estimated in small aliquots of urine by satisfactory colorimetric procedures. Such compounds, however, apparently only reflect the androgenic function of the adrenal cortex, and have not been

shown to be an index of the typical "corticosteroid" secretion. Therefore, it is not within the scope of the present thesis to review this subject further.

We have very little information of the metabolism of adrenal corticosteroids or of the chemical structure of the biologically active or inactive excretion substances. Therefore, the chemical isolation of steroids from urine has proven of academic interest to date, and no such method has been considered for the comparative study of numerous clinical cases of possible adrenal dysfunction.

DETECTION OF URINARY CORTICOIDS BY BIOLOGICAL ASSAY METHODS.

From 1931 to the present date numerous papers have appeared on the detection of cortin-like activity in urine extracts. Extraction procedures have become generally more refined throughout the years, rendering comparison of the methods somewhat difficult. Recent attention has been directed to the more specific function of glycogen deposition in the liver. However, it must be remembered that material of cortical origin without gluconeogenic activity but with life- maintaining properties may be present in urine. The previous section of the thesis demonstrated that compounds closely related to the pure active corticosteroids have not been completely characterized chemically, although Hoffman (9, 71) has suggestive evidence that 17-hydroxy, 11-dehydrocorticosterone acetate is present. The various bio-assay methods will be considered, methods depending on the deposition of glycogen in liver being grouped at the end.

(a) Older Methods of Bio-Assay:

In 1931 Perla and Marmorston-Gottesman (44, 45), first demonstrated cortin-like activity in human urine by a bio-assay depending on an increased resistance to histamine injections in adrenalectomized rats (43). They estimated that one litre of urine from normal young adults contained the equivalent in protective substances of one half-pound (225 grams) of beef adrenal gland excreted according to the method of Hartman (83). In 1933 Grollman and Firor (46) using life maintenance and growth of adrenalectomized rats found that one litre of normal urine contained only the equivalent of 0.5 grams adrenal gland extract.

Although both the above workers used benzene extracts of unhydrolysed fresh urine it must be noted that Perla (45) did not wash the benzene solution with water, whereas Grollman (46) washed with water and discarded the aqueous phase. Talbot (6) has shown that the aqueous phase contains all the 17-hydroxy, 11-oxysteroids and much of the other 11-oxysteroids under his own conditions; and Venning et al (9) showed by their fractionation procedure that biological activity by cold resistance and by glycogen deposition is confined to the aqueous phase after benzene partition. Therefore, Grollman may have washed out of the benzene extract with water much active material as well as impurities. Grollman's method of extraction was used by Anderson et al (10, 23) in testing various urine samples by survival of adrenalectomized rats (i.e. somewhat similar to Grollman's assay method). However, Anderson et al only injected the equivalent of 1% of a 24 hour urine collection into each rat daily while Grollman et al injected the extract of one litre of urine twice daily into each rat. This difference in dosage employed probably accounts for the fact that Anderson et al found no increased survival in rats treated with extracts of urine from a normal adult or from a patient with an adrenogenital syndrome. However, these workers succeeded in demonstrating in an extract from a 24-hour urine collection obtained from a case of Cushing's syndrome survival activity exceeding that of cortical extract equivalent to 400 grams of fresh gland.

The work of Venning et al (9) and of Talbot (6), discussed above, would suggest that benzene is not ideal for extracting all the cortin-like substances from an aqueous solution such as urine. On the other hand, the flow sheet on page 55 of Venning's (9) paper demonstrates that chloroform extracts all the activity from an aqueous mixture, and gives

strong evidence that ethylene dichloride is a satisfactory solvent for the extraction of urine. As early as 1937 Harrop and Thorn (8) made ethylene dichloride extracts of urine and assayed a saline suspension of the residue by the effect on sodium excretion in normal dogs. This method of assay gave activity when cortical extract was added to urine before extraction or when urine was collected from animals which had received massive doses of adrenal cortical extract intravenously. However, urine collected from normal men, normal or pregnant women, or pregnant animals failed to show activity by their method. In 1939 and 1940 Weil and Browne (12, 11, 13) showed that ethylene dichloride extracts of 24- hour urine collections of various patients contained activity when a watery suspension of the residue was given by gavage to adrenalectomized rats exposed to cold according to the procedure of Selye and Schenker (32). Weil and Browne (13) and Weil (55) found no detectable activity (i. e. less than 10 cold units per day) in the urine of normal people, but demonstrated activity in the urines of a case of Cushing's syndrome, several cases of hirsutism, four out of six cases of hypertension, and in the majority of cases of acute or chronic infections and of patients recently burned or subjected to surgical operations. The important implication of increased cortical activity following infection or trauma will not be discussed here. Although Weil and Browne were unable to detect activity in some urines from infected or traumatized individuals they believed this failure was due to interference by some toxic substance such as sulfonamide. It should be mentioned that the Selye-Schenker cold survival test is more sensitive than the ordinary rat survival tests and particularly so for the 11-oxy-corticosteroids. Thus Kendall (76) and Venning et al (9) found that only 10 gamma of corticosterone is equivalent to 1 cold unit but that 1.5 to 2.0 mg. of desoxycorticosterone

acetate is equivalent to 1 cold unit. On the other hand, Kuizenga et al (75) showed that desoxycorticosterone and its acetate are active on survival in rats in smaller doses than the various 11-oxycorticosteroids.

Between 1942 and 1944 Dorfman and various coworkers extended the work of Weil and Browne. Their earlier work (14, 16, 18, 38) used an ethylene dichloride extraction procedure similar to that of Weil and Browne and a slightly modified cold survival assay method. However, the chief modification was that larger urine volumes were extracted so that each rat might receive the equivalent of at least 5 or 6 hours of urine instead of the 2.4 hours urine equivalent used by Weil and Browne. In this way Dorfman et al showed that the urine of normal men and women contained daily, on the average, the cold survival activity equivalent to approximately 0.15 cc. Wilson's cortical extract (i. e. 11 grams fresh adrenal gland) or, in the terms of Venning et al (9), about 3 cold units per day. Dorfman et al (18), testing 15 extracts of urine from 7 patients with Addison's disease maintained on pellets of desoxycorticosterone acetate, found no detectable cold survival activity (with the exception of a single positive test). Extracts of urine of normal monkeys contained cold survival activity (38), whereas negative results were obtained from adrenalectomized monkeys. The same workers (18) also showed that a similar urinary extract of normal men contained substances active in the muscle-work test (65).

The later work of Dorfman et al made use of modifications in the original ethylene dichloride extraction similar to those of Venning et al (17, 9). The latter workers showed that washing with 0.1 N. sodium hydroxide resulted in no loss of activity but rather smaller residues of greater activity per mg. residue. They also showed that the most highly concentrated active residues occurred in the ketonic material using Girard's reagent T after separation into water from benzene. Using 7-day

pooled post-operative urines from a number of patients, Venning et al (9) demonstrated cold survival activity in the range of 10 - 30 C.U. per litre compared to the range of 1 - 3 C.U. per liter for normal men.

Dorfman and Horwitt (48) showed that the alkali-insoluble fraction of the ethylene dichloride extract of normal men's urine was active in a life maintenance test in rats. These workers (36) also showed that water retention and death after excessive hydration could be prevented by the alkali-insoluble fraction and still more completely controlled by the alkali-insoluble ketonic fraction. In 1945 Feil and Dorfman (50) showed that both the alkali-insoluble and alkali-insoluble ketonic fractions of extracts from normal men resembled adrenal cortical extract in their influence on potassium metabolism as shown by a potassium intoxication test in adrenalectomized rats. They also demonstrated less convincingly comparable actions in increasing sodium excretion in normal rats.

Venning et al (17, 9) also demonstrated that post-operative urine extracts exhibited life-maintaining properties in the adrenalectomized rat. It is of interest that their urinary extracts, when assayed by cold units and given in comparative doses of cold units as cortical extracts, contained less than half as much life maintaining activity as the cortical extract. The 11-oxy compounds are much more active than 11-desoxy compounds in the cold test (9) while the reverse is true for life maintenance (75). If one accepts Reichstein's isolation of 11-desoxycorticosterone from cortical extracts, one may speculate that a relative preponderance of 11-oxy compounds is excreted in urine. However the purification procedures (including benzene water separation) might remove whatever 11-desoxy compounds are excreted.

The recovery of cortical extract added to urine before the above mentioned methods of extraction has been seldom reported. Pfiffner et al (54) in 1934 state that added cortical extract is recoverable quantitatively according to a dog maintenance assay but the authors give no details as to the urinary extraction procedure. Harrop et al (8) by their ethylene dichloride extraction on unhydrolysed urine obtained a recovery of only 40% of added cortical extract when assayed by salt retention in the dog. It would be of considerable interest if data were available by the fractionation procedure of Venning et al (9) showing the comparative recovery of added cortical extract, when assayed by such diverse test methods as life maintenance, cold survival and glycogen deposition. The recovery of injected cortical extract will be discussed following a review of glycogen deposition assay methods, as will the effect of acid hydrolysis of the urine. All the above reported methods make use of unhydrolysed urine collections.

(b) Newer bioassay methods depending on glycogen deposition in the liver.

In 1942 Reinecke and Kendall (25) published a method for the bioassay of cortical extracts by the effect on glycogen deposition in the liver in the fasting adrenalectomized rat. Shortly thereafter Venning et al (17, 9) demonstrated that extracts of post-operative urine had a glycogen-deposition effect similar to cortical extracts. This was also demonstrated by Horwitt and Dorfman (15) in extracts of normal male urine. Work to develop assay methods using this activity has proceeded in four centres simultaneously. Much of this work was originaly published in the proceedings of the "Conference on Metabolic Aspects of Convalescence, Including Bone and Wound Healing" under the auspices of the Josiah Macy Jr. Foundation.

References to the subject occur as early as the third meeting in February 1943, and are well summarized in a symposium under the chairmanship of Dr. J. S. L. Browne in the tenth meeting in June 1945 (27). This symposium covers the work of (a) Venning and Kazmin, later published elsewhere (7, 35, 53, 82); (b) Dobriner, Lieberman and Eggleston of New York, also published elsewhere (31, 85) (c) Griswold and Forbes of Boston (40); and (d) Dorfman and coworkers of Cleveland recently published in detail (62, 64, 84). The methods of the four groups are essentially similar, and only a few of the major differences from that of Venning et al will be mentioned. All workers use white mice, approximately 20 grams in weight, because of their greater sensitivity than rats. With the exception of Dorfman et al (62, 64), who extract unhydrolysed urine, the urine is extracted after hydrolysis at room temperature with acid at pH 1.0. Venning et al (7) find that such acidification approximately doubles the eventual yield, which suggests the release of active steroids from conjugates. Venning et al (7) and the Dorfman group (62 64,) extract with ethylene dichloride or chloroform. The choice of ethyl ether as a solvent by the Dobriner group (31) is criticized by Talbot (6) on evidence of the extractibility of pure-17-hydroxycorticosteroids from water by the various solvents. All groups wash the extracts with weak alkali solutions, but none routinely carry the purification through to the preparation of pure Ketonic fractions. All use adrenalectomized mice as test animals. During the four to five days from operation to the commencement of the assay. The animals receive standard diets with the exception of Venning et al (17).

This group starves the animals for the final 16-17 hours, and a standard amount of glucose is injected along with each test extract in order to increase the sensitivity of the preparation to a controlled constant degree. Venning et al (7) inject a total of 6-8 hours urine equivalent per animal, whereas the other groups often inject less. The three other groups use oil as a vehicle for the injections of the urinary extracts in divided doses, whereas Venning et al use aqueous alcohol as a solvent, but this technical difference does not appear to be of much importance. There are no essential differences between the groups in the technique of removing the liver or determining the liver glycogen, which is then expressed in milligrams per 100 grams (or 10 grams) body weight of animal. The correlation of liver glycogen found and the equivalent in terms of known steroids is handled differently by the various groups. Dorfman (27, 64) and Dobriner (27, 85) use rather complicated statistical procedures, whereas Venning et al (27, 7), use a dosage-response curve.

The assay procedures of the Venning, the Dobriner and the Dorfman groups (27) give closely similar results for standard amounts of Kendall's compound E (17-hydroxy, 11-dehydrocorticosterone), averaging 10.2 mg. liver glycogen per 10 gram mouse for 50 micrograms of Compound E. Dobriner et al subsequently (85) have increased the sensitivity of their method, and have also outlined a modification involving the total fermentable sugar instead of glycogen in liver. The final results are expressed differently. The Venning group (27, 7) use glycogenic units in which one unit is equivalent to the activity of one microgram of 17-hydroxy, 11-dehydrocorticosterone. Dobriner et al (31) use rat units of Reinecke-Kendall, and they state on page 159 of the symposium (27) that 1 rat unit is approximately equivalent

to 40 micrograms of 17-hydroxy, 11-dehydrocorticosterone. Dobriner (85) later uses mouse glycogen units similar to the units of Venning (7). Finally the Dorfman group (62, 64) express their results in terms of the equivalent weight of 11-dehydrocorticosterone (Compound A) excreted. According to their data oh page 171, of the symposium (27) 17-hydroxy, 11-dehydrocorticosterone is approximately four times as active as Compound A on liver glycogen deposition, and Venning et al (7) show that it is at least 3 times as active. Therefore, the results of Dorfman et al (62, 64), can be approximately converted into the glycogenic units of Venning et al by multiplying by the factor $\frac{1000}{4}$. The methods of all the groups are thus essentially similar in principle. The published data of Venning et al (7, 35, 53, 82) covers a greater number of determinations over a wider range of clinical conditions, and therefore this work only will be reviewed in detail in this thesis.

Fourteen normal adult men showed excretions ranging from 40 to 85 glycogen units per 24 hours with an average value of 60 units. Fourteen adult women showed very slightly lower excretion, ranging from 25 to 65 glycogen units with an average of 41 units per 24 hours. Four adults over 70 years of age showed a slightly lowered excretion. No or only slight activity was detected in the equivalent of 12 hour urine excretion from new born children. Between the ages of $2\frac{1}{2}$ years and 16 years boys showed activity gradually approaching the adult level. It is of particular interest that the glycogenic steroids approach the adult range at a much earlier period than do the 17-ketosteroids, although the latter are apparently excreted at all ages

in a quantitatively greater amount than the glycogenic steroids, whether the latter be considered in terms of Compound E or A of Kendall. No changes were observed in the excretions of glycogenic steroids in relation to the menstrual cycle (2 cycles). It is of considerable importance to note that exercise in the form of a 4 mile route march by 14 healthy soldiers caused a three fold increase in the excretion per litre of pooled urine. This gives further support to the evidence of Venning (35), that bed patients tend to have a more even and lower output than ambulatory patients, which is further supported by patient C.D. in Table 3 or Shipley, Dorfman et al (62). Pregnancy in six normal women was shown (53) to result in increased excretion as early as the first trimester with values rising to three or four times normal before term, and falling promptly to normal after delivery. Infection, burns, accidental trauma and surgical operations cause an increased excretion of glycogenic steroids in previously healthy individuals (26, 29), but this work is not yet available in detail in the regular medical journals. Pathological adrenocortical states such as Addison's disease and panhypopituitarism are associated with low excretion levels (53) while cases of Cushing's syndrome tend to show abnormally high excretions of glycogenic corticosteroids.

(c) Biological Assays of Urinary Corticosteroids. Influences of Hormone Therapy and of Adrenalectomy.

Not all the bioassay procedures outlined above have been used for determining the urinary recovery of injected cortical material. Pfiffner et al (54) found less than 200 units present in a 53 hour urine collection of a normal dog which received 2340 dog units of cortical extract

subcutaneously in one dose; their assay method was by maintenance of the adrenalectomized dog. Harrop et al (8) using a salt retention method in dogs, found only 10% recovery after intravenous injections of 3000 dog units into normal dogs. Weil and Browne (13) state that an increase in cold protection activity is detectable in the urine of patients after injections of either adrenal cortical extract or desoxycorticosterone acetate but no actual experimental data is given from which to estimate the percentage of recovery. Weil (55) obtained a 25% recovery by cold protection test when 100 cc. cortical extract was given intravenously to a patient with Addison's disease. The most detailed work on this subject is reported by Venning et al (9). Three patients were used, whose urines during control periods were negative when assayed at low levels by the cold protection method. After treatment with 100 cc or more of cortical extract daily (nearly all intravenously) amounts varying between 7 and 12% were recovered in the urines, if one can assume that all the observed cold protection activity was derived from the exogenous hormone. None of the liver glycogen procedures have been tested in recovery experiments for cortical extract. Venning (29, f.) failed to detect an increased output of glycogenic activity in a patient given subcutaneously 20 mgms. on two successive days of crystalline 11-dehydrocorticosterone acetate. This is a small dose although, if 10% had been rapidly excreted unchanged, it would theoretically raise the urinary activity by 2000 micrograms divided by 4, i.e. 500 glycogen units daily. Such an increase would certainly have been detectable in an individual normally excreting less than 100 units daily. The probability is, therefore, that the crystalline steroid was not rapidly or completely enough absorbed from the injection site, and this failure in recovery can not be held as evidence against the efficacy of the biological assay. Subcutaneously injected 11-desoxycorticosterone acetate in another

individual also failed to result in an increased urinary glycogenic activity but this might be due to the weak carbohydrate activity of the steroid. The recovery by the glycogenic bioassay of injected cortical extract in the urine of a patient with complete adrenal insufficiency would be highly relevent, but such data is not available to the author at the present time.

Browne (28, b) reported increased urinary activity by the cold protection method for at least four days following the injection of an active pituitary corticotrophin preparation subcutaneously to a normal man. This fact affords strong support for the specificity of the cold protection method.

Dorfman et al (38) examined their rat cold protection method in adrenalectomized monkeys and thereby obtained some crucial information. Although the urine of monkeys with adrenals intact contained detectable activity in 15 collections out of 30 trials, in no case out of 6 trials was there a positive response in the urines of adrenalectomized monkeys. On the other hand the administration of adrenal cortical extract to the adrenalectomized animals invariably resulted in positive urinary activity in 6 experiments. The administration of desoxycorticosterone acetate to similar animals gave an equivocal result, as there was one positive response in three trials.

In summary, the recovery of injected cortical extracts, the effect of corticotrophin, and the effect of adrenalectomy in experimental animals have given solid support as to the specificity of cold protection methods for urinary bioassay. Such evidence has not yet been published for the glycogen deposition methods, although they undoubtedly have the advantage of much greater sensitivity for aqueous cortical extracts and for pure 11-oxycorticosteroids.

ESTIMATION OF CORTICOSTEROIDS IN URINE BY ASSAY METHODS DEPENDING ON CERTAIN CHEMICAL PROPERTIES.

As the α-ketol side chain of the corticosteroids endows them with rather specific chemical properties under certain conditions, attempts have been made by several workers to assay suitably purified urinary extracts by the quantitative determinations of these chemical properties. In regard to the applicability of such methods to pure steroids, consideration of their behaviour in certain extraction procedures and towards various assay reagents has already been presented earlier in this thesis. Four urinary assay methods will now be reviewed.

(a) Methods depending on periodic acid oxidation.

The effect of periodic acid oxidation on the biologically active pure corticosteroids and on other related steroids has been discussed above (see Table III). Fieser et al (86) suggested that this reaction should be useful in the detection in urine of those corticosteroids which are excreted with their highly oxygenated side chain intact. Talbot et al (51) separated urinary extracts after various hydrolysing procedures into ketonic and non-ketonic fractions by means of Girard's reagent T. The non-ketonic fractions were subjected to oxidation by periodic acid in the presence of sulphuric acid and the resulting 17-ketosteroid content determined. After correction for 17-ketosteroids present in the samples prior to periodic acid oxidation, estimations of "formed" 17-ketosteroids are obtained. Talbot et al (51)

found that normal individuals excreted about 0.4 mg. of formed 17-keto-steroids per day, whereas three cases of virilism due to adrenocortical hyperplasia excreted approximately 10 to 16 mg. of formed 17-ketosteroids daily. Results on studies in other types of clinical conditions are not given. Lieberman et al (87) made similar studies on the formation of 17-ketosteroids by periodic acid oxidation of non-ketonic extracts, but were not satisfied with their techniques as the quantitative results were variable and did not parallel the biological activity of the extract (presumably referring to the assay of Dobriner (26, 85).). Recently Lowenstein et al (70) have revised the use of the periodic acid oxidation on suitable urinary extracts. They measure the amount of formaldehyde released (instead of the 17-ketosteroid remaining), and thence calculate in terms of the weight of a known pure steroid, such as 11-dehydrocorticosterone. In terms of this steroid their preliminary communication (70) reports that the daily average excretion of normal males is 0.5 to 0.8 mg. whereas one case of Addison's disease excreted only 0.15 mg. and one of Cushing's Syndrome 21 mg. daily. Such wide variations in the diseased states from normal would, if consistently confirmed, make this assay method of considerable clinical value. Except for the evidence of Lieberman (87) correlation with biological assays has not yet been attempted, and it is theoretically probable that periodic acid would oxidize many steroids which would be inactive in glycogenic bioassay methods. Important objections to the periodic acid oxidation methods are the uncertainty as to the nature and origin of urinary steroids subject to this reaction, and that there are different types of reaction for various steroids as shown in Table III of this thesis. Therefore, it is felt that

considerable further testing and analysis of the methods depending on this reaction will be required before results obtained therefrom can be clearly interpreted from academic or clinical viewpoints.

(b) Methods depending on reactions with reducing reagents.

Urinary assay methods depending on reduction properties were first reported in 1945 simultaneously by Talbot et al (5) and Heard et al (3). They differ in the extent of purification of the extract and also in the choice of reducing reagent.

The following is an outline of the procedure of Talbot (6). A 24-hour (or 12-hour) collection of unhydrolysed urine is extracted by shaking four times with 15 volumes per cent of chloroform. Correction is made for loss in emulsification, and the combined chloroform extract is evaporated to dryness. The residue is redissolved in 100 cc. chloroform and washed three times with 10 cc. of 0.1 N aqueous NaOH and three times with 10 cc. of water. Back extraction with 10 cc. portions of chloroform is also carried out. The chloroform is again evaporated. The residue is dissdved in 30 cc. of benzene which is extracted ten times with 30 cc. lots of water. The aqueous extract is extracted four times with 45 cc. lots of chloroform. The combined chloroform extracts are evaporated to dryness ("crude fraction"). The crude extract is separated into ketonic and non-ketonic fractions by treatment with Girard's reagent T, and the non-ketonic fraction is discarded. A chloroform extract of the ketonic fraction is evaporated to dryness and freed from traces of chloroform by the addition and subsequent evaporation of three 5 cc. lots of methanol. An aliquot of the final methanol solution of the ketonic fraction is then subjected to the reducing procedure of Nelson (22), and the resulting colour density is estimated in an Evelyn macro-photoelectric colorimeter. The quantity of "oxy-corticosteroid" present is

calculated by reference to a calibration curve obtained on known amounts of pure corticosteroids (e. g. corticosterone). It may be noted here that corticosterone, 11-dehydrocorticosterone and 17-hydroxycorticosterone gave similar curves but that the available sample tested of 17-hydroxy, 11-dehydrocorticosterone gave a somewhat different calibration curve, which fact was disregarded. The estimations of urinary "oxycorticosteroids" reported in the later communications (6, 56, 39), were determined on the ketonic fraction, as were those in the preliminary communication (5) with the exception of one or two estimations on the "crude extract". The step of partition between benzene and water apparently does not yield complete recoveries of corticosterone and 11-dehydrocorticosterone, as illustrated by table II, earlier in this thesis. Therefore, it may not be assumed that Talbot's procedure necessarily yields a total estimation of urinary 11-oxycorticosteroids. This criticism has been offered by Heard et al (2). However, the benzene-water partition and the subsequent separation into a ketonic fraction remove considerable reducing material, much of which may not be of cortical origin. The results of Talbot et al (6), taken from their Table IV, are presented below in Table VI. It is seen that the results for some cases of Addison's disease, hypopituitarism and, surprisingly, also hypothyroidism are low, while those for all urines from cases of Cushing's syndrome and some of the burn and post-operative cases are high. Certain cases of Addison's disease and also some of the results reported on the traumatic cases, show an overlap with the normal range. Thus, the results can not always be interpreted for differential clinical diagnosis. On the other hand, there is strong suggestive evidence that the results reflect adrenal function, and therefore that the urinary substances measured were probably derived from the adrenal cortex.

TABLE VI.

EXCRETION OF 11-OXY-CORTICOSTEROIDS, EXPRESSED AS MG. PER 24 HOURS.

(Talbot et al)

CONDITION	NO. OF SUBJECTS	NO. OF DETERMINATIONS	URINARY CORTICOSTEROIDS Average	Range
Normal Adults	9	42	0.24	0.10-0.38
Addison's Disease	6	6	0.12	0.02-0.26
Hypothyroidism	3	4	0.09	0.06-0.13
Hypopituitarism	3	3	0.14	0.10-0.17
Cushing's Syndrome	3	4	4.20	0.90-12.0
Adrenal Cortical Virilism	2	3	0.43	0.15-0.57
Simple Hirsutism	2	2	0.28	0.23-0.32
Burn and Post-operative Cases	4	9	0.93	0.34-1.70

The effect of high protein intake in a normal individual was a somewhat increased excretion of "corticosteroids", but dietary changes did not alter the excretion of an Addison's patient, or cause significant lowering in a woman with Cushing's Syndrome (24). However, in two cases of Cushing's Syndrome of Wilkins et al (39) and in another of J. E. Howard's (24) the considerable reduction in protein intakes were associated with marked lowering of the previously high urinary "corticosteroids". The exact effects of diet on urinary "corticosteroids" and on nitrogen balance needs further elucidation, but the importance of the diet as a cuase of variations in urinary "corticosteroid" levels seems established, as it is unlikely that large variations in reducing substances of non-adrenal origin appear in the purified ketonic extracts.

The application of the reducing method of (4, 3, 1) Heard et al for determining corticosteroids in pure solutions to urinary extracts (4, 3, 2, 34) has already been considered from certain aspects in this thesis (Tables 3, 4, and 5). The techniques of Heard et al (2, 34) are now given in greater detail. Twenty-four hour urine samples are collected without preservative. An aliquot measuring 100 cc. or 50 cc. is acidified with 12 N sulphuric acid to pH 1.0 as determined by the Beckman glass electrode. This is then extracted with a 4 to 1 mixture of water-free re-distilled ether-chloroform (the ether reagent having been extracted twice with concentrated sulphuric acid). 40 cc. of the solvent mixture are first used followed by 20 cc. portions three times. The extracts are combined and a further 40 cc. of ether-chloroform is added to aid in breaking emulsions. The solvent solution is then washed five times with 10 cc. portions of 0.1 N sodium

hydroxide (without back-extraction) and then five times with 10 cc. amounts of water. The washed solvent is then freed of water by shaking with approximately 1.0 to 1.5 grams of anhydrous sodium sulphate. The solvent is then filtered through glass wool and evaporated to dryness under reduced pressure at room temperature. The residue is then transferred by approximately 2.5 cc. of ether into micro test tube (75 x 8 mm) from which the ether is blown off by a stream of nitrogen at room temperature. The evaporating flask is washed three more times with ether, the washings transferred to the test tube, and evaporated. The total residue in the micro test tube is dissolved in 2 cc. of the phosphomolybdic acid reagent (consisting of equal portions of glacial acetic acid and the phosphomolybdic solution A of Folin and Wu (21). The tube is suspended for 60minutes in a boiling water bath. Then the contents are cooled and decanted into an Evelyn macro test tube with the aid of a further 8 cc. of the phosphomolybdic reagent. After the elapse of 90 seconds for bubbles to disappear the colour intensity is determined in an Evelyn photoelectric colorimeter with 650 μμ filter, standardized with a simultaneously heated and diluted reagent blank. An estimation of the content in the urinary extract is made by reference to a standard curve for the optical densities of acetic acid dilutions of pure desoxycorticosterone with the reducing procedure.

The results of Heard et al (2) in their Table 1, are given here in Table VII in somewhat modified form. Results are expressed both as reducing equivalents of desoxycorticosterone and also of 11-dehydrocorticosterone (see data of Heard et al (1) and Table V in this thesis). It is found that the two cases of panhypopituitarism excreted distinctly lower than normal amounts of reducing material, while the case of Cushing's syndrome, two late pregnant women, and three early burn cases showed abnormally high

TABLE VII.

EXCRETION OF REDUCING SUBSTANCES UNDER VARIOUS CONDITIONS (Heard et al 2).

(Results expressed in mg. per day as the reducing equivalent of pure steroids: A=11-desoxycorticosterone: B = 11-dehydrocorticosterone)

SUBJECT	A(Desoxy) mg/day	B(Dehydro) mg/day
Normal Adult Women	1.0 to 2.0	1.1 to 2.3
" Average (9 subjects)	1.3	1.5
Normal Adult Men	1.1 to 2.1	1.3 to 2.4
" average (9 subjects)	1.5	1.7
Males, 2.5 to 3 years	0.3 & 0.5	0.4 & 0.5
" 5.5 to 7 years	0.7 & 0.8	0.8 & 0.9
Panhypopituitarism, female	0.4 & 0.5	0.5 & 0.5
" male	0.6	0.7
Cushing's Syndrome, female	4.8	5.5
Pregnancy, 9th month	2.5	2.9
" Case 2.	2.6	3.0
Burns 4th day, Male (2 cases)	3.0 & 3.4	3.4 & 3.9
" 24th " " 2 "	2.4 & 2.6	2.8 & 3.0
" 45th " " 2 "	0.84 & 0.85	1.0 & 1.0
" 4th " " (case 3)	2.7	3.1
" 24th " " " "	1.2	1.4

excretions. The two burn cases examined on the 45th. day showed lower than normal values, which is not at present explainable. With the exception of these late burn results, and somewhat lower values in the children, the variations from normal show a good correlation with the variations shown (2) on the same cases by the glycogenic bioassay method of Venning et al (7).

Heard et al (2, 34) attempted to show that the measurement of reducing substances in urine reflected adrenal cortical metabolism by certain experiments on dogs. In these dogs total daily urine outputs could seldom be obtained, and therefore reducing substances were determined in relation to the creatinine content of single samples, and thence proportional corrections made for the average daily total creatinine output. Because of dubious variations in total creatinine, and also as the acidity of the urine samples was not controlled before extraction, their first experiment on the effect of adrenalectomy will not be presented for consideration in this thesis. The urines were acidified in their second experiment (see reference 2, Figure 1). A single intravenous injection of 70 cc. of cortical extract resulted in a sharp increase in the excretion of reducing substances over the ensuing six hours, which increase represented a recovery of about 3 per cent of the material administered. Furthermore, two courses (5 and 3 days respectively) of subcutaneous injections of lyophilized anterior pituitary extract caused definite increased outputs of reducing substances over 17 days and 6 days respectively. It is probable that these increased values were due to corticotrophin affecting the animal's adrenals, though at the earlier injection site an abscess formed (non-specific damage) and the reducing power of the excreted metabolites

of the injected pituitary material itself is not know. However, it appears from these experiments that increased excretion of adrenal cortical products (exogenous or endogenous) is reflected by increased reducing substances in the urine. It is worthy of note that Talbot using his reducing method (6) did not find significant increases in "oxycorticosteroid" secretion in 12 patients or one normal subject who received 10 to 60 mg. of 11-dehydrocorticosterone acetate (synthetic) daily intramuscularly for 3 to 5 days (see Forsham et al 58).

Before attempting to compare the results of Talbot et al (6) with those of Heard et al (2), the influence of acidification of the urine prior to extraction must be considered. Heard et al (2) compared the effects of increased hydrion concentration on three different samples of normal male urine. Their Table III is shown in this thesis as Table VIII. It is apparent that extraction at pH 2.0 results in approximately three times the reducing material compared to extraction at pH 7.0. Data on urine No. 2 shows a considerable further increase at pH 1.0 and still more at pH 0.5. It is unfortunate indeed that an exact limit to this increasing effect is not found. However, Heard et al did find that urine residues subjected to boiling 10 minutes in 15% HCl lost about two-thirds of their activity, and that a residue exposed to 2% H_2SO_4 for one hour was also partially inactivated. On the other hand when urine No. 3 (Table VIII) was allowed to stand for 24 hours at pH 2.0 the extractable reducing material was further increased. Acidifying a urine sample to pH 2.0 and immediately neutralizing it to pH 8.0 before extraction resulted in the same value as untreated neutral urine. It seems unlikely that conjugates once hydrolyzed would reform, but perhaps the duration of exposure in the acid medium was insufficiently prolonged to cause hydrolysis.

It is not quite clear whether increasing acidity to at least pH 0.5 releases lipid soluble free substances from the less soluble parent conjugates, or merely increases the solubility of urinary steroids (free or conjugated) in the solvents. Both Venning (29, 7) and Dobriner (26, 85) noted increased glycogenic biological activity in urines extracted between pH 1.0 and 2.0 (approximately double the results on unacidified urines) which is slightly further increased on overnight standing. The effect of acidification to pH 0.5 is not reported for biological assay, and the assumption that acid simply leads to the hydrolysis of conjugates is not quite proven. It is concluded from the work of Heard et al (2) that excessive acidification actually disrupts the steroid substances, and this is supported by the biological assay of Harrop and Thorn (8). The final conclusion is that relatively mild acidification prior to extraction of urine leads to an increased yield of reducing substances, but (a) the mechanism is not clear; (b) the choice of pH 1.0 appears a convenient but arbitrary point, as it is not necessarily the point of maximum yield; (c) the effect of prolonged exposure to acidification (when emulsification leads to uncontrollable delays in complete extraction into the neutral solvent) is not known, and might cause some further increase in yield, and finally (d) the effect of acidification may be different (greater?) on the extraction of non-specific urinary reducing substances than on corticosteroid-like reducing substances. This last point is suggested by data in Table IV of Heard et al (2) where a urinary residue exposed to 2% sulphuric acid is not further destroyed by periodic acid, in contrast to a partial effect of acidification on desoxycorticosterone and

TABLE VIII.

EFFECT OF HYDRION CONCENTRATION ON EXTRACTION OF REDUCING SUBSTANCES FROM URINE.

URINE NUMBER	pH	REDUCING POWER gamma/100 cc
1	7.0	9
	6.0	6
	4.0	12
	2.0	33
2	3.0	9
	2.0	22
	1.0	42
	0.5	80
3	7.0	8
	2.0	20
	2.0 for 24 hrs.	39

(From Heard et al (2), Table III).

complete destruction by periodic acid.

It is, perhaps, presumptuous to attempt to compare two methods, when one claims to measure the urinary excretion of 11-oxycorticosteroids (Talbot 5, 6), and the other the excretions of total corticosteroid-like substances (Heard 2). The results of the latter may be recalculated into terms of 11-dehydrocorticosterone (see Table VI.) An attempt will be made to analyse the other factors of difference.

(1) Extraction at pH 1.0 yields ~~to~~ approximately three or four times as much reducing substances as at pH 7.0 (Heard and discussion above). Talbot does not control the acidity of the urine but the usual variations between pH 5 and 8 would not differ greatly from pH 7.0

(2) The solvent ether-chloroform (4 to 1) results in quantitative recovery of desoxycorticosterone from water or urine (Heard 2), so one may presume it is as satisfactory a solvent as pure chloroform, although both groups have evidence that pure ether is not a satisfactory solvent.

(3) Only Talbot partitions the urinary extract between benzene and water, by which some urinary reducing power is presumably removed. Any urinary corticosteroids without an oxygen atom at position 11, and a considerable portion of those 11-oxycorticosteroids without a hydroxyl group at position 17 would be discarded with the benzene solutions (see this thesis Table II; also Talbot 6). Therefore the final result of Talbot et al would be lower than that of Heard et al by an indeterminate value "x".

(4) Only Talbot fractionates the residue into a ketonic portion, which he states gives lower reducing activity than his crude residues, but does not provide an estimate as to the extent or variability of this decrease. Let this factor be "y".

The reducing reagent of Talbot is apparently specific for primary or secondary α-ketol groups (see this thesis Table III), whereas the phosphomolybdic reagent is less specific, reacting also with $\alpha\beta$- unsaturated 3-ketones. Possibly acting in the opposite direction is the fact that the reducing reaction of Talbot reaches completion within the 20 minute heating period, whereas that of Heard et al is not complete at the arbitrarily chosen heating period of 60 minutes (see this thesis Tables IV and V). Therefore, differences due to this factor may be represented by the symbol "z" which may be acting in a + or - direction.

(6) A symbolic formula for the comparison of results (in terms of 11-dehydrocorticosterone) of Heard's method to those of Talbot would thus be $\frac{3 \text{ or } 4}{1-x-y \pm z}$ (see Table IX).

TABLE IX.

SYMBOLIC FACTORS COMPARING RESULTS OF URINARY REDUCING SUBSTANCES.

(In terms of dehydrocorticosterone) by A, the method of Heard et al (2) and B, the method of Talbot et al (6).

	Method A	Method B	Effect as Ratio $\frac{A}{B}$
1. Acidification prior to extraction	acidified	not acidified	3 or 4 to 1
2. Choice of solvent	Ether-$CHCl_3$	$CHCl_3$	probably 1 to 1
3. Benzene-water partition	Not done	Partitioned	1 to 1-x
4. Ketonic fractionation	Not done	Ketonic	1 to 1-y
5. Choice of reducing reagent	Molybdic	Cupric	1 to $1 \pm z$
6. Combined factors above	-	-	$\frac{3 \text{ or } 4}{1-x-y \pm z}$

If this symbolic formula could be given a constant muerical value it is apparent that the ratio might actually be $\frac{3}{1}$ or even as high possibly as $\frac{7 \text{ or } 8}{1}$. With this uncertain figure in mind one may refer to Table X, in which certain of the results previously presented in Tables VII and IX are consolidated. It is seen that the results of Heard et al (2) exceed those of Talbot et al (6) by $\frac{6 \text{ or } 10}{1}$ for normal adults, about $\frac{5}{1}$ for hypopituitarism, possibly $\frac{3}{1}$ for normal adults, about $\frac{5}{1}$ for hypopituitarism, possibly $\frac{3}{1}$ for burn cases, and possibly slightly in Cushing's Syndrome. In the latter condition both methods give high results and the few cases do not admit of comparison. It might be suggested that a better comparison would be obtained by subtracting a figure 0.5 mgms. (representing factors x and y previously discussed) from all the results of Heard et al. This would still leave the results of Heard et al considerably higher than those of Talbot et al for normal adults, and for the burn cases.

TABLE X

URINARY REDUCING SUBSTANCES, EXPRESSED AS 11-DEHYDROCORTICOSTERONE IN MG/DAY.

	By Talbot (6)		By Heard (2)	
	Range	Ave.	Range	Ave.
Normal adults	0.10-0.38 (42)	0.24	1.1-2.4 (18)	1.6
Panhypopituitarism	0.10-0.17 (3)	0.14	0.5-0.7 (3)	0.6
Cushing's Syndrome	0.90-12.0 (4)	4.20	5.5 (1)	5.5
Burns and Post-Operative	0.34-1.70 (9)	0.93	-	-
Burns, 4th-24th days	-	-	2.8-3.9 (5)	3.3

Note: Figures in parentheses represent number of examinations.

CORRELATIONS BETWEEN BIOLOGICAL AND CHEMICAL METHODS OF URINARY ASSAY.

The biologically active corticosteroids so far isolated from adrenal tissue have been shown to have qualitative and quantitative differences, (a) in their biological effects in various assay procedures; (b) in their relative solubility in water and in various substances used in extraction; and (c) in their chemical reactions with certain reducing reagents. The fate of the secreted substances is not known. The chemical structures of the one or many biologically active steroids of cortical origin in urine are not known, (however, see Venning et al (9) and Hoffman (71)). The relative proportions of such active compounds to inactive metabolites, which may retain certain characteristic chemical properties are not known. Only a few inactive compounds (excepting 17-ketosteroids) which are presumed to be of adrenal origin have been completely characterized from urine; none have the characteristic primary α-ketol side chain; and it is presumbed that there are indeed other inactive compounds not yet identified. For such reasons as the foregoing, it can not be expected that the various biological and chemical methods of urinary assay should show exact degrees of correlation one with another. Attempts at such correlations are further complicated by the fact that the various methods are not standardized in terms of any single pure corticosteroid. Results of each assay method can only accurately be compared with the normal range for the particular method.

(a) General Correlations.- In the urine of adrenalectomized monkeys Dorfman et al (38) found no cold protection activity. The same workers (18) found no cold protection activity in the urines of patients with Addison's disease, and Venning and Browne (82) found extremely low glycogenic activity in the urines of Addison's and panhypopituitarism cases. The chemical

urinary assays of Heard et al (2), Talbot et al (6) and Lowenstein et al (70) all showed lower than normal values in such patients.

The injection of adrenal cortical extracts ~~to~~ in patients resulted in higher cold protection activity according to Dorfman et al (18) and Venning et al (9), and likewise in the dog increased reducing substances by the methods of Heard et al (2). The injection of pituitary corticotrophin material into a man by Browne (28,b) was associated with increased cold protection activity, while pituitary material resulted in elevated urinary reducing substances in a dog (Heard 2).

Cases of Cushing's Syndrome have shown high urinary biological activity by the life maintenance method of Anderson et al (10), cold protection method of Weil and Browne (13) and the glycogenic assays of Dobriner (26) and Venning (29, 82). Likewise, high excretions have been noted in such cases by the various chemical assays of Heard et al (2), Talbot et al (6, 5, 39) and Lowenstein et al (70).

Pregnancy has been associated with high glycogenic activity by Venning (53, 2) and with increased reducing substances by Heard et al (2). Burns, fractures, infections and operative conditions have been associated with high excretions of cold protecting substances according to Weil and Browne (11, 12, 13) and Shipley, Dorfman et al (62), and with unusually high values for glycogenic activity according to Venning (29), Shipley, Dorfman et al (62) and Dobriner (26). Similarly, Talbot et al (6) found elevated urinary values of reducing substances in burns and post-operative cases and Heard et al (2) noted elevations in three burn cases when examined at early periods.

Thus, there is a general correlation in most instances between the results of the various biological procedures, and the chemical assay methods. Published attempts to determine the correlation between a bioassay and a chemical assay by examining an identical group of urine samples have so far been limited to the relatively few reported by Heard, Sobel and Venning (2).

(b) Quantitative Correlations for urines from normal subjects.

The data of several workers on biological methods and several on chemical methods admits of recalculation in terms of the pure steroid, 11-dehydrocorticosterone. Venning et al (7) further state that acidification prior to extraction approximately doubles their glycogenic bioassay results , whereas Heard et al (2, 34) indicate that such acidification increased the urinary reducing substances three to four times. Finally, Talbot et al (5) state on page 211 that the ketonic values are about one-half to one-third of their crude values determined after benzene-water partition of urinary extracts. Thus, it has been possible to make approximate recalculations of several methods of urinary assay in terms of dehydrocorticosterone in gamma per day (see Table XI). The further unsupported assumption is made that urinary compounds, having reducing activity equivalent to a certain weight of dehydrocorticosterone,may be of sufficiently similar structure that they could be considered to have a biological activity equivalent to that same steroid. In fact, as mentioned several times above, we are ignorant of the exact chemical structures of the "corticosteroid" compounds in urine.

Table XI, shows that the chemical assay of the ketonic fractions of Talbot gives values approaching the low range of the glycogenic assay of Venning. On the other hand the alkali-insoluble fraction of Heard gives somewhat higher values, which perhaps can be comapred with those by the cold protection

assay of the Dorfman group (62).

TABLE XI.

URINARY ASSAYS ON NORMAL SUBJECTS, RECALCULATED FOR EXTRACTION AT NATURAL ACIDITY, IN TERMS OF MICROGRAMS OF 11-DEHYDROCORTICOSTERONE PER DAY.

AUTHOR	ACTIVITY	RESULT	REMARKS
Venning (35)	Glycogenic	50-150	(a. Cpd. A = 1/3 activity Cpd. E (b. Natural = ½ acidified urine
Dorfman (62)	Glycogenic	less than 400	
Dorfman (62)	Cold-protection	500 to 1800	
Talbot (6)	Reducing	100 to 380	-Ketonic
Talbot (6,5*)	Reducing	250 to 950	"crude" (benzene insoluble) (= 2 - 3 times ketonic)
Heard (2, 34)	Reducing	275 to 600	Total alkali-insoluble.

ADVANTAGES OF CHEMICAL ASSAY METHODS.

The relative advantages and disadvantages of the chemical and the bioassay methods must be considered from two aspects:

(1) The usefulness of the results, and

(2) The convenience, lengthiness and expense of the method.

From the first point of view, the bioassay methods are specific for corticosteroids and the glycogenic assay method has apparently high specificity for only the unsaturated, 11-oxygenated, α-ketol side-chain type of compound which acts on carbohydrate-protein metabolism. There is reason to assume that the unknown urinary compounds, which cause glycogenesis are closely related chemically to such a compound as 17-hydroxy, 11-dehydrocorticosterone, though there may be minor differences or additional conjugating radicles. (Consider the unidentified urinary compound of Venning et al (9), Hoffman (71). One may interpret the results of the glycogenic bioassay method as reflecting the actual secretion of cortical compounds of the group which influences carbohydrate protein metabolism.

The chemical methods offer the possibility of detecting inactive compounds as well as active. An advantage lies in their lessened specificity but this brings the danger that they detect also compounds formed elsewhere than the adrenal cortex. As so little is known about the metabolism of the active adrenal substances, it is obviously difficult, nearly impossible, to attach significance of clinical value to variations in the quantity of inactive compounds of questionable cortical origin. Yet further study of the corticoid chemical methods may be the means of learning more about cortical metabolism. Such a fundamental advance would more than justify the labor and the lack of any definite clinical value from the early results. As the method of Heard et al (1, 2) uses the total alkali-insoluble urinary extract, it may be measuring the following groups of compounds derived from the adrenal

cortex (as well as some reducing substances from other sources):

(a) active 11-oxy compounds;

(b) inactive 11-oxy steroids derived from the former;

(c) active 11-desoxy compounds (if present in urine)

(d) other inactive compounds of cortical origin.

An accurate estimation of the total of the four groups should be of considerable scientific value and might, after sufficient study, be of ultimate clinical value. The method of Talbot (6) includes purification to a ketonic residue, and apparently would remove compounds of group (c) and (d) above. Talbot only intends to measure the active glycogenic compounds and their most closely related 11-oxygenated inactive metabolites. Such a method might correlate closely with the bioassay method of Venning et al (7), but it would not have the possible advantage of measuring the wider range of excretion products of cortical origin.

From an academic viewpoint, economy of time or space or money may not be of primary importance. However, such are very important considerations for a clinical medical institution. The animal colony required for bioassays entails space, labor and considerable worry lest the supply fall below the demand. Alternatively, purchase of young animals is expensive. All biological methods suffer from the disadvantage of biological variation, requiring many test-animals. Seasonal variation is particularly important in the glycogenic bioassay, and some workers have found an expensive constant-temperature room necessary for satisfactory assays. Dorfman et al (63, 64) note considerable variation from group to group of animals subjected to the cold protection test, yet failed to determine the causes of this variation. Thus, large control

groups must be used each day an unknown assay is being done.

Even if the chemical method was as time consuming as the bioassay method it would have the considerable advantage of circumventing the use of rats and mice. The glycogenic assay method of Venning et al (7) requires approximately three days to assay a urine collection. A staff of three workers can complete about 16 specimens each week. On the other hand the method of Heard and Sobel (2) allows one technician to perform two analyses each day (two specimens or duplicates on one specimen). This is cheaper, and also allows the result to reach the clinician at an earlier date. The chemical method of Talbot (6) requires more than one full days work by a highly skilled technician for each urine tested. The only advantage from this viewpoint over the Venning bioassay is that animals are not necessary. Talbot's purification is laborious, requires considerable manipulation and transference of the material from one vessel to another. This offers chance for contamination but the successive steps probably remove such non-specific reducing contaminants.

Both the methods of Heard and Talbot require only a 24-hour collection of urine, but there is little added inconvenience in collecting the 48-72 hour samples required for the bioassay of Venning. The chemical methods have the disadvantage of being more sensitive than the bioassay methods to urinary contaminants, such as preservative, blood, or faeces. Heard (2) states these should be avoided, but there is often difficulty in ensuring the necessary clean collection on a busy general hospital ward. The presence of small amounts of blood or faeces in the urine apparently does not disturb the bioassay of Venning, though preservatives of the cresol type are extremely toxic to the mice and invalidate the assay.

In summary, then, in dollars and cents the chemical assay of Heard (2) is the cheapest, followed by Talbot's (6) assay, but the more costly glycogenic bioassay appears more specific, more accurate and more readily interpretable to the clinician at present.

SCIENTIFIC BASIS FOR INTERPRETING THE ASSAY OF URINARY CORTICOIDS AS A CLINICAL INDEX OF ADRENAL FUNCTION.

Undoubtedly the study of urine for the presence of substances of cortical origin, both quantitatively and qualitatively, is of great scientific interest and ultimately assures valuable aid to the clinical branch of medicine. But is there enough knowledge of the physiology and biochemistry of cortical secretions to interpret adequately the results of urinary bioassay? Some of the gaps in our knowledge will be emmerated in the following paragraphs.

(1) Many active and inactive compounds have been chemically isolated from the adrenal cortex, but Reichstein (20) emphasizes the lack of general agreement as to whether there is a single or many "hormones" actually secreted, and exactly what structure it (or they) has.

(2) Vogt (49) has evidence that the circulating blood rapidly inactivates the secreted hormone even when the kidney is short circuited, and therefore casts doubt on whether renal excretion reflects adrenal secretion.

(3) We do not have complete information as to the organs and tissues on which cortical hormones act. Increased glycogen in the liver might be due to accelerated protein break-down in the liver with simultaneous formation of new carbohydrate, or it might be merely the back-log effect of an inhibition of tissue oxidation of glucose, as suggested

from the work of Cori and coworkers (88). The influence on salt and water metabolism may take place on the resorptive mechanism of the renal tubular epithelium, but this is not proven. The adrenal androgens may be specifically formed or may only be degradation products of the larger "metabolic" hormones, and the degraded radicles may be androgenic only by chance. Dougherty and White (89) have indicated that the cortical hormone acts directly on lymphoid tissue causing cytolysis and the release of antibodies, but it is apparent that the cortical hormone must have other sites of action.

(4) The chemical sequence of oxidations, reductions and conjugations leading from the secreted hormone through unknown intermediate substances to those present in the urine is not known. Perhaps general diseases influence such metabolism, so that unusual cortical metabolites escape into the urine, even when the same quantity of identical cortical hormone as usual is being secreted. This influence might take place through the liver, which is known to normally inactivate progesterone (Masson and Hoffman 90).

(5) We do not yet have conclusive information as to the chemical structure of the active corticosteroids present in urine (refer Venning et al (9), Hoffman (71)). If one has this active urinary compound in crystalline form one might learn its stability in urine under various fluctuations of alkalinity and temperature and would discover the rate of its inactivation. Possibly highly alkaline or bacterially infected urines are low in biological activity without alteration in such a chemical attribute as reducing power, but this is not known.

(6) In conclusion, we do not know what proportion of the cortical hormone secreted in 24 hours appears as biologically active compounds, or, again, as biologically inactive compounds with various types of chemical characteristics such as reducing power. Evidence has been cited (9, 8), that only 10% of intravenously injected cortical extract appears in biologically active form in the urine. However, the continuously secreted endogenous hormone may reach the urine in greater or lesser proportions.

Despite these fundamental gaps in our knowledge the fact remains that experience has shown that urinary assay by the cold protection test of Weil and Browne (13) and more particularly by the glycogenic bioassay of Venning et al (29, 7, 35, 53, 82) do indeed provide a useful reflection of adrenal cortical function in humans. Thus, differential diagnosis, prognosis and therapy may be more accurately provided.

It is possible, though not certain, that the chemical urinary assay method of Talbot (6) provides data of equal clinical value. The relatively few correlations on clinical cases reported by Heard et al (2) suggest that this simpler, quicker chemical method might be clinically as valuable as the glycogenic bioassay. Even if the correlation was not perfect, it might be that this chemical method would give constantly altered results in some clinical conditions. Therefore, there is justification in attempting to apply the urinary assay of Heard (2) to a more extensive clinical trial, noting both correlation with the clinically valuable glycogenic bioassay, and also examining instances of negative correlation to see whether the chemical result be subject to useful clinical interpretation. Such is the purpose for which the following experimental data was obtained.

THE METHOD OF CHEMICAL ASSAY OF URINE EXTRACTS.

The method used is essentially that reported by Heard and Sobel (1, 2, 34) with certain modifications. The extraction of urine samples were prepared in two different ways, termed "Method A" and "Method B". All extracts were assayed for reducing power by one colorimetric method. Finally the preparation of standard curves for the reducing power of known solutions of pure steroids will be described.

I. Extraction Method A: Total 24 hour collections (or 2 to 3 day collections) of urine are made in chemically clean vessels without preservative. It should be free of feces, blood or disinfectants. It is kept cool (refrigeration if possible) during collection and storage. Extraction should not be delayed more than 24 or 48 hours. The collection is mixed and volume recorded. The specific gravity is noted. Duplicate aliquots (rarely triplicate or single samples) are pipetted into a beaker. Usually 50 cc is taken. The pH of the unacidified sample is measured by the Beckman pH meter (glass electrode). Each aliquot is adjusted to pH 1.0, by the cautious addition of 12 N H_2SO_4. The time is noted. The aliquots are decanted into separating funnels (500 cc funnels appear to allow less emulsion than 250 cc funnels). The stop-cocks must not be greased, but are moistened with distilled water. The urine is extracted with 40 cc of 4:1 ether - chloroform. The neck of the funnel is washed down with solvent from an all-glass wash-bottle. The mixture is shaken gently 200 times. (Vigorous shaking causes emulsification).

The urine is again extracted three times with 20 cc volumes of solvent. The urine is discarded. The solvent portions are replaced in the funnel. About five to ten minutes are allowed for trapped urine to separate. Usually some emulsion is seen. Now 40 cc of fresh solvent is added to the funnel, and it is shaken about 50 times vigorously which is almost always successful in breaking the emulsion. After a further ten minutes standing the emulsified layer should be not more than 5 cc. If larger emulsions are present, repeated vigorous shaking is often successful. Occasionally a further addition of 20 cc. fresh solvent is added and the funnel shaken once more. It was never found necessary to centrifuge to bfeak emulsions. The time is recorded, as the duration from acidification to this stage should be between 40 and 90 minutes. Now the extract is washed successively five times with 10 cc lots of 0.1 N NaOH. The first two washings are coloured, and there must be no emulsion remaining after the third washing. Occasionally persistant small emulsions require the alkali washings to be separated seven times. The clear extract is now washed with small volumes of distilled water (total 60 cc.). Finally as much water as possible is drained off. Three spatula points (about 2 grams) of anhydrous sodium sulfate is added and mixed well. The extract is decanted through a small glass funnel, containing glass wool as a filter, into a 250 cc standard taper Pyrex Erlenmeyer flask. Particles of sodium sulfate should not fall into the Erlenmeyer. The separating funnel is washed once with a small volume of solvent, and finally

the filter is also wahsed. Care is taken that the extract and washings do not touch the neck of the Erlenmeyer. A standard taper connecting tube with stop-cock and small side arm at 90° is inserted in the Erlenmeyer, and attached to the rubber connecting tube of a water suction set with trap. Care is taken to prevent bubbling of the extract, and also to prevent particles of rubber tubing being sucked into the flask when pressure is being discontinued. The flasks are inserted in water warmed no higher than 50°C., (which is actually cooler by the time the volume of extract is small). After evaporation the flask appears empty and dry. Such flasks may be stoppered and stored in the refrigerator overnight. Occasionally several days were allowed to lapse at this stage.

II. Extraction Method B: The extract is first prepared exactly as in Method A, but benzene-water separation is added. (N.B. the extract was not always dried with sodium sulfate, as water is later added to the evacuated Erlenmeyer flask). Ten c.c. of redistilled benzene is pipetted into the evacuated Erlenmeyer, shaken gently to make contact with the walls of the flask, and decanted into a clean separating funnel. Then 7 c.c. of distilled water is placed in the Erlenmeyer and the neck and sides washed down with a small volume of water from an all glass wash-bottle. The water is then decanted into the separating funnel, of which the neck is sprayed with water from the wash bottle. The total volume of water approximates 10 c.c. The benzene-water mixture is shaken, allowed to separate, and the aqueous extract carefully drained. Ten c.c. portions of water, which pass through the Erlenmeyer used for evacuation each time are shaken with the benzene for three more extractions as before. Care is taken that no traces of benzene are drained into the aqueous extracts. The pooled aqueous extract, approximately 40 c.c., is decanted into another clean separating funnel. This is extracted with 10 c.c. lots of redistilled chloroform four times. Sprays of water from the wash bottle are used to rinse the neck of the funnel, but the flask which has contained the aqueous extract is rinsed each time with the chloroform solvent. After these extractions the chloroform solutions are carefully drained into a standard taper Pyrex Erlenmeyer flask, which is immersed in water (not over 50° C), and carefully evacuated under reduced pressure.

III. Colorimetric Assay of reducing activity: A set of macro test tubes for the Evelyn Photo-electric colorimeter are standardized for light transmission. They are also calibrated at the meniscus line of 10 c.c. of water, and stored for use when chemically clean and dry. The residue from the evacuated Erlenmeyer is transferred to the Evelyn macro test tuve by the use of ether from an all-glass wash bottle. Four to five rinsings of the flask with a total of approximately 5 c.c. ether are transferred with the aid of a capillary pipette. Care must be taken not to allow the ether to come into contact with the rubber teat. The solvent should be delivered at the base of the tube. A final spray of ether washes down the sides of the test-tube. The level of the solvent should be approximately halfway from the base to the 10 c.c. line on the test-tube and must on no account be higher than the mark. The tube is then immersed in luke warm water and evaporated to dryness by a stream of compressed nitrogen, which must not cause splashing. All rubber connections must be treated in boiling solutions of 0.5% NaOH and 4% HCl successively, and rinsed till neutral before use. In addition, plugs of glass wool are inserted as filters near the terminal connections.

Five c.c. of freshly prepared mixture of equal parts of phosphomolybdic solution and glacial acetic acid reagent are added to each assay tube and also to one or two "blank" tubes. In addition, one or two standard solutions of 21-hydroxy pregnenolone in acetic acid are prepared in such a manner that the final 5 cc. reducing reagent consists of the usual equal parts of phosphomolybdic solution and glacial acetic acid (see "Standard Curves"). All tubes are immersed in a boiling water bath for one

hour, and the level of water must always be higher than the internal level of the solution. The tubes are covered by glass bubbles during boiling to prevent contamination and to allow condensation of internal vapour.

After the heating period the tubes are immersed in cool water for 3 minutes, and wiped clean and dry; then the mixed reducing reagent is added to the 10 c.c. mark in every tube. After mixing, each tube must stand about 1 minute to allow bubbles to disappear, but all tubes should be read within 10 minutes. (The colour changes only slowly during 30 to 60 minutes). The blank tubes may show a faint yellowish colour, but this is neutralized by adjusting the photo-electric colorimeter to give 100% light transmission with the blank. The same colour filter with transmission at 635 mμ was used in all experiments. The standard known solutions serve as a check that the heating and other conditions of the reducing reaction are constant. The per cent light transmission of each unknown solution is read on the galvanometer, and calculated as optical density from a table ($I_o = 2 - \log G.$).

Calculations: The reducing equivalent of the unknown (Z) is determined in terms of micrograms of 11-dehydro corticosterone (Compound A) from a standard curve (see Fig. 1). Alternatively this may be done by a mathematical formula. Let X be the optical density of the unknown sample, Y be the optical density of 100 micrograms of Compound A in the particular batch of reducing reagent, and Z be the reducing equivalent of the unknown in micrograms of Compound A. Thus the formula is:-

$$\frac{X}{Y} \times 100 = Z.$$

From the above finding, the final calculation is made as follows:

$$\frac{\text{Urine volume per 24 hrs. (in c.c.)}}{\text{aliquot assayed (in c.c.)}} \times \frac{\text{Z (in micrograms).}}{1000}$$

This answer is mg. Reducing Substance (equivalent of Compound A) per 24 hours.

IV. Preparation of Standard Curves: Dr. E. H. Venning had received a small quantity of Compound A through the kindness of Dr. E.C. Kendall. Due to other demands on this limited supply, only one suitable fresh solution of this material was available for the purpose of a standard curve after the technique of determining reducing power had been mastered. Dr. Venning supplied a fresh carefully prepared solution in alcohol of which 1 c.c. equalled 0.99 mg. The alcohol was evaporated under nitrogen at a low temperature. Four dilutions were made by standard pipettes with glacial acetic acid, so that 141, 85, 46 and 23 micrograms of Compound A, respectively, were contained in 1 c.c. The largest and the smallest amounts were taken in duplicate, and the two medium amounts in triplicate (totalling ten samples). Then 1 c.c. of the steroid solution was pipetted into a calibrated Evelyn macro test tube and 1 c.c. of undiluted phosphomolybdic reducing solution was added. Then 3 c.c. of a fresh mixture of equal parts of phosphomolybdic solution and glacial acetic acid was added, making a total of 5 c.c. These were heated for 60 minutes in the boiling water bath, diluted to 10 c.c., and reducing power determined as previously described. Batch "E" of the reducing reagent was used for this standard curve.

Figure 1 has micrograms of Compound A on the abscissa and optical density on the ordinate. The points shown were the average findings at each of the four dilutions tested. The line was drawn by taking an average of the optical densities of the ten samples tested, when each sample was corrected to a fixed quantity of steroid. It may be seen in Fig. 1 that the points fall close to the straight line, agreeing with Beer's Law.

Numerous standard curves were made with a sample of 21-hydroxy-pregnenolone, kindly supplied by Dr. S. Lieberman. On two occasions suitable dilutions of this steroid were prepared in glacial acetic acid. They were kept frozen between use and allowed to return to the liquid state by very gently warming. In this manner the reducing power was found to be constant over a period of several months. Standard curves with such an available steroid must be made with each new batch of reducing reagent. The variation in the reducing activity of various batches of reagent is shown in Fig. 2. From this data, a correction for each reagent batch is made, so that the standard curve of Compound A with Batch "E" of the reducing reagent may be applicable in each case. (See discussion later, and Table XII.).

Addendum: Purity of reagents: The whole extraction - colorimetric procedure must be performed on samples of 50 c. c. water at frequent intervals to demonstrate freedom of the reagent from reducing contaminants. This is most important whenever fresh solvents are prepared.

FIG. 1

STANDARD CURVE OF 11-DEHYDRO CORTICOSTERONE (Cpd. A) WITH REDUCING REAGENTS (Batch "E")

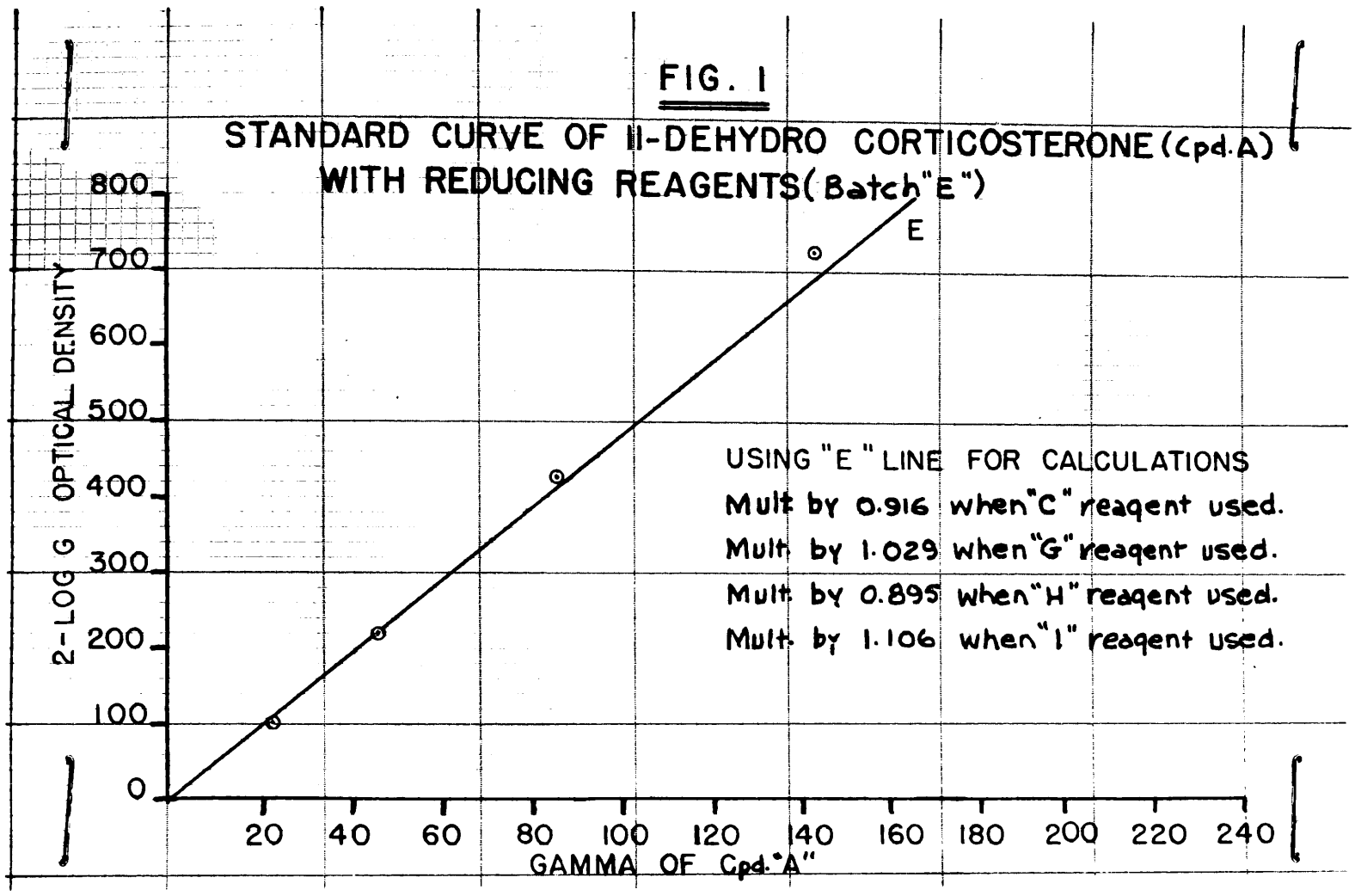
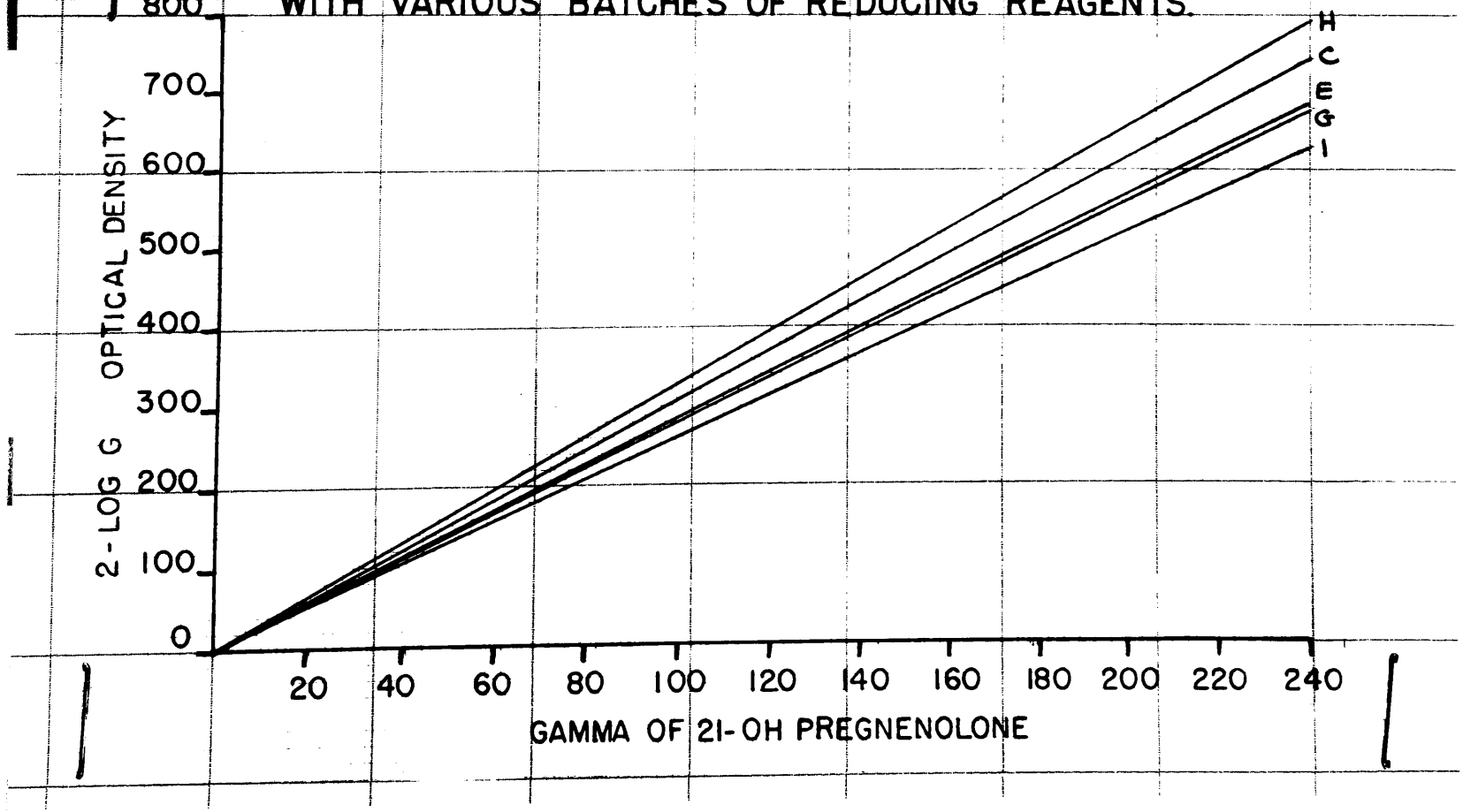


FIG. 2

STANDARD CURVES OF 21-HYDROXY PREGNENOLONE WITH VARIOUS BATCHES OF REDUCING REAGENTS.



COMMENTS ON THE METHOD:

1. Preparation of Reducing Reagent:-

One difficulty in preparing each batch of phospho-molybdic reagent is completely ridding the solution of ammonia. All supplies of molybdic acid crystals used were labelled as containing sizeable amounts of ammonia. This is gradually released during the heating procedure, but not completely during the 20 - 40 minutes boiling advocated by Folin and Wu (21). Therefore, boiling should be continued until the fumes are no longer alkaline to litmus paper, and even then one fears that traces of ammonia may sometimes remain, which may account for variations in activities of different batches of reagent. Although all batches are colourless after storage in the refrigerator, it was found that in most cases the blank tubes, prepared by equal mixture of the phospho-molybdic and acetic acid, became yellowish after sixty minutes heating. The density of this colour varies from batch to batch. It may account for variations in the reactivity with known steroids, and therefore a standard curve is necessary with each batch of reagent. The yellowish colour to the blank alters the centre setting when adjusting the colorimeter, and the centre setting adjustment possibly corrects for the effect of this yellow tinge on the colour developed by known steroids or unknown urinary extracts. As a further control of day to day alterations in the reagent itself, its dilution, and also the constancy of heating in the water bath, it seemed wise to always run through the colour development stage a known steroid simultaneously with unknowns. Only slight fluctuations were noted with rare exceptions.

Whatever be the reason, each batch of reducing reagent gave dif-

ferent standard curves with 21-hydroxy-pregnenolone solutions. Only two solutions of this known steroid were prepared, and these were compared on a certain batch of reducing reagent, so it was determined that the variations were due to the reagent batches themselves, and not solely to errors in making up the diluted standard solutions (see Fig. 2). The optical densities resulting from various concentrations of this steroid and each batch of reducing reagent were compared by averaging the results in each case for 100 gamma of steroid (see Table XII). A factor of comparison was then calculated, taking Batch E as the standard. It may be noted that the batches varied from +10% to -10% compared to E. These factors were used to estimate the reducing activity of each batch in terms of 11-dehydro-corticosterone (Compound A). Reagent E was used in a single standard curve with Compound A, but ~~the data in~~ the extreme right column of Table XII gives estimated optical densities for 100 gammas of Compound A with each batch of reagent.

TABLE XII.

COMPARISON OF VARIOUS BATCHES OF REAGENT BY THEIR REDUCING ACTIVITY WITH KNOWN DILUTIONS OF STEROIDS.

Reducing Reagent	Reaction with 21-OH Pregnenolone			Reaction with 11-dehydro-corticosterone	
	No. of Tests.	Optical Density for 100γ	Factor: $\frac{E}{\text{each batch}}$	Optical Density for 100γ	Calculated by Factor.
Batch C	12	.308	.916	not done	.532
Batch E	17	.282	1.000	.487	.487
Batch G	15	.274	1.029	not done	.473
Batch H	41	.315	.895	not done	.544
Batch I	24	.255	1.106	not done	.440

2. Contamination During Procedure:-

As the final reducing reaction is a non-specific one, the avoidance of all sources of contamination throughout the technique is essential. The glassware and rubber tubing should be scrupulously clean. Cleaning solution must be thoroughly removed. A final rinsing with distilled water was done by the writer himself almost invariably, even if a helper had already done so. The separating funnels were not usually put through cleaning solution between each extraction. Thorough cleaning in hot water (without soap) promptly after use, and repeated rinsing with distilled water just before reuse in recommended. Soap or soda were not used routinely except for storage flasks caked with urinary sediment. All apparatus used occasionally in the preparation of reagents must be thoroughly cleaned before use. The distillation apparatus, needed frequently for the solvent, should be reserved for this one technique. The benzene needed less often may conveniently be distilled elsewhere. There is danger of drops of water and particles of rubber being sucked into the Erlenmeyer during evaporation of the solvent. This may be due to careless closure of the water-tap before stop-cocks, but also unexpected changes in water pressure may occur, leading to back-pressure. Because of crowded facilities in the busy hospital laboratories it was not always possible to use the same sources of water-suction. The rubber tubing leading from the intervening trap to the Erlenmeyer is an occasional danger. This should be new tubing, recently cleaned, as previously directed. As the experimenter moved from room to room in search of available suction space, this rubber tubing was not always checked during these experiments, but if he were to repeat this work he would take care to overcome

this possible source of error. While blowing off the final ether solvent in the test-tubes with nitrogen there is some danger of dust from draughts falling into the exposed tubes, especially if near an open window.

These various sources of contamination of the apparatus and reagents with reducing substances may be checked by running distilled water blanks through the whole procedure including acidification, solvent extraction, alkali washing, etc. During the period in which the experimental data was collected (January - November, 1946) one dozen such water blanks were run. The eight blanks up to and including August read 97^{3} to 102 on the galvanometer scale, with an average of 99. This ruled out important sources of contamination. However, in the autumn (October 20th., 1946) the dispenser was no longer able to supply Merck's reagent ether for redistillation, and therefore Mallinkrodt's ether was used after redistillation. Four subsequent water blanks during the period gave colorimeter readings from 93 to 96^{1} (averaging 95). Therefore, an adjustment of reducing equivalent of 5 gamma of Compound A was necessary for all assays performed after October 20th. This is not a wholly satisfactory method of overcoming such a difficulty and it would be much better to have adequate supplies of suitable reagents constantly on hand.

3. Losses of the Solvent During Extraction:-

Some experience is necessary to overcome losses of the volatile solvents through the outlets during shaking the separating funnels or in turning the ungreased stop-cocks during extraction. Still more care is required when transferring the extract from the Erlenmeyer after evaporation into the test-tubes. During the preliminary work, when the micro-

test tubes advocated by Heard and Sobel (1, 2) were used, losses were encountered in decanting the solvent mixture. The macro tubes which fit the Evelyn colorimeter proved easier to handle. Finally, use was made of thin glass pipettes with rubber teats. This avodided losses of the material. Care must be taken not to allow the solvent to approach the rubber teat, and further rinsing of the pipette with a small amount of extra ether is performed. The same pipette was used for duplicate extracts, but a fresh pipette was always used for extracts of different urine collections.

4. Difficulties with Emulsions During Extractions:-

Emulsions tend to occur in certain urines, especially those that are highly concentrated, or those of an alkaline pH prior to acidification. Emulsion formed more often with 50 cc. of urine and the recommended total of 140 - 150 cc. of ether-chloroform solvent when a 250 cc. separatory funnel was used than when the larger 500 cc. funnel was employed. The latter size is accordingly recommended. Vigorous shaking should be avoided, especially at the first extraction. Slower, gentle shaking up to 200 times may be assumed to allow adequate extraction. The danger of emulsions is (a) that they may cause uncontrollable losses of time for part of the urine in the highly acid phase, and (b) that emulsion brought over with the solvent may carry non-steroid reducing substances which might go into solution and remain in the solvent during neutralization by the alkali washings. Talbot (6) uses centrifugation and Venning (7) uses salt to break the occasional emulsion. Both these were avoided here for fear of introducing extraneous contamination. The

addition of the extra 40 cc. of solvent to the pooled four extractions, followed by vigorous shaking, usually results in breaking the emulsion into two phases separated by a flocculent white emulsion of less than half an inch in diameter. Standing about ten minutes allows the emulsified layer to become smaller. The addition of a further 20 - 40 cc. of fresh solvent was sometimes used with success. Not more than a cc. or two of emulsion should be carried over into the alkali-washing stage and the line between the two phases should be quite clear by the third or fourth washing. This practise as recommended by Heard and Sobel (2, 34) was carried out. The impression is held, however, that it would be safer to discard all the emulsion before commencing washing with alkali. The loss of extract in the emulsified state would be minimal in most cases (if much emulsion occurs the extraction should be repeated on another sample). The error of carrying over considerable amounts of non-steroid reducing substances appears greater than that likely to occur from the loss of a little emulsified extract. Talbot (6) by using measured quantities of chloroform as a solvent, makes a correction for solvent loss in the discarded emulsion.

COMPARISON WITH THE METHODS OF HEARD AND SOBEL (1, 2).

Only minor alterations have been made in the original methods of Heard and Sobel (1, 2). Extraction method A is identical with the original extraction except that evaporation of the solvent under reduced pressure was conducted in a vessel containing water from the hot-water tap at not over 50° C. The internal temperature of the extract is obviously lower. Such heating results in no losses in biological activity (7, 9). The test-tubes to which the extract subsequently is transferred

during evaporation under nitrogen gas were also immersed in small volumes of warm water, thus speeding up the evaporation.

When using Merck's reagent grade ether, it was not found necessary to treat with concentrated sulphuric acid as recommended by Heard (2). This reagent ether did not separate into two layers with sulphuric acid. In any event, satisfactory water blanks were obtained using redistilled Merck's ether mixed with redistilled chloroform as the solvent. Possibly the sulphuric acid purification should have been employed when it became necessary to use Mallinkrodt's ether, but this was neglected. The extra expense of high grade ether is small compared with the unpleasant effort of mixing large volumes of ether with sulphuric acid, and during this process considerable heat is evolved.

The filter used in the photocolorimeter was 635$\mu\mu$ instead of the 650$\mu\mu$ filter used by Heard and Sobel. On the basis of a few comparisons, it is doubtful if this makes a significant difference in the results, provided the same filter is used for all assays.

11-dehydrocortiocsterone (Compound A) was chosen as the standard substance in place of desoxycorticosterone chosen by Heard et al (2). Theoretically, it is more logical to correlate the reducing substance assay method with the glycogenic bioassay method when an oxygenated corticosteroid is used for the standard in both methods. This statement is open to the objection that reducing power and biological activity are essentially different and therefore not directly comparable in terms of any pure steroid. In any event this modification in itself would not prevent comparison of the present data with that of Heard and Sobel (1, 2) as they supply comparative data on the reducing power on both the

steroids mentioned. Thus their data for urinary extracts admit of re-calculation in terms of Compound A.

The only modification of any importance is in the use of Evelyn macro test tubes in place of small test tubes during the heating with reducing reagent. The reasons for the change with the possible advantages and disadvantages, and some experimental data on the rate of colour development will now be considered in some detail. Decanting from an Erlenmeyer flask into the recommended micro test-tubes without loss of solvent proved tiresome and difficult. Further, the use of clips or clothes-pins to suspend such small tubes in the water bath led to certain initial difficulties, especially when six to ten tubes (including blanks, various concentrations of known steroids, etc.) are being heated concurrently. In early experience uneven heating of the micro tubes (possibly due to change in level of the boiling water) resulted in incomplete colour development. This experience prompted the trial of the Evelyn macro tubes, in which, the diluted reaction products are tested in the photo-electric-colorimeter. When transferring to such tubes from the Erlenmeyer flask care is taken never to exceed the 10 cc. line, even after washing down the sides of the tube with an ether spray. The transferred solvent is carefully delivered at the bottom of the tube with the aid of a capillary pipette. By such means it is felt that transfer is complete and that all the extract is at the bottom of the tube after evaporation, where it may react with the reducing reagent. Solutions of known steroids in acetic acid can be pipetted into the large tubes as advantageously. Because of the greater diameter of these tubes it was necessary to heat the extract or the known solutions in a total of 5 cc. reducing

mixture. This volume is made up to 10 cc. with reducing mixture after heating. This is quite different from the method of Heard and Sobel (1, 2), where only 2 cc. of reducing mixture are employed during heating, later diluted with a further 8 cc. after cooling. The large tubes rest upright on the bottom of the water bath during the sixty minutes heating period and the water level is higher than the internal level of the solution. Water in a similar tube was found to rapidly reach 98° C and to be maintained at this temperature throughout several experiments. It is felt that this method of heating the material with the reducing mixture is such that constant conditions can be maintained from day to day more easily than with the use of the small tubes suspended as recommended. This difference in technique is considered mainly responsible for the different optical densities obtained for known steroids heated according to the two methods. Thus 104 gamma of 11-dehydrocorticosterone resulted in an optical density of .598, and 100.5 gamma of 21-hydroxypregnenolone resulted in an optical density of .566 when heated for 60 minutes by the technique of Heard and Sobel (1, 34). With the technique of the writer comparable amounts of these steroids heated for sixty minutes resulted in optical densities of .505 in the case of dehydrocorticosterone, and only .284 for 21-hydroxy-pregnenolone. There were some differences observed in optical densities with various batches of reducing reagent as previously mentioned. The source (and possibly the preparation and melting point) of the 21-OH pregnenolone used by Heard and Sobel and by the writer was different. However, one may assume that the volumes of reagent heated and the methods of heating were the major factors in accounting for these differences in optical densities

observed. One may anticipate by suggesting that this difference in the colour developed by the standard steroids may account for the somewhat higher values for the reducing material in urinary extracts found in the present work than those reported by Heard and Sobel (2). In any event the differences in the technique of colour development prevent a direct comparison between the results of the two laboratories although they do not invalidate relative comparisons between normals and abnormals.

The effect of the duration of heating of the reducing reagent with 21-hydroxypregnenolone was studied on several occasions (see Table XIII). No significant differences were observed between heating times of 30 and 60 minutes. Two experiments showed considerable decrease in the optical density after 90 to 180 minutes of heating. This effect is different from that observed by Heard and Sobel (1, 34) for a wide number of steroids where increased heating up to three hours invariably led to greater optical densities. In Table XIII the first column gives average data prepared from Table III of Sobel's thesis (34). Whereas 60 minutes heating does not give maximal reaction with the dilutions and heating techniques of Heard and Sobel, it is apparent that by the writer's modification thirty to sixty minutes gives maximal density. Actually, thirty to forty minutes heating would appear preferable to the sixty minute period used in the experimental work of the present paper. The last column of Table XIII confirms another observation of Heard and Sobel (1) that water is not a satisfactory diluting fluid after heating the reaction mixture.

TABLE XIII.

THE EFFECT OF VARIOUS DURATIONS OF HEATING THE REACTION MIXTURE OF REDUCING REAGENT AND 21-HYDROXY PREGNENOLONE. COMPARISON WITH DATA BY THE TECHNIQUE OF SOBEL (34).

TIME OF HEATING MINUTES	100.5 gamma FROM SOBEL (34)	NO. 1 78 GAMMA REAGENT C	NO. 2 38 GAMMA REAGENT I	NO. 3 39 GAMMA REAGENT I	NO. 4 39 GAMMA REAGENT I (DILUTED BY WATER)
15	.40			-	-
20	-	.20	.09	-	-
30	.47	.24	.09	.08	.06
40	-	.25	.08	-	-
45	.53	-	-	-	-
60	.57	.25	.08	.08	.05
90	.61		.04	-	
120	.64		.04	.013	.00
180	.68			.00	

N.B. Data expressed as optical densities.

THE PROPER COLLECTION OF URINE.

In normal subjects, who may give careful attention to the collection of their own urine samples, there should be no difficulties from loss or contamination. Warning was given to avodi fecal contamination or paper tissue. Collections were not made during menstruation in any female subjects. Chemically clean collecting bottles were supplied directly to all normal subjects. The proper collection of urine from hospital patients is attended by several difficulties: (a) cleanliness of collecting bottles, (b) preservatives in bottles, (c) fecal contamination, especially likely in female bed patients, (d) loss due to negligence of orderly or nurse, if patient confined to bed, and (e) disinfectants in bed-pans. Only points (a) and (b) apply in the case of out-patients who usually can make complete 48-hour collections at their homes. Intelligent convalescent patients in hospitals are nearly as satisfactory, as they will notice and usually report if their own collecting bottle is inadvertently emptied by the maid or night orderly. Often collections were ordered by doctors in the local hospitals without first consulting the laboratory. Thus, routine ward collecting bottles sometimes have been used without previous cleaning, by the laboratory staff. Preservatives (as used in the collection of diabetic urine) may have not been washed out of these routine bottles. The writer thus sometimes reluctantly accepted urine specimens already collected without hir prior knowledge or supervision. In hospital bed-patients there are additional sources of error (c, d, e), which may occur even if the nurse-in-charge is warned personally and written orders are made, outlining all the necessary details. These errors are unlikely to occur on a special metabolic ward

which is highly trained, but most of the patients used for the present work were on general wards. During nights and week-ends, when the nurse-in-charge is absent, negligence and carelessness may occur unobserved. This is particularly so when the ward is busy, understaffed and only one complete urinary collection for research purposes is being made, while numerous routine morning samples are required from other patients. Fecal contamination, especially in females using bed-pans, cannot always be prevented, and unsupervised nurses may pour such contaminated urine into the total collection, rather than discarding the contaminated aliquot. The writer has recently learned that it is routine practice for nurses to rinse bed-pans with a little cresol to remove the odor of feces after washing the pans in hot water. The prevalence of this use of cresol was not previously appreciated by the writer who now suspects that it may account for several instances of high assays of reducing substances (see Table XIV). Cresol is also toxic to mice and interferes with accurate biological determinations in the glycogenic assay of Venning.

The following general observations made during the urinary extraction procedure suggest contamination by feces or cresol-like material. After the evaporation of the solvent there is always a uriniferous odor in the Erlenmeyer flask. This odor is still present in the test-tubes in extraction method A, in which benzene is not used. Such test-tubes usually show only a small amount of dry whitish material at their base. Fairly often, however, this has a definite brownish colour and a fecal odor, and in such cases rather high amounts of reducing substance were encountered. These brown discolorations also occurred in some urines

from normal subjects where the writer was assumred that no noticeable fecal contamination had taken place, so perhaps the brownish colour is sometimes due to substances of urinary origin. Rarely the solvent used to transfer the extract from the flask to the test-tube assumed a purplish colour, and the dried material was dark purple. The cause of the purple material was not ascertained, but might be foods (?beets) or medicaments (? phenol phthalein laxatives). These coloured substances in the dried residues which apparently led to falsely high assays of reducing substances prompted the writer to try the effect of benzene-water separation on the urinary extract (described previously as Extraction Method B). It was observed only occasionally that the benzene solution was actually coloured, but the rare purple colour was incompletely soluble in the benzene and slightly soluble in the water. The brownish colour was never present in the final dry residue of the test-tubes after method B. Indeed these test-tubes were quite odorless and contained scarcely visible amounts of nearly transparent residue. It was therefore felt that the benzene-water partition was an improvement in that it removed non-specific urinary reducing substances of fecal or other unknown origin. An experiment was therefore conducted to show the effect of fecal or cresol contamination on urines, extracted according to Methods A or B (see Table XIV). Two amounts of feces were added to 50 cc. samples of urine. In both instances the urine solution remained clear and the usual odor was unchanged, suggesting that considerable fecal contamination could occur without the laboratory worker noticing its presence by gross observation. When no benzene

was used (Method A) the dry residue in both cases (10.j and 10.k) was brownish and malodorous, especially when the larger fecal contamination was used. On the other hand, the dry residues were quite white and odorless after benzene-water separation (Method B). Determinations of the reducing substance by Method B showed they were unaltered from control values, but there was evidence of a slight increase with the larger fecal contamination (10.k) in the case of Method A. That small amounts of fecal contamination are not very important, even in Method A, is suggested by the data of 10.j, where the reducing substances equaled the control values. The data of 10.l shows that 0.5 cc cresol represent very large amounts of reducing substances, which would completely interfere with the proper interpretation of the results. Apparently 0.25 cc. cresol per 50 cc. urine (i.e. 5 cc. cresol per litre) would double the result of reducing substances by Method B, and obviously cause even greater interference with the original extraction Method A.

TABLE XIV.

EFFECT OF FECES AND OF CRESOL ON URINARY REDUCING SUBSTANCES.

NO.	TREATMENT	Reduc. Subst(Meth.A) gamma/50 cc.	Reduc.Subst (Meth.B) gamma/50 cc.
10. f	Control-untreated	121, 123	31
10. j	1 cc. pale fecal suspension added	121	30
10. k	1.4 cc. dark fecal suspension added	137	28
10. l	0.5 cc. cresol solution added	too dark to read	88

EFFECT OF VARIATIONS IN ACIDIFICATION PRIOR TO EXTRACTION.

The data in Table XV. is presented to illustrate two points: (i) the slight, though significant, effect of small increases in acidity beyond pH 1.0, and (ii) the considerable difference in reducing substances after extraction at pH 1.0 compared to pH 5.0. Except for some data in experiment I, all the other data was obtained with the original Method A. Experiments II and III were made after accidentally adding an excess of 12 N. H_2SO_4, resulting in pH values of 0.77 and 0.75. These excesses were in the order of ten drops and only occur after gross carelessness, in which case another sample of the urine specimen would

routinely be available for proper acidification. However, it is noted that such excess acidification caused an increase of only 10 to 20% in reducing substances, and indeed experiment I showed only a similar order of increase with acidity as great as pH 0.5. Thus it is concluded by the writer that an excess of one or even two drops of acid (which might occur more frequently accidentally, or result from minor errors in adjusting the Beekman pH-meter) would not cause significant differences in the observed reducing material.

The data of Table III in the paper of Heard et al (2) on the effect of hydrion concentration was previously mentioned in the introductory portion of this thesis (see also Table VIII here). They concluded that increases in acidification from neutral to pH 1.0 approximately quadrupled the observed urinary reducing substances. The technique of colour development used here is somewhat different from the original technique of Heard et al. Experiment I in Table XV., in which normal male urine collected without contamination was used, shows that acidification to pH 1.0 trebled the values obtained at pH 5.0, which is comparable to the data of Heard. However, further acidification to pH 0.5 resulted in less increase than Heard showed in his urine No. 2 (where pH 0.5 gave a value double that obtained at pH 1.0). It is apparent from experiment I in Table XV, that, with extraction Method B, more reducing substances are obtained after acidification at pH 1.0 than at pH 5.0, but here the further acidification to pH 0.5 gave no higher results than those at pH 1.0. This observation needs further confirmation, but nevertheless suggests that the reducing substances determined by extraction

Method B are less sensitive to variations in acidification. The nature of the additional reducing substances extractable from acidified rather than neutral urine has not been determined. If the excess reducing substances are not true corticosteroids, perhaps they are more soluble in benzene than in water and are thus removed in Method B. This might also be true if they were corticosteroids providing they were not of the highly oxygenated series.

Only one experiment was made to attempt to confirm the corticosteroid nature of the urinary reducing substance, and this was unfortunately only done using extraction Method A (see expt. IV, Table XV). Alkalinizing a urine sample to pH 10.0, then heating to 98°C for one hour, cooling, and acidifying to pH 1.0 before extraction gave results not significantly different from the control values. Alkali and heat have been previously shown to completely destroy the biological activity of corticosteroids. Either the inactivated material still retains its reducing power, or additional non-specific reducing substances have been formed by the procedure to compensate for the destruction of the original corticosteroids. This experiment may be compared with the effect of boiling with 15% HCl, which considerably decreased the reducing power according to Table IV of Heard et al (2). Their work, however, used urinary residue, dissolved in 50 ml. of 15% HCl, and refluxed, while the present writer used unextracted urine, which was alkalinized and heated. The writer's conclusion is that these indirect experiments do not afford definite proof whether or not all the extractable urinary reducing substances are corticosteroids.

TABLE XV

EFFECT OF VARIATIONS IN ACIDIFICATION OF URINARY EXTRACTS ON THE AMOUNT OF REDUCING SUBSTANCES ASSAYED. ALSO THE EFFECT OF HEAT AND ALKALI PRIOR TO EXTRACTION

NUMBER	TREATMENT	Aliquots Extracted in cc.	Reduc. Subst. (Method A) gamma/test	Reduc. Subst. (Method B) gamma/test
I. 10.h.	Acidified to pH 0.5	50	137	30, 31
10.f.	" to pH 1.0	50	121, 123	31
10.c.	" to pH 5.0	50	39, 40	12, 14
II. 11.c.	" to pH 0.77	25	55	not done
11.b.	" to pH 1.0	25	41	" "
III. 58.c.	" to pH 0.75	50	157	" "
58.a.	" to pH 0.92	50	134	" "
IV. 43.a.	" to pH 1.0	25	19, 20	" "
43.b.	Heated 60 min. at pH 10.0 Cooled. Acidified to pH 1.0	25	20, 23	" "

EFFECT OF STORING URINE PRIOR TO EXTRACTION.

Ideally all urine collections should be kept cool during collection, be refrigerated if necessary over-night or for 24 - hours or so, and should be extracted with a maximum delay of 36 to 48 hours after collection. This was almost universally done in the case of collections from the Montreal district. However, on several occasions samples were taken for re-check purposes two to ten days after collection and refrigeration and there was no evidence of significant alterations in reducing substances in these urines. However, some urines were accepted from Cleveland and from Baltimore, which entailed travel for one to two weeks without refrigeration. Five cc. chloroform per 100 cc. urine was used to reduce bacterial growth. All such specimens, as were so tested, were alkaline (approx. pH 9.0). One would suspect changes in corticosteroids and reducing power under such conditions. Actually the biological activity (when tested) varied from extremely high (in a case of Cushing's syndrome), to normal, and the reducing power likewise showed considerable variations. Therefore, the data on these transported urines will be presented but the delays in extraction will be noted appropriately in the text. The author would suggest the trial of toluol as a preservative in such cases, although this might interfere with the glycoganic bioassay if requested simultaneously. Toluol was used in urine No. 45.c. collected in Cleveland, and the results of both Methods A and B showed very high reducing substances in this case of Cushing's syndrome. Only one sample of normal urine was tested in a controlled experiment. One cc. of toluol in 50 cc. urine caused no significant differences in reducing substances by Method A or B when compared to untreated urine. In conclusion, however, one must state that

it would be safer to always extract urine within 24 - 36 hours of the completion of the collection, and that no preservatives should be used, unless the harmlessness of toluol is confirmed.

REPRODUCIBILITY OF RESULTS.

After allowing for apparent differences in the reactivity of different batches of reagents, it was found that repeated samples of 21-hydroxy-pregnenolone gave satisfactorily consistent results in the reduction reaction. As seen in Table XVI, only 4 poor checks (differences greater than 10%) were found out of 135 trials with this known steroid. However, with duplicate samples of urine extracted by Methods A or B, and then subjected to the reduction test less consistent results were found. Out of a total of 161 urine samples assayed in duplicate or triplicate, etc., 25 samples did not fall within the range ± 10% of the mean of the appropriate satisfactory results. When the difference between duplicates was greater than 10% both samples were counted as unsatisfactory. It is concluded that more errors occur in the extraction procedures than in the reduction reaction itself.

TABLE XVI

CONSISTENCY OF RESULTS WHEN TWO OR MORE SAMPLES TESTED.

	Known Steroids (Not extracted)	Unknown Urines (Extracted by Methods A or B)
Good (Differences < 5%	114	118
Fair (Diff. < 10% but > 5%	17	18
Poor (Diff. > 10%	4	25
TOTALS	135	161

SPECIFICITY OF THE PHOSPHOMOLYBDIC REDUCING REAGENT.

This subject has been considered in the earlier part of the thesis. Little additional data was obtained. On several occasions approximately 50 gamma of glucose was heated for 60 minutes with the reducing reagent, cooled and diluted as usual. Absolutely no colour change took place. When the phosphomolybdic solution(without acetic acid) is used for determining blood sugars a second solution containing copper is also used, which is apparently necessary for the reduction reaction with glucose. On the other hand small amounts of ascorbic acid crystals cause an intense colour with the present reagent. The data in Table XVIII, (see Nos. d, e, f) suggested that ascorbic acid does not pass into the final extract even by method A, for doses of 1.0 G. ascorbic acid daily to a normal male for 6 days did not cause any increase in urinary reducing substances.

The previous conclusion may be restated, that this reducing reagent is not specific and its suitability for the assay of urinary corticosteroids depends on the adequacy of the extraction-purification techniques in removing all non-specific urinary reducing substances before the extracted residue is subjected to the reduction reaction.

RECOVERY OF PURE STEROIDS FROM WATER OR URINE.

Satisfactory recoveries of desoxycorticosterone from water or urine are demonstrated in Table II of Heard et al (2). Using a comparable extraction technique (Method A) approximately quantitative recoveries of 21-hydroxy-pregnenolone from water, and of Compound A from urine were observed

(see Table XVII). In the case of the Compound A - solution used in these experiments it should be noted that this solution had been made up in alcohol and kept in the refrigerator for over six weeks. The retention of its original reducing power was not determined. Extraction Method B (which includes benzene-water partition) gave nearly negative results for 21-hydroxy-pregnenolone, and very poor but somewhat inconsistent results with Compound A. In support of these findings are the data of Talbot (6), showing that benzene-water partition resulted in all desoxycorticosterone and major portions of Compound A (11-dehydrocorticosterone) and corticosterone remaining in the rejected benzene fraction, while only 17-hydroxy compounds were quantitatively recovered from the water solutions. The present writer would therefore suggest that extraction Method A is satisfactory for all urinary steroids resembling the corticosteroid type, whereas method B extracts from urine only such oxy-corticosteroids as have the 17-hydroxyl group. Therefore, comparable results of urinary reducing substances would not be expected with the two methods of extraction. The relative advantages of greater or lesser specificity for highly oxygenated steroids are discussed elsewhere. It is again admitted that the writer's own experimental data on this subject is inadequate.

TABLE XVII.

RECOVERY EXPERIMENTS AFTER EXTRACTION OF PURE STEROIDS FROM WATER OR URINE.

	Reduc. Subst.(Method A) Gamma equiv. of Cpd. A	Reduc. Subst. (Method B) Gamma equiv. of Cpd. A
EXPT. (i) Aug. 13.		
38 gamma 21-OH pregnenelone; not extracted	20	20
No steroid. 50 cc water extracted	-	0
38 gamma 21-OH pregnenelone in 50 cc water	21	1
38 " " " "	27	1
No steroid - 100 cc. Urine No. 10.e. only	-	101
38 gamma 21-OH pregnenelone in 100 cc urine	-	108
EXPT. (ii) Aug. 30.		
19 gamma cpd. A in 50 cc. water	-	7
38 " " " " 50 cc "	-	6
38 " " " " 50 cc "	-	3
No steroid. 50 cc. Urine No. 10e only	192	51, 52
19 gamma cpd. A in urine	-	50
38 gamma cpd. A in urine	244	66

Notes (1) Approximately quantitative recoveries with Method A, but very poor recoveries with Method B.

(2) Cpd. A = 11-dehydro-corticosterone.

RECOVERY OF CORTICOSTEROIDS AFTER INTRAMUSCULAR INJECTION.

30 mg. of desoxycorticosterone acetate suspended in oil was injected intramuscularly for two successive days into a normal man (see Table XVIII, results i, j. k). Urine collections during the injections and on the two following days showed no significant alterations from control levels when assayed either biologically by the glycogenic method of Venning or by reducing activity after extraction Method A. This Method A should have extracted desoxycorticosterone from urine , but of course this steroid has little or no glycogenic biological activity. It has been shown that injected desoxycorticosterone appears in urine chiefly as pregnanediol. If as little as 3% of the 30 mg. injected appeared unchanged in urine one would expect that the resulting urinary reducing activity would have been increased by approximately 1 mg. daily, but this assumption cannot be corroborated from the results.(Table XIX) shows the effect of intramuscular injections of a preparation of 11-dehydrocorticosterone acetate in oil for two successive days into a debilitated patient. Unfortunately, only 20 mg. was available for each daily dose. Again, no significant alterations in urinary biological activity or reducing power were observed. This compound has a high glycogenic activity, and if present unchanged in urine, should have been extracted by the procedures used for both the bioassay and the chemical Method A. One may conclude either that dehydrocorticosterone is not excreted as such in amounts as high as 5% (which would be 1 mg. out of 20 mg.), or that the injection of this quantity of a corticosteroid suppressed the endogenous secretion to an

equivalent extent, so that the total biological and chemical activity of the 24 hour urine collection was unchanged. If such experiments could be repeated one might suggest the choice of a patient with adrenal insufficiency in whom the endogenous cortical secretion is at a minimum as determined by both methods of urinary assay.
(N.B. In 1946 Venning (29 f.) included these chemical assay results in a report on the effect of injected corticosteroids on the urinary bioassay values).

TABLE XVIII.

EFFECT ON URINARY CORTICOIDS OF TREATMENT WITH (1) ASCORBIC ACID BY MOUTH AND (2) DESOXYCORTICOSTERONE ACETATE INTRAMUSCULARLY IN A NORMAL SUBJECT (W. A.)

NO	DATE	TREATMENT	VOL. CC/DAY	CREATININE G/DAY	17-KETOS MG/ DAY	BIOASSAY GLYC.U/DAY	REDUC. SUBST.(Method A) Gamma/50 cc	MG/DAY	
4.a.	Apr. 11, 12	Control	1100	2.0	17.8	111	139, 156	3.2	2.8
b.	" 13, 14	Ambulatory	1000	1.9	14.5	71	140, 143	2.8	
c.	" 15, 16	Similar diet	1000	1.9	15.0	75	118, 124	2.4	
d.	" 17, 18	As above	1000	2.0	16.7	92	154, 159	3.1	2.8
e.	" 19, 20	plus 1.0 G. Ascorbic acid	1000	1.8	16.5	53	59 (25 cc) 139 (50 cc)	2.6	
f.	" 21, 22	daily, 6 days	960	1.7	15.8	82	60, 61, 65, 81, 86(all 25cc)	2.7	
g.	" 23, 24	Control	1000	1.8	19.2	Not done	52, 55 (25 cc)	2.1	2.6
h.	" 25	No Rx.	890	1.6	21.4	" "	82, 87 (25 cc)	3.0	
i.	" 26	DCA 30 mg.i.m.	1090	1.7	(16.4	(60	104 (50 cc)	2.3	2.7
j.	" 27	" " " "	1050(?)	2.2	(	(	74, 80 (25 cc)	3.2	
k.	" 28, 29	No Rx.	1015	1.8	17.0	80	58, 65 (25 cc)	2.4	

TABLE XIX.

EFFECT ON URINARY CORTICOSTEROIDS OF TREATMENT WITH 11-DEHYDRO CORTICOSTERONE ACETATE INTRAMUSCULARLY IN A DEBILITATED PATIENT (A.J)

NO	DATE	TREATMENT	VOL. CC/DAY	CREATI-NINE G/DAY	17-KETOS MG/DAY	BIOASSAY GLYC.U/DAY	REDUC. SUBST. (Method A) Gamma/50 cc	MG/DAY
26.a.	May 14	(Control.No Rx.	1315	1.1	10.5	(99	64, 64 (40 cc)	2.1
b.	" 15	(Diet = 2700 Cals.				(	76, 79 (50 cc)	2.4
		(Protein 162	1570	1.1	8.8			
c.	" 16	(Dehydro.Acetate	1530	1.0	10.2	(70	31, 45, 50(25 cc)	2.6
d.	" 17	(20 mg.i.m.daily	1462	0.8	9.1	(	43, 49(25 cc)	2.7
e.	" 18	Control. No Rx.	1440	1.0	9.2	(82	50, 52 (25 cc)	2.5
f.	" 19	" " "	1535	1.0	9.6	(	39, 43 (25 cc)	2.9

METHODS USED IN THE OTHER STUDIES ON THE URINE COLLECTIONS.

The experimental data to be presented includes the chemical assays for urinary corticosteroids, which were conducted by the author, and in addition other studies carried out by the members of the laboratory staff of the University Clinic, under the supervision of Dr. J. S. L. Browne and Dr. E. H. Venning. The glycogenic bioassays were performed by the method of Venning, Kazmin and Bell (7). 17-ketosteroids were performed by the method of Holtoroff and Koch (99) modified in that the time of colour development was increased to 105 minutes and a correction factor for non-steroidal chromagens was applied. Urine creatinine was determined by the method of Carphin -Birmingham modification of Folin's method (100)

EXPERIMENTAL DATA ON EXTRACTS OF URINE FROM NORMAL INDIVIDUALS AND SELECTED PATIENTS.

(a) Normal Individuals.

The results of the assays by the glycogenic bioassay method of Venning et al (7) and by the chemical methods described, together with certain other relevant data, are presented in tabular form in Tables XX - XXXXV inclusive. Summaries of the correlations between the clinical conditions and the respective methods of assay are shown in Tables XXXXVI and XXXXVII.

Tables XX to XXIII give data on numerous assays on ten normal young men, and Tables XXIV, XXVII, deal with the assays on ten normal women. Tables XXVIII, XXIX are concerned with assays on four elderly persons (apparently healthy) and on one pregnant woman. Including the latter, a total of twenty-five normal subjects were studied.

The 41 patients included one child (subject No. 66), but as no normal children were studied for comparison, the data in the child are only recorded at the foot of Tables XXXXII, XXXXIV, XXXXV, and are excluded in compiling the summary Tables XXXXVI, and XXXXVII. The 40 adult patients are divided into four groups on the basis of the urinary bioassay values. Thus, the group for which the bioassay values fell within a range of 21-99 glycogenic units per day, are shown in Tables XXX - XXXIII. Those with higher bioassay values are given in Tables XXXIV - XXXVII. Those with bioassay values lower than 20 g.u. per day in Tables XXXVIII - XXXXI. Finally, Tables XXXXII to XXXXV cover those patients on whom bioassays

were not performed, or if done, repeated bioassays gave widely varied results. The data in all these Tables will be considered in detail. Short summaries of the clinical findings on the patients appear in the appendix. It should be noted that each subject has been given an individual number and that letters are used to distinguish separate urine collections when several were obtained from one subject.

Table XX shows 20 urinary assays by chemical Method A on ten normal men, varying in age from 20 - 38 years. These men were laboratory workers or hospital physicians performing their regular duties and not on controlled diets. Height, weight, daily volume of urine, creatinine and 17-ketosteroids are also shown. It may be said that urinary specific gravity was also measured in all cases, but this only reflected the urinary volume and was not correlated with corticosteroid assay values. The pH of most samples was also studied before acidification but was universally between 5.0 and 7.0 in normal subjects of both sexes. Urinary reducing substances in all cases are shown in equivalent weights of Compound A. The actual values for each sample assayed are given, and the urine aliquots assayed were 50 cc. except as noted. If less than 50 cc. of urine was taken it was diluted to a total of 50 cc. by distilled water. When more than one sample was assayed from a single urinary collection the consistency of the results may be seen (such data has been previously summarized in Table XVI). For example both 1a and 1b show good checks whereas 4a is an example of a poor check. Therefore, the figure of 3.2 mg. per day shown for assay 4a is open to doubt, and about the same degree of doubt exists as to the exact value in assays 5 and 6.

Assays 10.d. show quite variable results when different volumes of urine were taken. Thus 122 gamma were found in 50 cc. only 49 gamma in 25 cc. and as much as 41 gamma in 12.5 cc. Presumably the latter assay suffered from contamination. No constant relationship was found in other cases where different volumes of urine were studied, and the extraction appears adequate for up to 100 cc. urine by the usual amount of solvents. However, a trial with 25 cc. quantities on numerous occasions (see later) showed no advantages and the impression is gained that assays on the larger quantities are more reliable. The only daily result which deserves comment is the highest one, i.e. 4.7 mg. on subject No. 7. This doctor felt poorly during the collection and developed a fever with coryza and generalized aches shortly thereafter. This result should therefore probably be excluded from comparison with the other normals, although it is interesting that the bioassay was only 37, which is in the normal range. Table XXI presents data on the same group of normal men when assayed by extraction Method B. Good duplicate checks were obtained in all these cases with exception of an apparently low value for a 25 cc. sample of urine 10.e. compared to values for 50 cc. and 100 cc. Results for 10.e. can be compared with those of 10.d. on Table XXI, and it is seen that the volume assayed does not in itself have a consistent effect. Once again it may be noted that the value on subject No. 7, is the highest obtained, and this result is accordingly disregarded in computing the average. The average result by Method B is 0.9 mg. equivalent per day compared to 2.8 mg. per day by Method A. Whether this difference is due to non-specific reducing substances extracted in Method A, or to benzene-soluble corticosteroids lost in Method B, has been discussed previously.

Table XXIII sets out the most important data so that the results of chemical assay and the glycogenic bioassay can be conveniently compared. With the exception of Assay 4.a. the bioassays fell within a normal range. This subject tends to have rather high values (see also Table XVIII, and also personal observations of Dr. Venning made in 1945). The twelve bioassays shown averaged 62 glycogenic units per day which may be compared with an average figure of 60 g.u. reported by Venning et al (35), for 14 normal men. Table XXIII was prepared to see whether more consistent results would be obtained if results for all assays were expressed per gram of creatinine excreted rather than in daily amounts. Only the assays on which satisfactory bioassay results are available are included. Columns A, B and C are obtained from the data of Table XXII after appropriate division by the figure for creatinine in grams. However, no significant improvement in the extents of the various ranges was observed. The two right hand columns give ratios showing the correlation between the results of each of the methods of chemical assay and the bioassay. As bioassay glycogen units are expressed in terms of Compound E which is approximately three times as active as Compound A, (Venning 7)) it was decided to divide the chemical assays (expressed as Compound A) by three times the bioassay values. This decision is really arbitrary and little is gained in practice, as the ratios would be equally expressive if the usual glycogen units had been employed as divisors. Excluding Assay No. 7, in which the values for chemical assay are considered abnormally high, it may be seen that the chemical assay with Method A gives values 9 to 33 times as high as the bioassay when both are considered in weights of Compound A. The smaller values obtained by Method B are only 4 to 7 times as high as the biological values.

TABLE XX

NORMAL MEN: DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD A

No.	Initials	Date	Age	Ht. Inches	Wt. lbs.	Volume cc./day	Creatinine G./day	17-Ketos. mg./day	Reduc.Subst.(a) Gamma per 50 cc.	Reduc.Subst.(A) mg./day
1a	I.M.S.	Feb.15,16	20	68	155	1025	1.7	25.8	86, 89	1.8
1b	I.M.S.	Mar.19,20	20	68	155	1192	1.2	14.6	81, 84	2.0
2	R.B.	Feb.15,16	35	70	160	1368	1.7	23.0	58, 63	1.7
3a	K.R.M.	Feb.19,20	34	67	170	1868	1.8	22.8	63, 66	2.4
3b	K.R.M.	Oct.30,31	34	67	170	1450	1.7	18.4	139	4.0
4a	W.A.	Apr.11,12	28	72	155	1100	2.0	17.8	139, 156	3.2
4b	W.A.	Apr.13,14	28	72	155	1000	1.9	14.5	140, 143	2.8
4c	W.A.	Apr.15,16	28	72	155	1000	1.9	15.0	118, 124	2.4
5	L.J.	June 5,6	30	72	200	1428	2.0	16.8	84, 94, 117	2.8
6	R.D.H.	June 26,27	37	68	108	2840	1.4	17.3	54, 65	3.4
7	E.R.	Oct.14,15	38	74	172	2535	1.6	18.5	93	4.7
8	J.S.	Oct.15,16	24	68	130	2105	1.3	22.3	41	1.7
9	A.D.	Oct.19,20	30	70	155	1300	1.6	24.0	136	3.5
10a	R.P.H.	Feb.4	33	75	195	915	1.8	-	116,124,126	2.2
10b	R.P.H.	Feb.28,1	33	75	195	1250	1.8	21.5	81	2.0
10c	R.P.H.	May 13,14	33	75	195	765	1.2	-	77 (25 cc.)	2.4
10d	R.P.H.	June 3,4	33	75	195	1330	2.1	19.0	122 (50 cc.)	(
10d	R.P.H.	June 3,4	33	75	195	1330	2.1	19.0	49 (25 cc.)	(3.2
10d	R.P.H.	June 3,4	33	75	195	1330	2.1	19.0	41 (12.5 cc.)	(
10e	R.P.H.	Aug.6,7	33	75	195	830	1.9	19.5	192	3.2
10f	R.P.H.	Nov.4	33	75	195	1640	1.8	17.4	121, 123	4.0
10g	R.P.H.	Oct.5,6	33	75	195	1538	2.0	16.3	130	4.0
	A v e r a g e (excluding #7)						1.7	19.1		2.8

TABLE XXI

NORMAL MEN: DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD B

No.	Initials	Date	Volume cc/day	Creatinine G./day	Reduc.Subst.(Method B) gamma per 50 cc.	Reduc.Subst.(Method B) mg./day
3b	K.R.M.	Oct.30,31	1450	1.7	34, 34	1.0
6	R.D.L.	Jun.26,27	2840	1.4	16, 17	0.9
7	E.R.	Oct.14,15	2535	1.6	60 (100 cc.)	1.5
8	J.S.	Oct.15,16	2105	1.3	15, 15	0.6
9	A.D.	Oct.19,20	1300	1.6	40, 44	1.1
10e	R.P.H.	Aug.6,7	830	1.9	45, 51, 52	0.8
10e	R.P.H.	Aug.6,7	830	1.9	101 (100 cc.), 18 (25 cc.)	0.8
10f	R.P.H.	Nov.4	1640	1.8	31	1.0
10g	R.P.H.	Oct.5,6	1538	2.0	27, 27	0.8
	A v e r a g e (excluding #7)					0.9

TABLE XXII

NORMAL MEN: COMPARISON OF THE RESULTS BY THE CHEMICAL METHODS AND BIOASSAY

No.	Initials	Volume cc./day	Creatinine G./day	Reduc.Subst.(Method A) mg./day (=mg. Cpd. A)	Reduc.Subst.(Method B) mg./day (=mg. Cpd. A)	Bioassay Glyc.Units/day (= gamma Cpd. E)
1a	I.M.S.	1025	1.7	1.8	-	51
1b	I.M.S.	1192	1.2	2.0	-	43
2	R.B.	1368	1.7	1.7	-	36
3a	K.R.M.	1868	1.8	2.4	-	52
3b	K.R.M.	1450	1.7	4.0	1.0	92
4a	W.A.	1100	2.0	3.2	-	111
4b	W.A.	1000	1.9	2.8	-	71
4c	W.A.	1000	1.9	2.4	-	75
5	L.J.	1428	2.0	2.8	-	Unsatisfactory
6	R.D.H.	2840	1.4	3.4	0.9	Unsatisfactory
7	E.R.	2535	1.6	4.7	1.5	37
8	J.S.	2105	1.3	1.7	0.6	60
9	A.D.	1300	1.6	3.5	1.1	56
10a	R.P.H.	915	1.8	2.2	-	Not done
10b	R.P.H.	1250	1.8	2.0	-	57
10c	R.P.H.	765	1.2	2.4	-	Not done
10d	R.P.H.	1330	2.1	3.2	-	Not done
10e	R.P.H.	830	1.9	3.2	0.8	Not done
10f	R.P.H.	1640	1.8	4.0	1.0	Not done
10g	R.P.H.	1538	2.0	4.0	0.8	40
R A N G E			1.2-2.1	1.7-4.7	0.6-1.5	36-111
#7 Excluded - Range			1.2-2.1	1.7-4.0	0.6-1.1	36-111
" - Average			1.7(19)	2.8(19)	0.9(7)	62(12)

N.B. Figures in parentheses represent numbers of results averaged.

TABLE XXIII

NORMAL MEN: ASSAY RESULTS EXPRESSED PER GRAM CREATININE

No.	Initials	A Red.Subst.(Meth.A) mg. Cpd. A /G. Creatinine	B Red.Subst.(Meth.B) mg. Cpd. A /G. Creatinine	C Bioassay gamma equiv. of Cpd. E /G. Creatinine	Ratio $\frac{A}{C \times 3}$	Ratio $\frac{B}{C \times 3}$
1a	I.M.S.	1.1	-	30	12	-
1b	I.M.S.	1.7	-	36	16	-
2	R.B.	1.0	-	21	16	-
3a	K.R.M.	1.3	-	29	15	-
3b	K.R.M.	2.4	0.6	54	15	4
4a	W.A.	1.6	-	55	10	-
4b	W.A.	1.5	-	37	14	-
4c	W.A.	1.3	-	39	11	-
7	E.R.	2.9	0.9	23	42	13
8	J.S.	1.3	0.5	46	9	4
9	A.D.	2.2	0.7	35	21	7
10b	R.P.H.	1.1	-	32	11	-
10g	R.P.H.	2.0	0.4	20	33	7
Range		1.0-2.9	0.4-0.9	20-55	9-42	4-13
#7 Excluded		1.0-2.4	0.4-0.7	20-55	9-33	4-7
" Average		1.5(12)	0.55(4)	36(12)	15(12)	5(4)

N.B. Figures in parentheses represent numbers of results averaged.

Tables XXIV and XXV give the detailed data by the chemical assays on ten normal young women, arranged similarly to Tables XX and XXI, for men. The checks between duplicate assays done by Method A were satisfactory except for a rather wide variation in assay No. 18, and again inconsistent values were found when different volumes were taken in assay No. 16. In Table XXV it is seen that one exceptionally low value of 6 gamma per 50 cc. was found in assay No. 15.b. which is omitted from the calculations as it is considered in error. The result of 3.4 mg. reducing substance by Method A for assay No. 19, is unexplainably higher than all the other results, and is accordingly omitted when computing the averages. The results for the women average somewhat lower than those for the men, and this relationship is best seen at the foot of Table XXVI. The latter Table is similar to Table XXII, for the men, summarizing the results by the chemical procedures and the bioassay. Thus, by Method A the average for 11 assays on women was 2.0 mg. per day, compared to a figure of 2.8 mg. for 19 assays on men. Five assays by Method B averaged 0.6 mg. per day for women, while seven assays on men averaged 0.9 mg. Finally, ten bioassay values for women averaged 45 g. u. compared to the average of 62 g.u. for twelve bioassays on men.

Table XXVII, gives the data for women in a similar manner to Table XXII for men. The most interesting finding is that when all the urinary assays are expressed in relation to 1.0 G. of creatinine the averages for women in each case are almost identical to the averages for men. Even the bioassays average 36 g.u. per gram creatinine in both sexes. This strongly suggests that the apparent sex difference in the daily excretion of urinary corticoids is due to difference in total muscle mass (as related to creatinine excretion) rather than to some inherent sex difference.

TABLE XXIV

NORMAL WOMEN: DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD A

No.	Initials	Date	Age	Ht. Inches	Wt. lbs.	Day of Cycle	Volume cc./day	Creatinine G./day	17-Ketos. mg./day	Reduc.Subst.(A) gamma/50 cc.	Reduc.Subst.(A) mg./day
13a	M.K.	May 9,10	23	68	155	8	1015	1.3	13.2	85, 91	1.8
13b	M.K.	Oct.21,22	23	68	155	12	1485	1.2	12.3	73	2.2
14	R.F.	Apr.9,10	22	65	132	22	1115	1.2	17.1	89, 94	1.9
15a	Y.B.	Apr.10,11	22	62	130	25	1000	1.1	14.5	94, 99	1.9
15b	Y.B.	Oct.8,9	22	62	130	15	1228	1.1	9.2	32	0.8
16	Y.C.	Apr.18,19	30	64	140	48p.p.	1000	1.1	10.3	116,43(25 cc)	2.1
17	D.K.	May7,8	27	70	160	18	2240	1.6	15.9	58, 62	2.7
18	R.K.	May 13,14	27	71	150	9	1440	1.4	23.3	88, 99	2.7
19	L.S.	June 11,12	27	68	130	15	1502	1.2	14.7	114	3.4
20	M.H.	Oct. 9,10	36	66	130	11	1428	1.0	15.1	51	1.5
21	L.D.	Oct. 16,17	24	67	134	19	1550	1.7	9.0	64	2.0
22	E.B.	Oct.28,29	22	66	140	15	2080	1.2	11.2	42	1.8
	A v e r a g e (excluding #19)							1.3	13.8		2.0

TABLE XXV

NORMAL WOMEN: DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD B

No.	Initials	Date	Age	Ht. Inches	Wt. lbs.	Day of Cycle	Volume cc./day	Creatinine G./day	17-Ketos. mg./day	Reduc.Subst.(B) gamma/50 cc.	Reduc.Subst.B mg./day
13b	M.R.	Oct.21,22	23	68	155	12	1485	1.2	12.3	22	0.6
15b	Y.B.	Oct.8,9	22	62	130	15	1228	1.1	9.2	17(omit 6)	0.4
20	M.H.	Oct.9,10	36	66	130	11	1428	1.0	15.1	15, 20	0.5
21	H.D.	Oct.16,17	24	67	134	19	1550	1.7	9.0	24, 27	0.8
22	E.B.	Oct.28,29	22	66	140	15	2080	1.2	11.2	13, 15	0.6
		A V E R A G E									0.6

TABLE XXVI

NORMAL WOMEN: COMPARISON OF THE RESULTS BY THE CHEMICAL METHODS AND BIOASSAY

No.	Initials	Volume cc./day	Creatinine G./day	Reduc.Subst.(A) mg./day (=mg. Cpd.A)	Reduc.Subst.(B) mg./day (=mg. Cpd.A)	Bioassay Glyc.Units/day (=gamma Cpd.B)
13a	M.A.	1015	1.3	1.8	-	Unsatisfactory
13b	M.A.	1485	1.2	2.2	0.6	40
14	A.F.	1115	1.2	1.9	-	39
15a	Y.B.	1000	1.1	1.9	-	47
15b	Y.B.	1228	1.1	0.8	0.4	50
16	Y.C.	1000	1.1	2.1	-	42
17	D.K.	2240	1.6	2.7	-	26
18	A.K.	1440	1.4	2.7	-	47
19	H.S.	1502	1.2	3.4	-	59
20	M.H.	1428	1.0	1.5	0.5	24
21	E.D.	1550	1.7	2.0	0.8	65
22	E.D.	2080	1.2	1.8	0.6	71
R A N G E			1.0-1.7	0.8-3.4	0.4-0.8	24-71
#19 Excluded - Range			1.0-1.7	0.8-2.7	0.4-0.8	24-71
" - Average			1.3(11)	2.0(11)	0.6(5)	45(10)
Men - Average			1.7(19)	2.8(19)	0.9(7)	62(12)
Men & Women - Average			1.5(30)	2.5(30)	0.75(12)	54(22)

1. Figures in parentheses represent numbers of results averaged.

2. Venning et al[35] found 14 normal women to average 41 glycogenic units and 14 normal men to average 60 glycogenic units.

TABLE XXVII

NORMAL WOMEN: ASSAY RESULTS EXPRESSED PER GRAM CREATININE

No.	Initials	A Red.Subst.(Meth.A) mg. Cpd. A /G. Creatinine	B Red.Subst.(Meth.B) mg. Cpd. A /G. Creatinine	C Bioassay Gamma Cpd. E /G. Creatinine	Ratio A C x 3	Ratio B C x 3
13b	M.K.	1.8	0.5	33	18	5
14	R.F.	1.6	-	32	17	-
15a	Y.B.	1.7	-	43	13	-
15b	Y.B.	0.7	0.4	46	5	3
16	Y.C.	1.9	-	38	17	-
17	D.K.	1.7	-	16	35	-
18	R.K.	1.9	-	34	19	-
19	H.S.	2.8	-	49	19	-
20	M.H.	1.5	0.5	24	21	7
21	H.D.	1.2	0.5	38	11	4
22	E.B.	1.5	0.5	59	8	3
Range		0.7-2.8	0.4-0.5	16-59	5-35	3-7
#19 Excluded		0.7-1.9	0.4-0.5	16-59	5-35	3-7
Diff. Average		1.5(10)	0.5(5)	36(10)	16(10)	4.5(5)
Men - Average		1.5(12)	0.55(4)	36(12)	15(12)	5(4)
Men & Women "		1.5(22)	0.5(9)	36(22)	16(22)	5(9)

The ranges for the ratios of reducing substances to glycogenic activity are likewise almost the same for women as for men.

Table XXVIII, shows the data on two elderly men and two post-menopausal women, all four living at home and in their usual health. The only clinical fact of note is that assays No. 24a and 24b were done at a time when this individual was suffering from a succession of acute emotional shocks which may have caused the elevated bioassay value of 112 g.u. and likewise effected the chemical assays. Subject No. 24, also suffers from diabetes. No known clinical facts are available to explain the higher chemical assay for 11.b. than for 11.a., but collection 11.b. was refrigerated for a week before this assay was begun. On the whole, the few assays for the elderly men and women are somewhat lower than those for younger individuals, but again, this may be partially explained by low creatinine excretions (see also Table XXIX, where the results are expressed per gram creatinine).

Four urine collections from the third trimester of a case of normal pregnancy are also shown in Tables XXVIII and XXIX. The urinary pH prior to acidification was noted to be 7.5 in No. 65.c. and 6.8 in No. 65.d).

It may be noted that urinary corticoids determined by the chemical assay (Method A) or by the bioassay are approximately twice normal, and indeed, when expressed per gram creatinine excreted, are approximately three times normal. That this effect is due to the pregnancy and not to some inherent abnormality in this subject may be found by comparing these values with assay No. 20, in Tables XXVI to XXVII, for the latter values were obtained

six months after the delivery of a normal male child. Duplicate checks with the chemical extraction Method A, in all assays shown in Table XVIII, are satisfactory with the exception of those on No. 24.b. Unfortunately, none of these urines were studied by chemical method B. This might be particularly interesting in pregnancy. In the case of keto-steroids it has been shown by Venning (53) that the high results by the Zimmerman reaction in pregnancy (Note 26.6 mg. for 65.d. here) are due to keto-steroids other than the 17-ketosteroids. The reducing reagent has been shown by Heard et al (1) to react with conjugated 3-ketosteroids. Such compounds might be present in unusually high quantities after extraction Method A, and yet might not be present in the final residue after benzene-water partition such as in Method B.

TABLE XXVIII

ELDERLY SUBJECTS AND A CASE OF PREGNANCY: DATA RELEVANT TO THE CHEMICAL METHOD A AND THE BIOASSAY

No.	Initials	Date	Age	Ht. Inch.	Wt. lbs.	Day of Cycle	Volume cc/day	Creatinine G/day	17-Ketos. mg./day	Reduc.Subst.(Method A) Gamma/50 cc.	Mg./day	Bioassay Glyc.U/day
A. Elderly Men												
11a	W.W.	Feb.18,19	81	68	160	-	1000	1.2	10.6	47,52	1.0	Low? 20
11b	W.W.	Mar.10,11	81	68	160	-	1000	1.1	8.7	41(25 cc.)	1.6	19
12	S.	Feb.20,21	77	72	150	-	1000	1.1	10.0	43,45	0.9	37
Average elderly men								1.1	9.8	-	1.2	25
Average normal younger men								1.7	19.1	-	2.8	62
B. Elderly Women												
23	L.S.	Feb.20,21	74	60	155	-	1000	0.7	8.2	49,51	1.0	26
24a	B.R.	Feb.27,28	55	65	156	-	1525	1.1	14.2	35,37	1.1	112 (?)
24b	B.R.	Mar.19	55	65	156	-	1030	1.1	8.2	69,77	1.5	Not done
Average elderly women								1.0	10.2	-	1.2	?
C. Case of Normal Pregnancy (third trimester)												
65a	M.H.	Feb.5,6	36	66	150	214	1605	1.0	-	93,93	3.0	131
65b	M.H.	Feb.9,10	36	66	150	218	1342	1.1	-	142,146	3.9	Not done
65c	M.H.	Feb.23,24	36	66	150	232	1800	1.2	-	72,73	2.6	Not done
65d	M.H.	Mar.30,31	36	66	150	267	1275	1.0	26.6	215,217	5.5	115
Average 4 samples from pregnancy								1.1	-	-	3.7	123
Average Normal Young Women								1.3	13.8	-	2.0	45

TABLE XXIX.

ELDERLY SUBJECTS AND A CASE OF PREGNANCY. ASSAY RESULTS EXPRESSED PER GRAM CREATININE.

NO.	INITIALS	DAY OF CYCLE	A RED. SUBST.(Meth.A) mg. Cpd. A / G. creatinine	C BIOASSAY Gamma Cpd. E /G. creatinine	RATIO A / C x 3
A. ELDERLY MEN					
11.a	W.W.	-	0.8	17	16
b	W.W.	-	1.5	17	29
12	S.	-	0.8	34	8
Average Younger Men			1.5	36	15
B. ELDERLY WOMEN					
23	L.S.	-	1.4	37	13
24.a.	B.R.	-	1.0	100?	3?
24.b.	B.R.	-	1.4	Not done	-
C. CASE OF NORMAL PREGNANCY (third trimester)					
65.a.	M.H.	214	3.0	131	8
65.b.	M.H.	218	3.5	Not done	-
65.c.	M.H.	232	2.2	Not done	-
65.d.	M.H.	267	5.5	115	16
Average this pregnancy			4.7	123	12
Average normal women			1.5	36	16

(b) Data on Patients.

Data on the various groups of patients will now be considered. The previous discussion as to the difficulties in assuring complete and uncontaminated urine collections on hospital patients should be borne in mind. For these reasons the relationship of the chemical assay values to the biological assays is probably more significant than that between chemical values and clinical diagnosis. However, contamination by such reducing substanees as feces or cresol might not affect the bioassay as much as the chemical assays. Attention will be drawn to any apparent evidence of such contamination. Asterisks before assay numbers distinguish those assays which are regarded as definite instances of incomplete collections, based usually on low creatinine values. The figures for urine volume in cc. per day are misleading in this regard for all collections of less than two litres for 48 hours were made up to that amount by adding distilled water. For example collection No. 39a. was only actually 800 cc. urine in 48 hours and No. 40.a. measured only 1130 cc. in 48 hours (i.e. 565 cc. per 24 hours).

Tables XXX - XXXIII show data on those patients on whom bioassay values greater than 20 g.u. per day but less than 100 g. u. per day were recorded. Table XXX shows details relevant to the chemical assay Method A on 5 assays for 4 such males, and on 16 assays from 14 females. Duplicate values were satisfactory except in assays No. 39.b., 40, 42.b., 41 and also in at least one of the multiple values obtained in No. 44 and 42.a. Most of those listed as unsatisfactory were obtained when only 25 cc. urine was taken for assay. Table XXXI shows the results of assays by chemical method B on urine from 3 men and 8 women. With the exception of the urine of two of the men, the same

collections had been also assayed by chemical Method A. Duplicate checks are not available for the results by Method B with a single exception. Creatinine determinations are missing for assays No. 28, 29, and 30.

The urinary assay results shown in Tables XXX and XXXI are condensed in Table XXXII. The exceptionally high chemical assay value for No. 28, is included in the Table, in order to point out certain difficulties in assaying such urine collections. The urine was collected in the Notre Dame Hospital, and had been stored in the refrigerator for one week before the chemical assay was attempted (the bioassay extraction had been done promptly). Due to summer vacations, this extraction was performed by a competent technician according to the author's instructions. However, considerable tenacious emulsion developed, and it is extremely doubtful if adequate sodium hydroxide washing is possible in such cases. The residue after evaporation was a purple-brown syrup. Attempts to dissolve the discoloration in benzene were not completely successful. The coloured syrupy material undoubtedly accounts for colorimeter values of less than one division after subjecting the residue to the reducing reagent. The inferences drawn are that urine samples in which severe emulsification occurs should be discarded, but that this difficulty might not have occurred if the specimen had been promptly extracted. The exact nature of the reddish-brown reducing contaminant is not known but proper extraction would probably have prevented its appearance in the final residue. (N.B. The tecnician made the extraction by Method A on a total of 5 assays reported).

Table XXXII, also shows chemical assays by Method A which are higher than the range for normal women in the cases of No. 35, 39.b. 40 and 44. Urines in these cases were assayed promptly. Patients No. 35, and No. 40, were bed patients on general wards of the Royal Victoria Hospital, while No. 39, and 44 were clinic out-patients. The latter usually gather complete collections, but the vessels used to obtain the voidings (prior to transfer to the clean collecting bottles) may have been contaminated. This is suggested for patient No. 39, where the high assay b. was found on urine at a pH of 8.3 prior to acidification while the lower chemical value on No. 39.a. was found on urine at a pH 6.2. There was no history of cystitis in this young girl, so the highly alkaline urine was probably due to contamination. Urines No. 35 and 44 were normally acid prior to collection, while this was not measured in No. 40. Except for the unsupported suggestion that one or all of urines No. 35, 40 and 44 may have been contaminated during collection, no other reason can be offered for these high chemical assay values. Two of these samples under discussion (No. 35 and No. 39.b.) were also assayed by chemical Method B, and found to be 1.8 mg. and 1.2 mg. respectively. These are also higher than the range of 0.4 to 0.8 mg. found by this method on normal women. The value of 0.4 mg. by Method B. for No. 29, which was collected in Cleveland from a man with apparently arrested Cushing's Syndrome is somewhat lower than average. This patient, however, had had the whole left adrenal and part of his right adrenal removed in 1943, as well as courses of deep x-ray therapy to his pituitary region. Thus, the normal bioassay value and the lower than normal chemical assay value may be explainable in this case.

Table XXXIII, shows the various assay values from the same group of patients, when calculated per gram of creatinine excreted (where the latter is known. Higher than average values occur in the same instances as discussed above and thus require no further elaboration.

PATIENTS ON WHOM THE BIOASSAY VALUES RANGED BETWEEN 21 AND 99 G.U. PER DAY

DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD A.

NO.	INITIALS	DATE	DIAGNOSIS	pH	VOL. cc/DAY	CREATININE G/DAY	17-KETOS MG/DAY	REDUC. SUBST.(Method A) Gamma/50 cc	mg/day
a. Male patients									
25	J.I.	Apr.30 May 1	Hypogonadism	6.3	1605	1.2	6.7	86, 89	2.8
26.a.	A.J.	May 14	Bronchiectasis	6.1	1315	1.1	10.5	64, 64 (40 cc)	2.1
b.	A.J.	" 15	"	6.4	1570	1.1	8.8	76, 79	2.4
27	M.J.	Nov.1,2,3,	Hypopituitarism (treated)	6.2	1233	1.4	10.2	68	1.7
28	E.B.	Jun.23-25	Disseminated Sclerosis	-	986	-	-	Too dark to read	very high?
Average Normal Men						1.7	19.1		2.8
b. Female patients.									
31	A.L.	Apr.10,11	Hirsutism, (simple)	6.4	1018	1.2	20.5	120, 127	2.5
32	F.H.	Jun.19,20	Hypothyroid Hirsutism	6.4	1000	1.0	16.5	109	2.2
33	C.G.	Jun.22-24	Question Addison's	6.4	1573	0.7	5.5	43, 45	1.4
34	W.B.	Aug.27-29	Fatiguability	6.3	1090	0.9	13.6	83	1.8
35	L.	Sep.22-23	Anorexia Nervosa	6.8	2225	1.0	18.0	107	4.8
36	W.	Oct.1-2	Scleroderma	5.5	1390	1.2	8.1	58	1.6
37	M.	" 7, 8	Luteoma, Virilizing	7.0	1500	1.1	12.5	35	1.0
38	D.V.	Oct.18-20	Acanthosis nigricans	7.3	1493	1.0	8.4	55	1.6

TABLE XXX (CONTINUED)

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/DAY	CREATI-NINE G/DAY	17-KETOS MG/DAY	REDUC.SUBST (Method A) Gamma/50 cc	mg/day
*39a	E.N.	Jun.13,14	Precocious puberty	6.2	1000	0.7	4.8	33, 37 (25 cc)	1.4
39b	E.N.	" 23,24	"	8.3	1240	1.0	9.1	172, 194	4.5
*40	A.Ge.	Mar.28,29	Loss body hair Hyperthyroid	-	1000	0.6	5.1	157, 206	3.6
41	N.S.	Apr.28,29	Hypertension	5.3	1688	1.3	12.0	31, 35 (25 cc)	2.2
42a	L.F.	May.19,20	"	6.3	1795	1.2	19.3	24,30,36,37 (25 cc)	2.3
42b	L.F.	Jun.6, 7	"	6.3	1460	1.2	17.2	41, 53 (25 cc)	2.8
43	R.V.	May 31, - June 2	Hypertension Diabetes	5.1	4240	1.3	17.1	19,20,22 (25 cc) & 36, 38	3.3
44	E.Mc.	Jun.4,5	Renal hypertension	6.1	1345	1.2	15.6	81,88,90 (25 cc)	4.6
Average Normal Women						1.3	13.8		2.0

TABLE XXXI.

PATIENTS ON WHOM THE BIOASSAY VALUES RANGED BETWEEN 21 AND 99 G.U. PER DAY. DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD B.

NO.	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATININE G/day	17-KETOS mg/day	REDUC.SUBST.(Method B) Gamma/50 cc	mg/day
a. Male patients									
27	M.J.	Nov.1,2 3	Hypopit.(treated)	6.2	1233	1.4	10.2	25, 27	0.6
29	D.F.	Jun.29,30	Cushing's (arrested)	-	1322	-	11.0	13	0.4
30	G.T.	Jun.26	Cushing's "	-	3028	-	-	25	1.5
Average Normal Men						1.7	19.1		0.9
b. Female patients									
32	F.H.	Jun.19,20	Hypothyroid Hirsutism	6.4	1000	1.0	16.5	33	0.7
33	C.G.	Jun.22-24	Question Addison's	6.4	1573	0.7	5.5	16 (38 cc)	0.6
34	W.B.	Aug.27-29	Fatiguability	6.3	1090	0.9	13.6	20	0.4
35	L.	Sep.22-23	Anorexia Nervosa	6.8	2225	1.0	18.0	41	1.8
36	W.	Oct.1,2	Scleroderma	5.5	1390	1.2	8.1	27	0.8
37	M.	Oct.7,8	Luteoma,virilizing	7.0	1500	1.1	12.5	11	0.3
38	D.V.	Oct.18-20	Acanthosis nigricans	7.3	1493	1.0	8.4	17	0.5
39b	E.N.	Jun.23,24	Precocious Puberty	8.3	1240	1.0	9.1	45 (45 cc)	1.2
Average Normal Women						1.3	13.8		0.6

PATIENTS ON WHOM THE BIOASSAY VALUES RANGED BETWEEN 21 AND 99 G.U.PER DAY.
COMPARISON OF THE RESULTS BY THE CHEMICAL METHODS AND THE BIOASSAY.

NO.	INITIALS	DIAGNOSIS	VOL cc/day	CREATI-NINE G/day	REDUC.Subst.(A) mg/day (= mg.Cpd.A)	REDUC.SUBST.(B) mg/day (= mg Cpd.A)	BIOASSAY Glyc. Units/day (= gamma Cpd.E)
A. Male patients							
25	J.I.	Hypogonadism	1605	1.2	2.8	not done	61
26a	A.J.	Bronchiectasis	1315	1.1	2.1	not done	(99
26b	A.J.	"	1570	1.1	2.4	not done	(99
27	M.J.	Hypopit.(treated)	1233	1.4	1.7	0.6	45
28	E.B.	Dissem.Sclerosis	986	-	very high?	not done	35
29	D.F.	Cushing's (arrested)	1322	-	not done	0.4	42
30	G.T.	Cushing's "	3028	-	Not done	1.5	96
	Range Normal Men			1.2 - 2.1	1.4-4.7	0.6-1.5	36-111
	Average Normal men			1.7	2.8	0.9	62
B. Female Patients.							
31	A.L.	Hirsutism	1018	1.2	2.5	not done	29
32	F.H.	Hypothyr. Hirsut.	1000	1.0	2.2	0.7	44
33	C.G.	Question Addison's	1573	0.7	1.4	0.6	61
34	W.B.	Fatiguability	1090	0.9	1.8	0.4	24
35	L.	Anorexia Nervosa	2225	1.0	4.8	1.8	47
36	W.	Scleroderma	1390	1.2	1.6	0.8	47
37	M.	Luteoma,virilizing	1500	1.1	1.0	0.3	46
38	D.V.	Acanth.nigr.	1493	1.0	1.6	0.5	36
39a*	E.N.	Precoc. puberty	1000	0.7	1.4	not done	30
39b	E.N.	" "	1240	1.0	4.5	1.2	27 (?)
40*	A.Ge.	Hair loss. Hyperthy.	1000	0.6	3.6	not done	55
41	N.S.	Hypertension	1688	1.3	2.2	"	53
42a	L.F.	Hypertension	1795	1.2	2.3	"	81
42b	1.F.	"	1460	1.2	2.8	"	59
43	R.V.	Hypertens.Diabet.	4240	1.3	3.3	"	73
44	E.Mc.	Renal hypertens.	1345	1.2	4.6	"	51
	Range Normal Women			1.0-1.7	0.8-2.7	0.4-0.8	24-71
	Average Normal Women			1.3	2.0	0.6	45

PATIENTS ON WHOM THE BIOASSAY RESULTS RANGED BETWEEN 21 AND 99 G.U. PER DAY.

ASSAY RESULTS EXPRESSED PER GRAM CREATININE.

NO	INITIALS	DIAGNOSIS	A RED. SUBST. (Meth. A) mg. Cpd. A. /G. Creatinine	B RED. SUBST. (Meth. B) mg. Cpd. A /G. Creatinine	C BIOASSAY Gamma Cpd. E /G. Creatinine	RATIO A / C x 3	RATIO B / C x 3
A. Male Patients							
25	J.I.	Hypogonadism	2.4	-	51	23	-
26a	A.J.	Bronchiectasis	1.9	-	(90	7	-
26b	A.J.	"	2.2	-	(	8	-
27	M.J.	Hypopit. (treated)	1.2	0.4	32	13	6
28	E.B.	Dissem. Sclerosis	High?	-	normal	high?	-
29	D.F.	Cushing's (arrested)	-	?	?	-	3
30	G.T.	Cushing's (arrested)	-	?	?	-	5
		Range normal men	1.0 - 2.4	0.4 - 0.7	20 - 55	9 - 33	4.7
		Average normal men	1.5	0.55	36	15	5
B. Female Patients							
31	A.L.	Hirsutism	2.1	-	24	29	-
32	F.H.	Hypothyr. Hirsut.	2.2	0.7	44	17	5
33	C.G.	Question-Addison's	2.0	0.9	87	8	3
34	W.B.	Fatiguability	2.0	0.4	27	25	5
35	L.	Anorexia Nervosa	4.8	1.8	47	34	13
36	W.	Scleroderma	1.3	0.7	39	11	6
37	M.	Luteoma, virilizing	0.9	0.3	42	7	2½
38	D.V.	Acanth. nigr.	1.6	0.5	36	15	5
39a	E.N.	Preco. Puberty	2.0	-	43	16	-
39b	E.N.	" "	4.5	1.2	27(?)	56?	15?
40	M.Ge.	Hair loss. Hyperthyr.	6.0	-	92	22	-
41	N.S.	Hypertension	1.7	-	41	14	-
42a	L.F.	Hypertension	1.9	-	68	9	-
42b	L.F.	"	2.3	-	49	16	-
43	R.V.	Hypertens. Diabetes	2.5	-	56	15	-
44	E.Mc.	Renal Hypertension	3.8	-	43	29	-
		Range Normal Women	0.7-1.9	0.4-0.5	16-59	5-35	3-7
		Average Normal Women	1.5	0.5	36	16	4.5

Tables XXXIV to XXXVII deal with patients on whom glycogenic bioassay results exceeded 100 g.u. per day. There are only two males and two females in this group, and the clinical diagnosis are given either as Cushing's Syndrome or as adrenal tumor. Only in one of the cases was the 17-ketosteroid determination high and this is a value of 26.1 mg. per day for the female patient No. 47.

In Table XXXIV it may be seen that the results of chemical assay by Method A only slightly exceeded the normal range in assays No. 45b. 46, 48a, 48b. In the latter three instances the collections were from local hospitals, were extracted promptly and were recorded as having pH values below 6.5 prior to acidification. Collection No. 45b was made in Cleveland, Ohio, shipped to Mohtreal, and the pH was not recorded on arrival. On the other hand, collections No. 45c and No. 47 were also collected in Cleveland, received in Montreal 9 to 12 days later, when their pH readings were, respectively, 8.4 and 9.0. The latter two urines, after the usual extraction Method A, showed such high reducing powers that they were too dark to read in the colorimeter. Thus, it is possible that these two very high results were partly due to alkalinization during the transportation without refrigeration.

Table XXXV shows that the results by the chemical Method B, are rather more satisfactory. By this method all the results are definitely higher than the normal range with the exception of No. 46, and this man is an undoubted case of Cushing's syndrome, studied thoroughly in the metabolic ward of the Royal Victoria Hospital. This exception is extremely disappointing, and difficult to explain (even by a complicating cystitis)

as mumerous bioassays exceeded 400 glycogenic units. The collection No. 46, studied by chemical assay was normally acid. By Method B, however, urine No. 48b showed 2.9 mg. Cpd. A equivalent per day which is 3 to 4 times the normal value. The three specimens collected in Cleveland No. 45a, 45c and 47 all showed satisfactorily high results, but again the part played by the delay in extraction with intervening alkalinization is not known.

Table XXXVI summarizes the above results, and shows that all the bioassay results were high, particularly so in the two males. In Table XXXVII the results are expressed per gram of creatinine excreted. It is of interest that the Royal Victoria Hospital, patient No. 46 whose diagnosis of Cushing's Syndrome is beyond doubt, shows more definitely elevated values for both the chemical methods A and B, when the results are expressed in this manner. The various ratios of chemical assay results over the bioassay values are scattered and do not reveal any consistent or unusual pattern.

PATIENTS ON WHOM BIOASSAY VALUES EXCEEDED 100 G.U. PER DAY. DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD A.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATININE G/day	17-KETOS mg/day	REDUC.SUBST.(Method A) Gamma/50 cc	mg/day
A. Male Patients.									
45b	H.C.	Jun.25	Cushing's S.	-	1420	-	19.0	122	3.6
45c	H.C.	Jul.27,28	" "	8.4	1190	0.7	-	?,too dark to read	very high
46	H.R.	Oct.22,23	Cushing's S.	6.0	1022	1.4	12.8	173	4.5
	Average Normal Men					1.7	19.1		2.8
B. Female Patients.									
47	J.S.	Aug.1,2	Cushing's S.	9.0	1280	-	26.1	too dark to read	very high
48a	A.Ge	May 18,-20	Adrenal tumor	6.0	1127	1.4	15.7	55,66(25 cc)	2.7
48b	A.Ge.	May 31, June 1,2	" "	6.5	1420	1.1	17.6	54,59 (25 cc)	3.2
	Average Normal Women					1.3	13.8		2.0

PATIENTS ON WHOM BIOASSAY VALUES EXCEEDED 100 G. U. PER DAY.

DATA RELEVANT TO THE CHEMICAL ASSAY METHOD B.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATI-NINE G/day	17-KETOS mg/day	REDUC.SUBST(Method B) Gamma/50 cc	mg/day
A. Male Patients.									
45a	H.C.	Jun.24	Cushing's Syndrome	-	2000	-	19.0	70	2.8
45c	H.C.	Jul.27,28	" "	8.4	1190	0.7	-	-243, 274	6.2
46	H.R.	Oct.22,23	" "	6.0	1022	1.4	12.8	36	1.2
	Average Normal men					1.7	19.1		0.9
B. Female Patients.									
47	J.S.	Aug. 1, 2,	Cushing's Syndrome	9.0	1280	-	26.1	190	4.9
48b	A.Go.	May 31, June 1, 2	Adrenal Tumor	6.5	1420	1.1	17.6	102	2.9
	Average Normal Women					1.3	13.3		0.6

PATIENTS ON WHOM BIOASSAY VALUES EXCEEDED 100 G.U. PER DAY. COMPARISON BETWEEN THE RESULTS BY THE CHEMICAL METHODS AND THE BIOASSAY.

NO	INITIALS	DIAGNOSIS	VOL cc/day	CREATI-NINE G/day	REDUC.SUBST.(A) Meth. mg/day (= mg.Cpd.A)	REDUC.SUBST.(B) Method B. mg/day (= mg.Cpd.A)	BIOASSAY Glyc.Units/day (= gamma Cpd.E
A. Male Patients							
45a	H.C.	Cushing's Syndrome	2000	-	Not done	2.3	383
45b	H.C.	" "	1420	-	3.6	Not done	218
45c	H.C.	" "	1190	0.7	Very high	6.2	Not done
46	H.R.	Cushing's Syndrome	1022	1.4	4.5	2.9	400
	Range Normal Men			1.2-2.1	1.7 - 4.0	0.6 - 1.1	36-111
	Average Normal Men			1.7	2.8	0.9	62
B. Female Patients.							
47	J.S.	Cushing's Syndrome	1280	-	very high	4.9	137
48a	A.Go.	Adrenal tumor	1127	1.4	2.7	not done	121
48b	A.Go.	" "	1420	1.1	3.2	2.9	102
	Range Normal Women			1.0 - 1.7	0.8-2.7	0.4-0.8	24-71
	Average Normal Women			1.3	2.0	0.6	45

PATIENTS ON WHOM BIOASSAY VALUES EXCEEDED 100 G. U. PER DAY.

ASSAY RESULTS EXPRESSED PER GRAM CREATININE.

NO	INITIALS	DIAGNOSIS	A RED. SUBST. (Method A) mg Cpd. A /G. creatinine	B RED. SUBST. (Method B) mg. Cpd. A /G. creatinine	C BIOASSAY gamma Cpd. E /G. creatinine	RATIO A / Cx3	RATIO B / Cx3
A. Male Patiehts							
45a	H.C.	Cushing's S.	Not done	?high	?very high	-	2½
45b	H.C.	" "	?high	not done	?very high	5½	-
45c	H.C.	" "	Very high	8.9	300 est.	?	10
46	H.R.	Cushing's S.	3.2	2.1	286	4	2½
	Range Normal Men		1.0-2.4	0.4-0.7	20-55	9-33	4-7
	Average Normal Man		1.5	0.55	36	15	5
B. Female Patients							
47	J.S.	Cushing's S.	very high	?high	?high	?	12
48a	A.Go.	Adrenal tumor	1.9	not done	86	7½	-
48b	A.Go.	" "	2.9	2.6	93	10½	9½
	Range Normal women		0.7-1.9	0.4-0.5	16-59	5-35	3-7
	Average Normal Women		1.5	0.5	36	16	4.5

Tables XXXVIII to XXXXI, show the data on several patients on whom bioassay results of less than 20 g.u. per day were found. Table XXXVIII, shows data relevant to several assays by the chemical Method A, on two such male and four female patients, and Table XXXIX, gives detailed data by chemical method B on the same group. A few duplicate checks were done, and these were satisfactory except for those on No. 52a by Method A. The alkaline pH values of 8.5 and 8.6 obtained on collections No. 49b and 51 are of interest. These urines were obtained from two patients of proven Addison's disease receiving desoxycorticosterone acetate therapy, but no cortical extract. The specimens were collected in July, while the writer was out of town, and were consequently refrigerated for approximately two weeks before being processed. Presumably the alkaline reactions had developed during this time and it may be noted that an earlier collection from one of these patients No. 49a was acid. (N.B. The bioassays on No. 49b and 51 were done on urines volumes which had been promptly extracted). The results by both Methods A and B on No. 51, were approximately normal rather than low, and such apparently increased results might be presumed to be due to the effect of the delay and resultant alkalinization of the urine, as has been suggested in cases discussed hitherto. In contrast the results by both chemical methods on urine No. 49b were very low, which indicates that the delay and alkalinization did not materially increase the amount of reducing substances in this instance. This finding thus throws some doubt on the suggested effect of alkalinization in allowing the formation of non-specific extractable reducing substances. Urine No. 49a, was extracted

promptly while the urine was normally acid, and the results by both chemical Method A and the bioassay were low. Urines No. 50a and 50b were collected at Notre Dame Hospital from a cooperative male bed patient with a destructive pituitary tumor and generalized signs of panhypopituitarism. There is little doubt that the specimens were incomplete, but repeated enquiries from his medical attendants elicited assurances that no alkalinizing medications were prescribed and that no preservatives were used in the urine collecting bottles. Both these specimens were found to be at pH 9.0 the day following their collection and they fizzed and foamed when acid was added to bring aliquots to pH 1.0. The writer, therefore, suggests that some exogenous alkali was present in the urines, possibly due to some material used in cleaning the urinals, or possibly due to some laxative or carminative prescribed by the nursing staff without the doctor's knowledge. These remarks are made to possibly explain rather than to excuse the high values for reducing substances by both chemical methods in this case, when both the clinical history and the extremely low bioassay values would lead one to expect low results for urinary reducing substances.

The clinical diagnosis on patients 52, 53, and 54 also shown in Tables XXXVIII to XXXXI do not indicate clinical adrenal insufficiency, yet the available bioassay values were very low. Case No. 52, had had several previous low bioassay determinations. Nevertheless, apparently satisfactory urine collections from this ambulatory outpatient gave normal chemical assay results by both methods. Urine No. 53a was collected on the general wards of the Royal Victoria Hospital and was apparently complete and almost

exactly neutral. No reason can be advanced for the very high value of 5.7 mg. per day by chemical Method A in contrast to the low bioassay result. A second sample on this patient, 53b, was taken while she was attending the out-patient clinic and receiving ammonium chloride therapy. Low normal values for both chemical assay methods were obtained then, but unfortunately the bioassay was unsatisfactory due to a technical mischance. Urine No. 54, was collected on the general wards of the Montreal General Hospital from an elderly, bearded, Italian woman with poor renal function and hypertension. The clinical impression was rather against the possibility of Cushing's Syndrome. Biological assays on this urine No. 54, were indeed low, and no technical reasons (delay, alkalinization, etc.) can be advanced to explain the higher than normal results by chemical Methods A and B.

Table XXXX gives a summary of the above group of urinary assays and Table XXXXI shows the results when calculated per gram creatinine excreted. The latter calculation does not help to demonstrate any better correlation between the chemical and biological assays than noted already. The two columns on the right in Table XXXXI show ratios of reducing substances to biological active material which are almost universally high. This is in line with the general conclusion for this group with low bioassay values that the chemical methods appear rather unsatisfactory in that they do not show comparable low results.

PATIENTS ON WHOM BIOASSAY VALUES WERE LESS THAN 20 G.U. PER DAY.

DATA RELEVANT TO THE CHEMICAL ASSAY METHOD A.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATI-NINE G/day	17-KETOS mg/day	REDUC. SUBST. (Method A) gamma/50 cc	mg/day
A. Male Patients									
49a	W.L.	May 23,24	Addison's Disease	6.1	1170	0.9	2.4	48, 49	1.1
49b	W.L.	Jul.20,21	tbc. " "	8.5	1450	0.8	-	32 (40 cc)	1.2
50a	A.M.	Jun.12-14	Hypopituitarism (tumor)	9.0	800	0.33	1.9	207	3.3
50b	A.M.	Sept.10-12	" "	9.0	400	0.17	2.4	too dark to read	very high
	Average Normal Men					1.7	19.1		2.8
B. Female Patients.									
51	C.M.	Jul. 25	Addison's Disease	8.6	1300	0.6	1.6	97	2.5
52a	A.E.	Apr.24,25	Scleroderma	6.5	1625	1.0	8.3	52, 70	2.2
52b	A.E.	Aug.21,22	"	6.5	1580	1.0	12.6	61	1.9
53a	C.V.	Apr.6	Hypertension	7.1	2235	1.5	9.9	115, 134, 134	5.7
53b	C.V.	Jun.17,18	"	5.9	1505	1.2	10.7	48	1.4
54	A.B.	Oct.26,27	Hirsutism, Hypertension	7.1	2390	0.7	6.9	81	3.9
	Average Normal Women					1.3	13.8		2.0

TABLE XXXIX.

PATIENTS ON WHOM BIOASSAY VALUES WERE LESS THAN 20 G.U. PER DAY.

DATA RELEVANT TO THE CHEMICAL ASSAY METHOD B.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATI-NINE G/day	17-KETOS mg/day	REDUC.SUBST.(Method B) Gamma/50 cc	mg/day
A. Male Patients									
49b	W.L.	Jul.20,21	Addison's Disease Tbc.	8.5	1170	0.9	2.4	6, 10 (40 cc)	0.3
50a*	A.M.	Jun.12-14	Hypopituitarism (tumor)	9.0	800	0.33	1.9	71	1.0
50b*	A.M.	Sept.10-12	"	9.0	400	0.17	2.4	227	1.8
	Average Normal Men					1.7	19.1		0.9
B. Female Patients									
51	C.M.	Jul.25	Addison's Disease	8.6	1300	0.6	1.6	42	1.1
52b	A.E.	Aug.21,22	Scleroderma	6.5	1580	1.0	12.6	17	0.5
53b	C.V.	Jun.17,18	Hypertension	5.9	1505	1.2	10.7	17	0.5
54	A.B.	Oct.26,27	Hirsutism, Hypertension	7.1	2390	0.7	6.9	32, 32	1.5
	Average Normal Women					1.3	13.8		0.6

TABLE XXXX.

PATIENTS ON WHOM BIOASSAY VALUES WERE LESS THAN 20 G.U. PER DAY.

COMPARISON OF THE RESULTS BY THE CHEMICAL METHODS AND THE BIOASSAY.

	INITIALS	DIAGNOSIS	VOL cc/day	CREATININE G/day	REDUC.SUBST.(A) mg/day (= mg.Cpd.A)	REDUC.SUBST.(B) mg/day (= mg.Cpd.A)	BIOASSAY Glyc.Units/day (= gamma Cpd.E)
A. Male Patients							
49a	W.L.	Addison's Disease	1170	0.9	1.1	Not done	< 10
49b	W.L.	" "	1450	0.8	1.2	0.3	< 10
50a*	A.M.	Hypopituitarism	800	0.33	3.3	1.0	< 10
50b*	A.M.	"	400	0.17	Very high	1.8	< 10
	Range Normal men			1.2 - 2.1	1.7 - 4.0	0.6 - 1.1	36 - 111
	Average Normal Men			1.7	2.8	0.9	62
B. Female Patients							
51	C.M.	Addison's Disease	1300	0.6	2.5	1.1	< 10 (previously twice ~~x 2~~)
52a	A.E.	Scleroderma	1625	1.0	2.2	not done	< 10
52b	A.E.	"	1580	1.0	1.9	0.5	< 10
53a	C.V.	Hypertension	2235	1.5	5.7	not done	< 10
53b	C.V.	"	1505	1.2	1.4	0.5	Unsatisfactory
54	A.B.	Hirsut.Hypertension	2390	0.7	3.9	1.5	< 20
	Range Normal Women			1.0-1.7	0.8-2.7	0.4-0.8	24-71
	Average Normal Women			1.3	2.0	0.6	45

PATIENTS ON WHOM BIOASSAY VALUES WERE LESS THAN 20 G.U. PER DAY.

ASSAY RESULTS EXPRESSED PER GRAM CREATININE.

NO	INITIALS	DIAGNOSIS	A RED. SUBST (Method A) mg. Cpd. A /G. creatinine	B RED. SUBST. (Method B) mg. Cpd. A /G. creatinine	C BIOASSAY gamma Cpd. E /G. creatinine	RATIO $\frac{A}{C \times 3}$	RATIO $\frac{B}{C \times 3}$
A. Male Patients							
49a	W.L.	Addison's Disease	1.2	Not done	< 11	> 37	-
49b	W.L.	"	1.5	0.4	< 12	> 40	> 10
50a	A.M.	Hypopituitarism	10.0	3.0	? < 30	> 110	> 33
50b	A.M.	"	very high	10.1	? < 58	? 500	> 60
	Range Normal Men		1.0-2.4	0.4-0.7	20-55	9-33	4-7
	Average Normal Men		1.5	0.55	36	15	5
B. Female Patients							
51	C.M.	Addison's Disease	4.2	1.8	< 17	> 83	> 36
52a	A.E.	Scleroderma	2.2	Not done	< 10	> 73	-
52b	A.F.	"	1.9	0.5	< 10	> 63	> 16
53a	C.V.	Hypertension	3.8	Not done	< 7	> 190	-
53b	C.V.	"	1.2	0.4	Unsatisf.	?	?
54	A.B.	Hirsut. Hypertens.	5.6	2.1	< 28	> 65	> 25
	Range Normal Women		0.7-1.9	0.4-0.5	16-59	5-35	3-7
	Average Normal Women		1.5	0.5	36	16	4.5

Data on the final group of patients, on whom no bioassays were attempted, or when performed showed variable results on successive trials, may be seen in Tables XXXXII to XXXXV. Many of the assays by chemical Method A on this group were done in duplicate or triplicate as shown in Table XXXXII. In the case of each of the three collections on No. 56, one of the triplicate samples was unsatisfactorily lower than the other two comparable samples. The probable explanation is that the low triplicates were assayed 10 to 20 days after refrigeration of these acid urines, while the first two aliquots were extracted promptly. Also No. 57b and 58b showed rather poor checks between duplicates. The five urines on the two male patients, 55 and 56, were collected on the wards or through the out-patient clinic of the Royal Victoria Hospital and were all apparently complete and of the usual acidity prior to prompt processing in the laboratory. The two collections on the female patient No. 57 were made on the hospital wards while she was febrile with acute rheumatic fever and under salicylate therapy. Undoubtedly, the collections were incomplete for only 400 - 435 cc. were obtained for each 48 hour period (diluted to approximately 2000 cc. for assaying) and the specific gravities for these collections were only 1.020 (a normal person with fever and perspiring freely should concentrate urine to more than 1.030). These samples were assayed promptly so the slight alkalinity of No. 57b might have little effect. The creatinine levels also support the contention that they were incomplete collections. Thus, the low results in No. 57a and 57b obtained by chemical Method A might be explained, but as shown in Tables XXXXIV and XXXXV, these

low results are quite out of line with the relatively high result obtained by bioassay in No. 57a. It should be stated that the reason for including subject No. 57, with this miscellaneous group of unsatisfactory bioassays is solely the doubt as to the completeness of the urine collections. The urines on patient No. 58 were collected at the Notre Dame Hospital. They were of adequate volume and satisfactory acidity, but the low creatinine values on No. 58a and 58b suggest that these collections were incomplete. The correlations with the bioassay values in this patient will be discussed below. The collection on No. 59, was apparently satisfactory and the chemical assay was done on the fourth day after refrigerating the specimen which remained slightly acid. The collections on the four female patients No.61, 62, 63, 64, were made in Baltimore. Aliquots containing a few cc. of chloroform as an anti-bacterial agent, were expressed to Montreal. They spent approximately three weeks in transit in mid-summer. It will be noted that all four specimens were highly alkaline on arrival, which fact, together with the associated time loss, probably accounts for the very high results by chemical method A on patients No. 62 and 63, and the high normal result on No. 64. These last three patients were all Addison's disease cases controlled by desoxycorticosterone acetate therapy and not in crisis during collection. The other Baltimore patient No. 61, is diagnosed as Cushing's syndrome so the high chemical assay is welcome though probably the delay and alkalinity of the specimens contributed to the high result. The last assay shown on Table XXXXII was done on urine No. 66, from a three year old girl with rheumatoid arthritis. The present paper has no basis of comparison with such a young normal subject but Heard et al (2) showed that

two 3 year old boys excreted less than a third of the average normal adult quantity of reducing substances. The value found in No. 66 was 1.3 mg. per day and, by analogy with Heard's results, one might be able to consider this result as 1.3 x 3 = 3.9 mgms. per day for purposes of comparing the result to those of normal adult woman. The bioassay on No. 66 was 77 g.u. per day which is high for a three year old girl. However, it has seemed wiser to exclude this child from the remainder of the data when summarizing the correlations between the two types of assays and the clinical histories.

Table XXXXIII shows the results with the chemical assay Method B on five of the women already discussed, and on No. 60. Urine from this middle aged woman with acromegaly was collected through the out-patient Department. It was apparently complete, of usual acidity, and processed promptly. A normal chemical assay result was found, but the biological test was unsatisfactory due to some misfortune with the animals. Somewhat more interesting results were found on the Baltimore group of patients by this Method B. The high results found in No. 61, 62, 63 by Method A were not confirmed in all by Method B, but the value of 5.3 mg. on No. 61, who suffers from Cushing's Syndrome, suggests that this is indeed a true reflection of increased cortical secretion. Likewise, the very low value of 0.3 mg. found in No. 62, who has Addison's disease, is pleasing. This result should be compared with the low result on No. 49b (see Table XXXIX) and it may be noted that alkalinity and delay in extraction did not cause unexpectedly high results in these two cases of Addison's disease. On the other hand the values by Method B done on old alkaline urine in No. 63 and 64 were abnormally high, and certainly not in accord with the diagnosis of adrenal insufficiency.

t should be recalled that injections of desoxycorticosterone acetate had o effect on reducing substances (as determined only by Method A) in a ormal adult man (see Table XVIII). Unfortunately, no biological assays ere done on the four Baltimore subjects.

Table XXXXIV, shows the results of the chemical assays and the ioassay on this group of patients. It is seen that the males, No. 55 nd 56, each showed one normal and one high result by bioassay. Because f this unexplained circumstance these patients are grouped here. The hemical assays (Method A) parallel the variations in the bioassay in the ase of No. 55, but showed reverse trends in the case of No. 56. They both uffered from hypertension but there is no definite evidence from the results f other patients with this condition studied in the present work (or at other imes by the bioassay method of Venning (29)), that hypertension is consistently ssociated either with high or normal excretions of corticoids. Subject No. 59, hows a bioassay value of 54 g.u. per day, which is lower than expected for the th month of pregnancy, and is not correlated with the high excretion of reducing ubstances by Method A (see TableXXVIII and refer to Venning (53). Patient o. 58, was admitted to the Notre Dame Hospital in collapse with hypotension. provisional diagnosis of Addison's disease was made, and she received adrenal ortical extracts and intravenous saline with satisfactory response. Urine ollected during this first 72 hour period showed extremely little biological ctivity and yet higher than normal values for reducing substances by Method A. f the collection was incomplete, as suggested by the very low creatinine utput, the bioassay result may be explained, but this does not account for the ack of correlation between the biological and chemical methods.

For the following few weeks this subject was maintained on desoxycorticosterone acetate therapy without the use of cortical extract. The bioassay at this time on 55c and 55d combined gave a normal value of 44 g.u. per day. If this is indeed the true value one must feel that the earlier very low bioassay value was not a true indication of this subject's condition, perhaps due to incomplete urinary collection alone. The chemical assays, 58c by Method A and 58d by Method B, are both higher than normal, which fact is not correlated with the normal bioassay value, or explained by any proven and controlled data for the effect of desoxycorticosterone therapy in other subjects yet available. The subsequent history of this subject No. 58 casts some doubt on her clinical diagnosis as she had no mucosal pigmentation, suffered no further collapse, and was discharged from hospital without hormonal therapy and has not again reported to her hospital during the intervening five months. Thus, the data on No. 58 is generally confusing.

The final Table XXXXV. for this group of patients shows the calculation of the assays per gram of creatinine. That the collections 57a, 57b, 58a and 58b, were probably incomplete was already mentioned but the present data shows a definite lack in correlation between the results of the chemical and biological assays. The results on No. 56 and No. 59, also remain uncorrelated. The divergent high and normal results on No. 55a and 55b show a correlation between the two methods of assay. No attempt was made to compute the ratios between the results of the chemical and biological assays in this group of patients on account of the unsatisfactory or missing data.

PATIENTS ON WHOM BIOASSAY RESULTS WERE INCONSTANT OR WERE NOT DONE.

DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD A.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATININE G/day	17-KETOS mg/day	REDUC. SUBST. (Method A) Gamma/50 cc	mg/day
A. Male Patients									
55a	W.D.	Apr.4	Hypertension	5.6	2155	1.2	9.6	83,89,89	3.7
55b	W.D.	Jun.16,17	"	5.3	1460	1.1	9.9	60,63	1.8
56a	L.Mc.	Apr.16,17	Buerger's Dis. Hypertens.	6.2	1088	1.7	11.0	186,203(50 cc) & 81 (25 cc)	4.0
56b	L.Mc.	Apr.23,24	" " "	5.6	1000	1.3	13.2	57,67,67 (25 cc)	2.5
56c	L.Mc.	Apr.25,26	" " "	6.0	1520	1.6	18.5	28,40,41 (25 cc)	2.2
		Average Normal Men				1.7	19.1		2.8
B. Female Patients									
57a	V.R.	Apr.7,8	Acute rheumatic fever	6.6	1000	0.4	6.8	24, 25	0.5
57b	V.R.	Apr.9,10	" " "	8.0	1070	0.5	5.5	21, 27	0.5
58a	E.D.	Jun.7	Quest.Addison's (Rx Cortin)		1700	0.4	-	134	4.5
58b	E.D.	Jun.7-9	" " "	6.5	1370	0.3	4.4	81, 90	2.3
58c	E.D.	Jun.15-16	" " (Rx. D.C.A.)	6.3	1040	0.7	7.4	185,190	3.9
59	H.	Jun.8,9	Pregnant (6th mos.) Hydrarthrosis	6.5	1330	1.4	11.3	201, 204	5.4
61	S.	Jul.11,12	Cushing's Syndr.	9.2	1840	-	-	204	7.5
62	R.M.	Jul.16,17	Addison's (Rx.DCA)	9.1	1300	-	-	239	6.2
63	A.	Jul.15	Addison's (Rx.DCA)	9.0	400	-	-	too dark to read	very high
64	F.C.	Jul.24,25	Addison's (RX.DCA	8.7	1900	0.8	-	75	2.8
66	D.G.	Apr.27,28	Rheumatoid Arth.(age 3)	7.0	350	1.4	0.9	94,96 (25 cc)	1.3
		Average Normal Women				1.3	13.8		2.0

PATIENTS ON WHOM BIOASSAY RESULTS WERE ~~,~~ INCONSTANT OR WERE NOT DONE. DATA RELEVANT TO THE CHEMICAL ASSAY BY METHOD B.

NO	INITIALS	DATE	DIAGNOSIS	pH	VOL cc/day	CREATI-NINE G/day	17-KETOS mg/day	REDUC.SUBST.(Method B) Gamma/50 cc	mg/day
A. Male Patients (None tested)									
B. Female Patients									
58d	E.D.	Jun.17,18	Quest. Addison's (Rx. DCA)	7.1	1160	0.7	7.4	64, 64	1.5
60	E.M.	Jun.27,28	Acromegaly	6.0	1400	1.4	13.1	25,30	0.8
61	S.	Jul.11,12	Cushing's Syndr.	9.2	1840	-	-	144	5.3
62	R.M.	Jul.16,17	Addison's (Rx DCA)	9.1	1300	-	-	11	0.3
63	A.	Jul.15	Addison's " "	9.0	400	-	-	166	1.3
64	F.C.	Jul.24,25	Addison's " "	8.7	1900	0.8	-	45	1.7
	Average Normal Women					1.3	13.8		0.6

PATIENTS ON WHOM BIOASSAY RESULTS WERE INCONSTANT OR WERE NOT DONE.

COMPARISON OF THE RESULTS BY THE CHEMICAL METHODS AND THE BIOASSAY.

NO	INITIALS	DIAGNOSIS	VOL cc/day	CREATI-NINE G/day	REDUC.SUBST.(A) mg/day (= mg.Cpd.A)	REDUC.SUBST.(B) mg/day (= mg.Cpd.A)	BIOASSAY Glyc.Units/day (= Gamma Cpd.E)
A. Male Patients							
55a	W.D.	Hypertension	2155	1.2	3.7	Not done	117
55b	W.D.	"	1460	1.1	1.8	" "	42
56a	L.Mc.	Buerger's Hypertens.	1088	1.7	4.0	" "	63
56b	L.Mc.	" "	1000	1.3	2.5	" "	162
56c	L.Mc.	" "	1520	1.6	2.2	" "	Unsatisfactory
	Range Normal Men			1.2-2.1	1.7-4.0	0.6-1.1	36-111
	Average Normal Men			1.7	2.8	0.9	62
B. Female Patients							
57a*	V.R.	Ac.Rheum. Fever	1000	0.4	0.5	Not done	41
57b*	V.R.	" " "	1000	0.5	0.5	" "	Not done
58a*	E.D.	?Addison's (Rx Cortin)	1700	0.4	4.5	" "	(10
58b*	E.D.	" "	1370	0.3	2.3	" "	(10
58c	E.D.	" (Rx.DCA)	1040	0.7	3.9	" "	(44
58d	E.D.	" "	1160	0.7	Not done	1.5	(
59	H.	Pregnant, Allergic	1330	1.4	5.4	Not done	54
60	E.M.	Acromegaly	1400	1.4	Not done	0.8	Unsatisfactory
61	S.	Cushing's Syndr.	1840	-	7.5	5.3	Not done
62	R.M.	Addison's Rx.DCA	1300	-	6.2	0.3	" "
63	A.	Addison's Rx.DCA	400	-	very high	1.3	" "
64	F.G.	Addison's Rx.DCA	1900	0.8	2.8	1.7	" "
65	D.G.	Rheumatoid Arth.age 3	350	1.4	1.3	not done	77
	Range Normal Women			1.0-1.7	0.8-2.7	0.4-0.8	24-71
	Average Normal Women			1.3	2.0	0.6	45

PATIENTS ON WHOM BIOASSAY RESULTS WERE INCONSTANT OR WERE NOT DONE.

ASSAY RESULTS EXPRESSED PER GRAM CREATININE.

NO	INITIALS	DIAGNOSIS	A REDUC. SUBST. (Method A) mg. Cpd. A /G. creatinine	B REDUC. SUBST. (Method B) mg. Cpd. A /G. creatinine	C BIOASSAY Gamma Cpd. E /G. creatinine
A. Male Patients					
55a	W.D.	Hypertension	3.1	Not done	97
55b	W.D.	"	1.6	" "	38
56a	L.Mc	Buerger's Hypertens.	2.4	" "	37
56b	L.Mc	" "	1.9	" "	125
56c	L.Mc	" "	1.4	" "	Unsatisfactory
		Range Normal Men	1.0-2.4	-	20-55
		Average Normal Men	1.5	-	36
B. Female Patients					
57a	V.R.	Ac. Rheum. Fever	1.2	Not done	102
57b	V.R.	" " "	1.0	" "	Not done
58a	E.D.	?Addison's (Rx. Cortin)	11.2	" "	33
58b	E.D.	" "	7.7	" "	
58c	E.D.	" (Rx. DCA)	5.6	" "	63
58d	E.D.	" "	Not done	2.1	
59	H.	Pregnant, Allergic	3.9	Not done	39
60	E.M.	Acromegaly	Not done	0.6	Unsatisfactory
61	S.	Cushing's Syndrome	?high	?very high	not done
62	R.M.	Addison's (Rx. DCA)	?high	?low	"
63	A.	Addison's (Rx. DCA)	?very high	?high	"
64	F.G.	Addison's (Rx. DCA)	3.5	2.1	"
66	D.G.	Rheumatoid Arthr. age 3	0.9	not done	55
		Range Normal Women	0.7—1.9	0.4—0.5	16 - 59
		Average Normal Women	1.5	0.5	36

CORRELATION BETWEEN THE VARIOUS ASSAYS AND WITH THE CLINICAL DIAGNOSIS.

Clinical diagnosis are not invariably accurate, and on further study they may be modified. We do not know the usual state of adrenal cortical function in all clinical conditions. Furthermore, cortical function probably varies fairly widely from time to time with alterations in diet, increased physical activity or the presence of various types of non-specific stress. Therefore, the results of even the most specific and accurate measure of cortical function would not be expected to show an invariable correlation with the clinical diagnosis.

The glycogenic bioassay of Venning et al (7) has been shown to give a very satisfactory correlation with the clinical condition in normal individuals (35), pregnant women (53), various endocrinological disorders (82), and general conditions of stress, such as burns, surgical operations, etc. (29). The data presented in this thesis has been examined from a rather strict and arbitrary standard of the range which may be accepted as normal. The data is shown in Table XXXXVI. In the first place, the glycogenic bioassay is not always technically successful due to biological factors, such as mortality of the adrenalectomized mice during the test from toxic material in the extract, or other causes. Thus, 6 of the 82 bioassays attempted were unsatisfactory and could not be interpreted for reporting. Of the remaining 76 assays 71 showed an excellent correlation with the clinical impression. The five exceptions include No.4a which was 111 g.u. per day on a normal young man, who excreted 2.0 G. of creatinine per day, and who had shown a high result the previous year. The second exception was 112 g.u. per day on No. 24a, who was a middle aged diabetic woman who had recently undergone considerable emotional trauma, thus possibly influencing her cortical function. The third exception was the finding of less than 10 g.u. per day

on No. 58b, which specimen was probably incomplete. In this patient there remains doubt concerning the possible clinical diagnosis of Addison's disease, and a normal bioassay of 44 g.u. per day was subsequently found. Therefore, explanations may be advanced for the abnormal results in the two supposedly normal subjects and a less definite but plausible explanation is advanced also for the patient No. 58. No explanations can be advanced for the abnormally high bioassays in No. 55a and 56b, as normal results were obtained in both these hypertensive men on other occasions. The possibility of course remains that these two men may have had temporarily increased adrenal cortical secretion at the time of the two urine collections concerned. The conclusion from all the evidence at hand is that the glycogenic bioassay of urinary extracts gives a very satisfactory picture of adrenal cortical function in the normal individuals and patients studied.

The results obtained by neither the chemical assay methods A or B show good correlations with the bioassay results or the clinical diagnosis. 94 assays were made by Method A on a total of 62 subjects, of whom only 53 subjects also had bioassays performed (see Table XXXXVII (a)). If one allows a normal range of 1.7 - 4.0 mg. of Cpd. A equivalent for men and 0.8 - 2.7 mg. per day for women, it is found that one man No. 7 and one woman No. 19 had abnormally high results despite normal bioassay results. The 37 chemical assays (Method A) on the 28 patients showed a much poorer correlation with the available bioassay results. 13 poor correlations were found. These consisted of 4 high results (No. 28, 35, 39b and 44) out of 21 chemical assays on subjects with normal bioassay values; two normal results (No. 48a, 48b) out of 6 assays on patients with high bioassay values; and 5 high normal results (No.50a, 51, 52a, 52b and 54) and 2 very

high results (No. 50b, 53a) out of 10 chemical assays attempted on patients showing low bioassay values. The correlation is thus poorest in this latter group of patients showing low bioassay results. Possible explanations (contaminated urine collections, delays and associated alkalinization of the urine prior to extraction) have been put forward in the preceding text, but the fact remains that approximately one-third of the chemical assays by Method A, on these patients showed poor correlation with the bioassay results. In addition 15 chemical assays were done on 9 patients on whom inconstant or no bioassay determinations were available. There is thus no satisfactory standard of reference, but 3 (No. 62, 63, 64) high results were obtained on urines shipped from Baltimore collected from women with Addison's disease. A similar analysis of the correlation of the results by chemical Method B with the bioassay is tabulated in Table XXXXVII (b).

The ranges allowed as normal by this method are 0.6 - 1.2 mg. of Cpd. A, equivalent per day for men and 0.4 - 0.9 mg. for women. Of the 13 assays made on 11 normal subjects there was 1 exception (1.5 mg. on No. 7). However, in the 11 assays made on 11 patients with normal bioassays there were 3 instances of abnormal chemical results (0.3 mg. on No. 37, 1.5 mg. on No. 30 and 1.8 mg. on No. 35). In the 5 assays made on 4 patients with high bioassay values there was 1 uncorrelated result found (1.2 mg. on No. 46). In the 7 assays made on 6 patients with low bioassays 4 normal or high results were obtained (No. 50a, 50b, 51 and 54). Thus, there were a total of 8 instances of poor correlation out of the 23 assays performed on 21 patients with available bioassay results. This seems a very unsatisfactory correlation and it cannot be explained in every instance by poor collection or delayed extraction of the urines (see text). There were also 6 assays by Method B made on patients without

satisfactory bioassay results available, and of these there were 3 high results on patients diagnosed as Addison's disease (No. 63, 64, 58b).

The totals show that there were 18 unsatisfactory results out of 94 chemical assays by Method A, and 12 unsatisfactory results out of 42 assays by Method B. One may add that the calculations of the results per gram of creatinine did not significantly alter the proportions of unsatisfactory correlations between the chemical and biological assays.

TABLE XXXXVI.

THE CORRELATION BETWEEN THE RESULTS OF THE GLYCOGENIC BIOASSAY AND RESULTS EXPECTED FROM THE CLINICAL DIAGNOSIS.

	NO. OF ASSAYS ATTEMPTED	NO. OF ASSAYS REPORTED	GOOD CORRELATION	NOT CORRELATED
25 NORMAL SUBJECTS	34	31	29	2
36 PATIENTS	48	45	42	3
TOTAL 61 SUBJECTS	82	76	71	5

TABLE XXXXVII.

THE CORRELATION BETWEEN THE RESULTS OF THE CHEMICAL ASSAYS (METHODS A AND B) WITH THE BIOASSAY, OR WITH CLINICAL DIAGNOSIS IF NO SATISFACTORY BIOASSAY AVAILABLE FOR COMPARISON.

	NUMBER OF ASSAYS	GOOD CORRELATION	NOT CORRELATED
a) Method A			
25 Normal subjects	42	40	2
28 Patients with bioassays	37	24	13
9 Patients(with clinical diagnosis only)	15	12	3
TOTAL - 62 subjects	94	76	18
b) Method B			
11 Normal subjects	13	12	1
21 Patients with bioassays	23	15	8
6 Patients with clinical diagnosis only	6	3	3
TOTAL 38 subjects	42	30	12

FURTHER DISCUSSION OF THE CORRELATION BETWEEN THE ASSAY METHODS.

The unsatisfactory correlation found between the chemical assays and the biological assay may be due to a number of causes, which have already been mentioned. First of all, there are purely technical difficulties, which might lead to errors in the chemical results. The number of poor checks when duplicate samples of urine were assayed simultaneously (see Table XVI) should be considered in this regard. Secondly, there are important difficulties in the collection of uncontaminated urine from patients, for faeces and such bed-pan disinfectants as cresol and Dakin's solution (Javelle water) introduce reducing material. Also delays in transporting specimens prior to extraction may allow an increase in non-specific urinary reducing substances. These hazards are much less likely to occur in collections from normal subjects, especially those at work near the laboratory, but they are a very real problem when dealing with hospital patients. Their importance was not fully realized before this study was undertaken. The third difficulty is more theoretical and it has been stressed earlier that the phospho-molybdic reducing reagent is not specific, and thus would react with certain non-corticoid urinary reducing substances if they passed into the final extract residue. It was to limit this possible source of error that the benzene-water partition of Method B was introduced but it must be admitted that the correlation with the bioassay was not improved. Another possible way to reduce this error would be to use Nelson's sugar reagent of Talbot (6), which does not react with the α-β-unsaturated 3-keto group alone, which might be easier to prepare and standardize than the phosphomolybdic reagent, and which is said to give a complete reaction within twenty minutes of heating.

The fourth and final reason for the lack of correlation may be simply that the amount of inactive corticosteroids excreted bears no constant relation to the amount of active glycogenic corticosteroids. This is the conclusion which would be reached from the results if the first three possible sources of error could be eliminated. It is indeed a very interesting possibility, which future work on the biochemistry and physiology of the adrenal cortex might support and explain. However, this point cannot be expanded in further detail at present, as the difficulties in urinary collections and in some technical details of the method should first be overcome.

The writer is still greatly troubled about the effect of acidification in increasing the quantity of extractable reducing substances. If this were simply a matter of hydrolysis it should not be reversible (as indicated by Table III of Heard et al (2)), and one might expect to find an end-point to the effect of acidification at a higher pH than 0.5. One might suggest that the acidification may be increasing the extractability of non-specific reducing substances rather than of corticosteroids. Talbot (6) does not use acidified urine, but it would be most interesting to learn whether in his more highly purified extraction method (including Girard's reagent) acidification increased the reducing power of either the ketonic or the non-ketonic fractions.

Finally, as indicated in the earlier portions of this thesis, the gaps in our knowledge of the physiology and the biochemistry of adrenal cortical hormones, particularly the part played by renal excretion of such substances, impose difficulties on the interpretation of all urinary corticoid assays. The urinary glycogenic bioassay of Venning et al (7, 35, 53, 82, 29)

(also see Table XXXXVI) gives results which correlate satisfactorily with our present knowledge of cortical function in various disorders and conditions of stress. This knowledge is not static; the future may bring some modifications and improvements in the exact interpretation of the bioassay method. However, we know still less about the exact nature or the significance of the urinary substances measured by the reducing reactions - what proportions may be various biologically active corticosteroids or inactive corticosteroids or other non-specific substances. Therefore, an exact correlation between urinary glycogenic activity and lipid soluble alkali-insoluble total reducing substances might not be obtainable in every case.

SUGGESTIONS FOR FURTHER WORK.

The work presented leaves many unanswered questions, but a few suggestions for future work on urinary corticoids may be enumerated.

(1) Urine collections from patients must be constantly supervised so that there is no contamination with reducing substances such as feces or disinfectants (such as cresols, etc.). Collections which do not meet the laboratory specifications should not be accepted for extraction. However, the use of toluol as a preservative should be considered and rechecked, as preliminary observations suggest that this substance does not act as a reducing substance or interfere with the extraction. (2) The effect of short periods of refrigeration at pH 9.0 should be re-checked in comparison with aliquots at the usual pH 6.C. (3) As the attempt to use small volumes of urine for extraction led to unsatisfactory checks between duplicates,

a trialshould be made to reduce possible contamination or loss during extraction by the use of larger aliquots, say 500 cc. urine. Talbot (6) extracts such volumes ~~and larger~~. In addition, further search should be made for the actual source of contamination (rubber, glassware, source of distilled water, dust, etc).

(4) Other volatile solvents or greater proportions of solvent to urine might be tried in order to reduce the amounts of emulsions. (5) The relative amounts of the alkali insoluble reducing substances, which are in the benzene soluble and in the water-soluble ketonic and non-ketonic fractions should be ascertained for urines from several normal and a few abnormal subjects. This might lead to the adoption of the full extraction technique of Talbot (6). (6) As there are difficulties with the phospho-molybdic reducing reagent and as it is certainly less specific than Nelson's copper reagent used by Talbot, the substitution of the latter reagent is recommended. (7) The selection of an untreated patient with Addison's disease or panhypopituitarism in whom the urinary assays of glycogenic corticoids and reducing substances are low would be an ideal subject to study the recovery of injected cortical extract and pure corticosteroids. (8) Similar urine with low reducing power would be an excellent material to study the comparative effects of strong alkali, strong acids and graded increases of acidity from pH 5.0 to 0.5. The results of these changes should be compared with those occurring in further samples with normal reducing power. Thus, indirect evidence would be obtained as to the nature of the urinary reducing substances, and the relevant importance of the hydrolysis of conjugated corticoids might be ascertained.

(9) Finally, extractions of urine without acidification (controlled at a standard pH, say 5.0) might be found to give better correlations between the chemical assay and the bioassay.

SUMMARY.

A large number of crystalline steroids have been isolated from adrenal cortical extracts, and six of these substances have been shown to have biological activity similar to whole cortical extracts. These pure corticosteroids have certain characteristic solubilities in organic volatile solvents and in water, thus permitting their extraction and concentration. Various satisfactory biological assay methods have been developed for cortical material. The crystalline substances may also be assayed chemically by their reducing activity (Heard (1), Talbot (6)) or by oxidation with periodic acid.

Certain clinical disorders and other clinical states of trauma and stress have been shown to resemble closely the experimental biological effects of adrenal cortical insufficiency or overdosage with potent cortical material. Therefore, attempts have been made to assay fluids from human subjects in order to assess quantitatively adreno-cortical function. The huge quantities of blood required for such assays have discouraged the use of this fluid, and the work of Vogt (49) suggests that the cortical hormones present in the suprarenal veins are rapidly inactivated in the circulating blood. Therefore attention has been directed to the study of urine.

No biologically active corticosteroids or even inactive substances with the characteristic highly oxygenated side-chain have been chemically identified in crystalline form from urine. However, Hoffman (71) has recently obtained almost complete evidence for the iden-

tification of the acetate of 17-hydroxy, 11-dehydrocorticosterone (Compound E of Kendall) in a biologically active extract of human post-operative urine. On the other hand, numberous workers, by various methods, have demonstrated in urine biological activity similar to cortical extracts. These methods are reviewed. The protection of adrenalectomized animals against cold-exposure has been successfully applied to urine extracts. More recently, methods based on the deposition of glycogen in the livers of adrenalectomized mice have been used for the assay of urine extracts. This reaction is sensitive and specific for 11-oxycorticosteroids. A series of communications by Venning et al (29, 7, 35, 53, 82) has demonstrated good correlation between the results of the glycogenic bioassay and the clinical findings in a wide range of conditions. Published reports are not yet available of the successful application of this method to the quantitative recovery of active cortical substances which have been injected into suitable subjects. Therefore, some theoretical doubts remain as to the applicability of urinary assay methods in reflecting alterations in cortical hormone secretion, though in clinical practice the results of Venning and her coworkers give strong evidence to dispel these doubts.

Methods have been adapted to urinary extracts for assaying corticosteroid-like contents by means of characteristic but non-specific chemical reactions. Talbot (51) and others (87) used the periodic acid reaction to detect formed ketosteroids, and, in a recent preliminary communication, Lowenstein et al (70) used the same periodic reac-

tion but quantitatively estimated the formaldehyde released. However, there is evidence to suggest that periodic acid may react in an irregular manner with a wide range of possible urinary steroids. The glucose-like reducing properties of corticosteroids have been used in assaying urine extracts by Talbot (6) and Heard and Sobel (2). Talbot uses the ~~unhydrolysed~~ unhydrolysed ketonic fraction of urine extracts. Heard and Sobel acidify urines prior to extraction and only purify to the alkali-insoluble stage. Their reducing reagent is somewhat less specific than Talbot's but both reagents react with many steroids which lack cortical biological activity. This non-specificity of the reducing reagnets means that these methods must depend primarily on the completeness of the extraction and on proper purification if the results are to be specific for cortical metabolites. Although it is established that the acidification of urine, as performed by Heard, increases the extractable reducing substances, neither the mechanism of this increase nor its specific relation to urinary corticoids is clearly established. The suggested advantages of these chemical assays over bioassays are (i) that they are quicker and cheaper and (ii) that the estimation of total inactive plus active urinary corticoids might be a more accurate (or at least an additional) reflection of adrenal cortical function.

The experimental data consists primarily of comparisons between Venning's bioassay (7) and slight modifications of the chemical method of Heard on urines from a group of normal individuals and from forty selected patients. Heard's method has been modified principally in the manner in which the reducing reagent is allowed to react with the

urine extracts. Thus the reaction is shown to be maximal within one hour, whereas Heard's original reduction proceeds at least for three hours. Quantitative comparison between the results of Heard (2) and those here reported is rendered impossible by this modification; this is termed Method A. A second modification, named Method B, was sometimes used, and it consists in the additional purification of the extract by benzene-water partition. There are also other slight differences in the methods used here from the original method of Heard. The results are expressed in equivalent weights of 11-dehydro-corticosterone (Compound A) instead of 11-desoxycorticosterone.

Good recoveries of 21-hydroxypregnenolone and of 11-dehydrocorticosterone from water or urine were obtained by Method A, but this was not true when Method B was used. Neither desoxycortiocsterone nor dehydrocorticosterone were recovered as excess reducing substances by Method A after intramuscular injections of small doses of these steroids into two different individuals.

The reduction reaction gave fairly constant results with repeated trials of known steroids. However, extractions of urine samples in duplicate or triplicate by either Method A or B resulted in a rather large proportion of unsatisfactory checks, which indicate incompleteness of extraction or the introduction of contaminants.

In 19 assays on urine from nine normal men chemical Method A gave an average daily excretion of 2.8 mg. (as equivalents of Compound A), and in 11 assays on urine from nine women an average of 2.0 mg. Chemical Method B showed averages of 0.9 mg. per day for five men and 0.6 mg. for

five women. The glycogenic bioassay showed averages of 62 units for men and 45 units for women (1 unit is the equivalent of one microgram of Compound E). When the results of each assay were calculated per gram of creatinine no sex difference was found, the averages being 1.5 mg. by Method A, 0.5 mg. by Method B, and 36 glycogenic units by the bioassay.

Four elderly subjects were studied by the chemical Method A and by the bioassay. In general the results were slightly lower than average by both methods. Four assays by Method A on one pregnant woman averaged about three times as high as normal, which was correlated with high bioassay values.

Urine specimens from forty patients were assayed by some or all the methods. A total of 48 bioassays were performed on thirty-six patients. By chemical Method A, 52 assays were done on thirty-seven patients, and by Method B, 29 assays were made on twenty-seven patients. Summaries of the clinical histories are found in the appendix. The results are tabulated in groups according to whether the bioassay values were normal, high or low. A fourth group consists of those patients on whom no bioassays were attempted and those on whom the bioassay results were considered unsatisfactory.

Out of 45 technically satisfactory bioassays reported, 42 showed a good correlation with the clinical findings. However, at least one third of the assays made by each of the chemical methods failed to show correlations with the bioassay results. For some, but not all, of these unsatisfactory chemical results plausible explanations may be suggested, such as contamination during collection or delays between col-

lection and extraction. In particular, some urines were found to be highly alkaline prior to the usual acidification and extraction, and often such specimens showed high reducing activity. The difficulties in assuring proper urine collections from hospital patients are stressed.

The other suggested reasons for the poor correlations of the chemical assays with the bioassay are: 1. Technical errors, 2. the extraction of urinary non-corticoid reducing substances which may not be completely removed by the purification procedures adopted, and 3. that the chemical methods presumably measure biologically inactive as well as active corticoids.

Several suggestions for further work are made. The effect of storage delays, especially at an alkaline pH should be studied. Larger volumes of urines should be extracted to reduce technical errors. The proportions of ketonic reducing substances to total reducing substances should be compared on a number of patients to see whether either has consistent advantages over the other in clinical conditions. There are certain disadvantages to the phosphomolybdic reducing reagent, which might be overcome by replacing it by Nelson's reagent used by Talbot. Urine of low biologically active corticoid content and low reducing power (e.g. from a case of panhypopituitarism) might be a useful medium to study the effect of increasing degrees of acidity on the extractable reducing substances. A comparison between the effect of acidification to pH 1.0 or pH 5.0 should be made on urines collected from patients with varied clinical disorders. Finally adrenal cortical substances should be

injected into patients with known adrenal insufficiency and recoveries estimated simultaneously by the glycogenic bioassay and by a variety of modifications of the chemical reducing activity methods.

C O N C L U S I O N.

The results obtained by the chemical methods used in the present work did not show satisfactory correlation with the results of the urinary glucogenic bioassay, nor can the chemical results be interpreted solely in relation to the clinical histories of the patients studied.

APPENDIX I.

REAGENTS AND APPARATUS REQUIRED FOR THE MODIFICATION OF THE CHEMICAL ASSAY DESCRIBED.

Reagents:

Ether (reagent grade) is distilled in all-glass apparatus.

Chloroform (reagent grade) is distilled likewise.

Extraction mixture consists of 4 parts ether and 1 part chloroform.

Sodium hydroxide (reagent grade pellets). 0.1 N solution in distilled water is prepared.

Distilled water as prepared for routine laboratory use in the metal still was used.

Sulfuric Acid (reagent grade) - 12 N solution is prepared.

Molybdic Acid (reagent grade) - N.B. contains ammonia.

Sodium tungstate (reagent grade)

Phosphoric acid (85%) - (reagent grade)

Acetic Acid (glacial) (reagent grade)

Phospho-molybdic solution (refer Folin and Wu 21) is prepared as follows:

Molybdic Acid	-	70 gram
Sodium tungstate	-	10 "
10% sodium hydroxide	-	400 cc
Distilled water	-	400 cc

The above are mixed in a chemically clean 4 litre beaker and boiled until the odor of ammonia has disappeared, and the fumes are neutral to moist litmus paper. This may require one hour or more. The beaker is covered and allowed to stand overnight. The solution is filtered to remove the fine white precipitate. The filter is washed once with a small amount of

distilled water. 250 cc of phosphoric acid (85%) are added, and the solution made up to one litre with distilled water in a volumetric flask. The final solution should be clear and colourless. It is stored in the refrigerator.

Reducing Reagent: This is prepared fresh each day. Equal parts of the phospho-molybdic solution and glacial acetic acid are mixed so that the transient white precipitate is redissolved. The mixture is colourless.

Benzene (reagent grade) is distilled. This is required only in the modification described as Method B.

Nitrogen (gas) - compressed in cylinder

STEROIDS FOR STANDARD CURVES.

Note: Pure samples of 11-dehydrocorticosterone and of 5 - pregnene, 3-21-diol, 20-one (21-hydroxy-pregnenolone) were used. The writers appreciation for these steroids is expressed to Dr. E. H. Venning and particularly to the original donors, Dr. E. C. Kendall and Dr. S. Lieberman.

APPARATUS:

For the chemical method of urinary corticoids the following are required:

Beckman pH meter.

Photoelectric colorimeter (e.g. Evelyn type).

Macro test tubes for colorimeter. (graduated at 10 cc).

Distilling apparatus with all glass connections.

Water suction apparatus.

Water bath, with perforated cover to hold test-tubes.

Test-tube racks.

Separating funnels, preferably 500 cc. size (at least six needed)

Stands for funnels.

Volumetric flasks, one 1000 cc.

" " several 25 cc. and 50 cc.

Erlenmeyers flasks (Pyrex) with standard taper necks and glass stoppers. At least one dozen 250 cc. size.

Connecting tubes (standard taper) with stop-cocks. (e.g. CanLab A82-430).

Pipettes, volumetric, sizes 1 cc., 3 cc., 5 cc., 10 cc., 25 cc. 50 cc.

Pipettes, graduated 10 cc.

Pipettes, capillary, with rubber teats.

Cylinders, graduated, assorted, especially 200 cc. and 2000 cc.

Erlenmeyer flasks, assorted, especially 200 cc. and 500 cc. sizes.

Beakers, small and one 4000 cc.

Glass wool.

Glass tubing.

Filter paper.

Wash-bottles, all glass, 500 size (at least two needed)

glass bottles for reagents.

Two litre glass bottles for urine collections.

Rubber-tubing, black (N.B. Internal surfaces of tubing must be scrubbed. Then tubing placed in boiling solutions of 0.5% NaOH and 4% HCl successively for 5 minutes each. Then rinsed in tap water till neutral to litmus; rinsed in distilled water) .

A P P E N D I X - II.

CLINICAL SUMMARIES OF PATIENTS STUDIED.

No. 25 - J.I. (male) - Admitted wards of Montreal General Hospital, in April 1946, under care of Dr. E. S. Mills. Hospital No. 2355-46.

Diagnosis: Hypogonadism.

Age 40 years. Height 70 inches. Weight 150 lbs. Blood pressure 100/62. No libido and weakness for 8 years and hot flashes for 4 years. Showed pale skin, absence of facial and axiallary hair with scant pubic hair. Phallus normal but testes small and soft. No treatment during urine collection in hospital.

No. 26 - A. J. (male) Royal Victoria Hospital ward, No. 46-4702. Admitted April 4th, 1946. Died on July 25th, 1946. Diagnosis - Bronchiectasis, bilateral with malnutrition. Age 25 years. Height 70 inches. Weight 98 lbs. Cough with expectoration since childhood. In 1939 hospitalized and diagnosed as bronchiectasis, weight then 102 lbs. On re-admission in 1946, weight 88 lbs. No fever. Blood pressure 120/70. Penicillin inhalations and high protein diet given with gradual weight gain. Was on metabolic ward during time of all urine studies which are considered complete. Later died during thoracotomy operation.

No. 27 - Royal Victoria Hospital (M.J. male) - ward No. 46-15031. Admitted October 1946.

Diagnosis: Panhypopituitarism (chromophobe adenoma) - Age 24 years; Height 74 inches. Weight 190 lbs. When 19 years old he was 70 inches tall and weighed 144 lbs; best weight of 242 lbs. reached April 1946. Dry skin 3 years

Shaves beard only once weekly in past year. Headaches, blurred vision for 18 months. In June 1946, first admitted, weighing 214 lbs. Blood pressure 110/70. Optic fields and discs normal. X-ray sella markedly enlarged. Flat glucose tolerance curve. B. M. R. minus 32. Blood cholesterol 182 mg%. Urine of June 26, 1946, showed 17-ketosteroids of 9.5 mg. per day and glycogenic corticoids only 15 units. Received 3 weeks intense X-ray therapy in July to the pituitary region. Thyroid extract thereafter. Headaches improved before re-admission in October 1946. No change in outline of sella turcica or in level of B. M. R. Urine collections made while up-patient in ward in October 1946, while he was receiving 40 mg. methyl testosterone daily.

No. 28 - E. B. (male). This patient was in Notre Dame Hospital, Montreal, No. 4893, under the care of Dr. C. E. Grignon.

Diagnosis: Disseminated sclerosis. Questionable acromegaly.

Age 37 years; Height 65 inches; weight 135 lbs. Blood pressure normal. Symptoms of about 3 years duration included weakness of the legs, speech difficulties, poor memory. There was optic atrophy. X-ray of skull showed a moderately enlarged sella but no definite erosion. The mandible was prominent. The glucose tolerance curve was flat. A definite clinical diagnosis of acromegaly could not be made. Urine cultures showed B.coli and staphylococci. Several urines were alkaline. The treatments were soda bicarbonate and aspirin. The urine collection assayed was collected on the hospital wards and was considered complete. No reason was elicited to explain the low creatinine observed. The 17-ketosteroid excretion was 17.7 mg. at the time of corticoid assay, and it was 26.6 mg. at a later date.

No. 29 - D. F. (male)- Cleveland Clinic, Cleveland, Ohio.

Diagnosis: Cushing's Syndrome (treated). Age 27 years; Height 63 inches; weight 140 lbs. Obesity and plethora for 15 years. Blood pressure 210/140. Urea clearance impaired; serum proteins low. Glucose tolerance normal. Mild osteoporosis of spine. X-ray therapy to pituitary region in May - June 1943. Left adrenal and part of right adrenal removed in October 1943, but no abnormalities found. Clinically unchanged since operation. No endocrine therapy recently or during urine collection.

No. 30 - G.T. (male) - Cleveland Clinic, Cleveland, Ohio.

Diagnosis: Cushing's Syndrome (treated). Age 39 years. Backpains and raised blood pressure noted in 1937. Marked osteoporosis of spine and multiple fractured ribs. Blood pressure 164/116. Mild diabetes. X-ray therapy to pituitary in 1937 and 1938. Serum globulin low in 1943. Since 1943, treated with testosterone and 150 g. protein diet. In 1946, appearance still resembled Cushing's Syndrome, but was moderately improved and he could do light work. Receiving testosterone proprionate 25 mg. three times a week during the urine collection.

No. 31 A. L. (female) - Royal Victoria Hospital - O. P. D. No. F-4912.

Diagnosis: Hirsutism, simple. Age 23 years. Height 65 inches, weight 125 lbs. Some hair on shins for 10 years and few hairs on chin and breasts for 3 years. Supra-pubic mid-line hair growth. Habitus and attitude feminine. Normal menses. Blood pressure 130/80. No clittoral hypertrophy. Breasts normal. Pregnanediol present in urine at 14th - 15th days of cycle. Urines for corticoid assays collected as out-patient.

No. 32 - F. H. (female) Royal Victoria Hospital Ward No. 46-14631. Admitted June 1946.

Diagnosis: Hirsutism. Age 27 years; Height 62 inches; weight 126 lbs. Excessive hair on legs since puberty with gradual increase in supra-pubic area and over nipples. Menses regular except for two scanty flows in previous month. Was treated as hyperthyroid until 1943, when B. M. R. normal, and no treatment since. Now B. M. R. minus 12, and cholesterol 197 mg.%. Pelvic examination normal. Pulse 80. In June urine collection made while up-patient on ward. In September 1946, appendectomy, and uterus and ovaries were normal except for small cyst of left ovary.

No. 33 - C. G. (female). Patient in Notre Dame Hospital, No. 5300-46, under the care of Drs. G. Hebert and C. E. Grignon.

Diagnosis: Pigmentation, probably not Addison's Disease. Age 36 years, Height 60 inches, weight 108 lbs. Blood pressure 115/80. Pigmentation of hands, face, lips and mouth noted for 18 months, and weight loss of 22 lbs. in the last 12 months. She had salt craving. Normal menses. No asthenia. No crises. Normal hair distribution. B. M. R. minus 20. Haemoglobin 76%. Blood sugar and sodium normal. Kepler "water test" normal. No hormone therapy at any time. Urine collection made while in hospital.

No. 34 - W. B. (female) Private patient of Dr. J. S. L. Browne.

Diagnosis: Fatiguability. Age 43 years. Height 65 inches, weight 140 lbs. Blood pressure 125/90. Lives in Brazil. Had thyroidectomy in 1933, apparently for hyperthyroidism. Complained of fainting spells and general weakness for several years. Was still having some menstrual cycles but no pregnanediol was excreted at any time of cycle. Showed Krauritus vulvae. No stigmata of Addison's disease. Present B. M. R. minus 17. All urine collections made as an out-patient. On July 12-13, 1946, the 17-ketosteroids were 16.6 mg., creatinine 1.1 mg. and glycogenic bioassay was less than 10 g.

On August 27-29, the 17-ketosteroids were 13.6 mg. the creatinine 0.9 mg., and the glycogenic bioassay was 24 g.u. A later collection on September 16-18 showed a glycogenic bioassay of 36 g.u. and it was concluded that she did not have Addison's Diseas.e

No. 35 - E. L. (female) Royal Victoria Hospital Ward No. 46-12827. Admitted September 1946.

Diagnosis: Anorexia secondary to malnutrition and anxiety.

Age 30 years. Height 62 inches; weight 88 lbs. Blood pressure 138/80. Normal weight 115 lbs. Worries and poor appetite for about 12 months. Miscarriage in April 1946, and skin became generally darker thereafter, but no buccal pigmentation. Hair normal. Blood sodium 140 m.eq./l; potassium 5 m.eq./l; Sugar curves within normal limits; N.P.N. 22 mg%. B. M. R. minus 14, on September 17th. Improved on high-protein diet. Weight 97 lbs. on October 18th. Urine collected September 22-23, while bed-patient on ward.

No. 36 - (female)F. W. - Royal Victoria Hospital O. P. D. No. B-6396. Age 41 years; height 60 inches; weight 115 lbs.

Diagnoses: Scleroderma and possible Addison's disease? Bronchiectasis? Stiffness and brownish pigmentation of skin began in 1940. Chronic cough. Claw-hands. Cold feet. Blood pressure 110/76. No mucosal pigmentation. Blood sodium 141 m.eq/l. Glucose curve was low normal. Urine glycogenic bioassay normal in 1945. Unimproved by testosterone or stilboestrol therapy. No endocrine therapy during urine collection made September 1946 as out-patient.

No. 37 - D. M. (female) Royal Victoria Hospital Ward No. 46-13601. Admitted September 25, 1946.

Final Diagnosis: Hypernephroid tumor of left ovary. Age 46 years; height 66 inches; weight 148 lbs. Amenorrhoea for 11 years. Masculine growth of hair on face, limbs, abdomen for 6 years. Apical baldness increased muscular strength, though readily fatiguable and lost 12 lbs. weight. Clittoral hypertrophy. Husky voice. Haemoglobin 17.2 G%. Blood sugar curve normal. X-rays of chest, sella and spine normal. 17-ketosteroids slightly high (max. 22.8 mg. per day).

The urine collection studied by chemical "corticoid" method was collected on October 7th - 8th, in hospital ward, but daily creatinine was only 1. 1 G. compared to her usual level of 1.6 G. daily. On October 24th, exploratory laporatory showed the left ovary weighed 10 G. and contained a "hypernephroid tumor". The left adrenal was also removed, but proven normal histologically. Satisfactory post-operative course.

No. 38 - D. V. (female) Patient in Notre Dame Hospital No. 8622-46, under the care of Dr. C. E. Grignon.

Diagnosis: Acanthosis nigricans. Age 23 years; height 65 inches; weight 125 lbs. Blood pressure 100/60. She complained of slight asthenia, and showed only a generalized brownish pigmentation which did not involve the mucosa. Normal hair growt' and menses. No crises. Blood sugar and sodium were normal. The Kepler "water test" and the Wilder test were normal. She had received no hormone therapy prior to the urine collection in the hospital wards.

No. 39 - E. N. (female) Royal Victoria Hospital O. P. D. No. F-6056.

Diagnosis: Over-development obesity syndrome, without tumor. Age 10 years; height 56 inches; weight 138 lbs. Axillary hair, breast development and obesity began over 18 months ago. Menses began January 1946 (aged 10 years). Now breasts and hair like adult. Bone age of 17 years. X-ray sella normal. Fields of vision were generally constricted (cause?). Both urine collections for assays made through out-patient department.

No. 40 - A.Ge. (female) Royal Victoria Hospital Ward No. 46-5635. Admitted March 1946,

Diagnosis: Hyperthyroidism. Age 49 years; height 60 inches; weight 114 lbs. Amenorrhoea since last pregnancy in 1934. Thyroidectomy in 1936, for nodular toxic goitre which later recurred. Brownish pigmentation of skin, but none on mucous membranes. No axillary hair, scant pubic hair. Recent weight loss and nervousness. Blood pressure 120/70; B. M. R. +46. Cholesterol 106 mg%. Serum Na 142 m.e.g./l. Rx. thiouracil 0.2 G. b.i.d. started March 25th. Urine collection from ward four days later. On April 23, 1946, re-admitted for surgical removal of recurrent nodular goitre.

No. 41 - N. S. (female) Royal Victoria Hospital O. P. D. No. F-2726.

Diagnosis: Essential hypertension; Age 46 years; height 64 inches; weight 184 lbs. No definite symptoms prior to 1941 when blood pressure 192/100. Systolic cardiac murmur. X-ray chest negative. Urine, trace albumin. E. C. G. impaired. Ammonium chloride therapy 6 g. daily begun in April 1946, prior to urine collection as an out-patient. Present Blood pressure 210-230/100.

No. 42 - L. F. (female) Royal Victoria Hospital Ward No. 46-6595. Admitted May 1946.

Diagnosis: (1) Probable adenoma of pituitary (2) Hypertension.

Age 37 years: Height 62 inches: weight 146 lbs. Blood pressure 220/140. Left temporal headache since December 1945. Menstruated only between ages of 16 and 23 years. Not since. Urinalysis and Mosenthal test normal. Hb. 13.2 G%. Blood sugar curve normal. X-rays of chest and spine normal X-ray of sella was large, suggesting an expanding intrasellar lesion. Left papilloedema. Fields normal. Electro-encephalogram and pneumo-encephalogram did not localize the intracranial abnormality well. Urine collections studied were obtained while an up-patient on the ward.

No. 43 - R. V. (female). Patient in Notre Dame Hospital, No. 5346-46, under the care of Dr. C. E. Grignon.

Diagnosis: Obesity. Diabetes mellitus.

Age 49 years; height 66 inches; weight 298 lbs. Blood pressure 220/100. Symptoms were polyuria, polyphagia and gain in weight. Still having regular menses when studied. No virilism. No osteoporosis. Hair normal. Fasting blood sugar 420 mg%. X-ray sella normal. Clinically not Cushing's Syndrome. Urine collection made while on ward before the glycosuria was controlled.

No. 44- E.McK. (female), Royal Victoria Hospital Ward No. 46-16214 (November 1946).

Diagnosis: Malignant Hypertension. Age 24 years; height 67 inches; weight 135 lbs. Albuminuria began in 1941, and hypertension in 1943. Right nephrectomy in February 1943, showed malignant nephrosclerosis. Bilateral supra-diaphragmatic splanchnic neurectomy performed in December 1943. Blood pressure again rose to 220/120. Mosenthal test showed poor variation in specific gravity. NPN 45 mg%. No treatment during urine collection through O. P. D. in June 1946.

No. 45 - H. C. (male). Cleveland Clinic, Cleveland, Ohio.

Diagnosis: Cushing's Syndrome. Age 11 years; height 52 inches; weight 119 lbs. For past year plethora, moon-face, obesity. Osteoporosis of lumbar spine; striae of skin; scant pubic and axillary hair. R. B. C. 6.25 million. X-ray of sella normal. No endocrine therapy prior to urine collections. Later bilateral explorations of adrenals made, but no gross abnormalities found and no tissue removed.

No. 46 - H. R. (Male). Royal Victoria Hospital No. 46-142221. Admitted to wards October 8, 1946.

Diagnosis: Cushing's syndrome. Age 31 years; height 71 inches; weight 286 lbs. In 1939 he weighed 185 lbs., gained to 252 lbs. in 1941, and reached 335 lbs. in 1945 when reducing diet instituted. A child was born in 1941. Libido and general strength have decreased. Urinary tract infection noted early in September 1946. Examination showed plethora, round face, red striae on body, normal hair. X-rays of skull and long bones normal. Blood pressure 156/104. Fasting blood sugar 95, one hour 215; two hour 212; three hour 99. Urine collection here reported was collected in metabolic ward while patient was not under endocrine therapy. Several other collections also showed very high results by glycogenic bioassay.

No. 47 - J. S. (female). Cleveland Clinic, Cleveland, Ohio.

Diagnosis: Cushing's Syndrome. Age 39 years. Hypertension and protuberant abdomen noted one year ago. Plethora and facial hirsutism in past 6 months. Blood pressure 200 systolic. Diabetic type of glucose tolerance curve. Little bony changes. Serum sodium 147 - 154 m.eq/l, and potassium 2.3 - 3.9 m.eq/l. X-ray of sella normal. The right adrenal was removed and was $2\frac{1}{2}$ times normal size, containing an adenoma. The urine collection was two weeks post-operative.

No. 48 - A.Go. Patient in Notre Dame Hospital, No.4260-46. under the care of Dr. C. E. Grignon.

Diagnosis: Adrenal tumor. Chronic myocarditis. Age 41 years; height 61 inches weight 187 lbs. Blood pressure 170/110. Mother and sister shave daily. Patient began to shave at 15 years. Married at 22 years. Never pregnant and little libido though menses always regular. Weighed about 150 lbs. at age 22 years. Five recent hospital admissions for cardiac failure. Voice is deep. Habitus and musculature are masculine. Has excessive arm, trunk and suprapubic abdominal hair, but no apical baldness. No acne. No striae. Breasts poorly developed. Moderate clittoral hypertrophy. X-ray showed no osteoporosis. E. C. G. showed left bundle branch block. Blood glucose curve was normal. B. M. R. plus 7. No operations have been done. Both urine collections studied were made on wards and no preservatives were added. She was not under endocrine therapy though under digitalization.

No. 49 - W.L.(male). Bed patient in Royal Edward Laurentide Sanitarium.

Diagnosis: Genito-urinary and pulmonary tuberculosis. Addison's Disease. Age 48 years; height 70 inches; weight 150 lbs. Blood pressure 120/60. Long history of generalized tuberculosis, but only acutely ill during past year. Generalized and mucosal Pigmentation apparently of recent development led to suspicion of Addison's disease. No treatment prior to collection (a) in May 1946, which was extracted promptly after arrival. He received desoxycorticosterone 10 mg. daily, but no cortical extract, prior to and during collection (b) in July 20 - 21. The latter collection was stored in the refrigerator for two weeks and became alkaline prior to extraction. He died on July 28, 1946 and autopsy showed complete destruction of both adrenal glands by tuberculosis.

No. 50 - A. M. (male) Patient in Notre Dame Hospital No. 4801-46, under the care of Dr. C. E. Grignon.

Diagnosis: Pituitary tumor with panhypopituitarism. Age 49 years; height 65 inches; weight 140 lbs. Blood pressure 108/82. Married at age 32. No children. Loss of libido and weakness, began 9 years ago. Marked weakness for one year. No pubic or axillary hair. Moderately developed genitalia. Optic atrophy and hemianopsia. X-ray showed enlarged sella turcica. B. M. R. varried from minus 33 to minus 22. Cholesterol 229 to 347 mg%. Blood sugar 100 mg%. Sodium 136 m.eq. per litre. Testicular biopsy showed atrophy of tubules and interstitial cells. He developed a crisis when not on adreno-cortical therapy. Collection (a) without added preservatives was made in June on the hospital wards when he was receiving no hrmonal or other therapy. Early in September 1946, he had fever and received penicillin. During collection (b) on September 10-12 he was receiving thyroid, methyl testosterone, and also desoxycorticosterone 5 mg. every second day. He was not receiving cortical extract or alkali. The alkalinity of both the above collections remains a mystery, but was possibly due to accidental contamination during collection, as a urine sample in November was said to show pH 5.5.

No. 51 - C. M. (female) Royal Victoria Hospital ward No. 46-10569. Admitted July 1946.

Diagnosis: Addison's Disease. Age 32 years. Height 61 inches; weight 96 lbs. Onset weakness and brownish pigmentation of skin and mucosa in 1944. Best weight about 105 lbs. Childbirth in 1938. Urinary corticoids (bioassay) less than 10 units on 4 occasions since 1944, and 17-ketosteroid likewise low (between 2.0 and 4.5 mg. daily). Blood pressure 80/48. Blood sodium

126 m.eq/l. Blood glucose curves flat. X-ray chest clear. Receiving Adrenal Cortical Extract 2 cc. (hypo) daily during collection of urine, as an up-patient in hsopital.

No. 52 - A. E. (female) Private patient of Dr. J. S. L. Browne. Diagnosis: Scleroderma. Age 32 years; height 63 inches; weight 122 lbs. Onset of scleroderma in 1932, aged 16 years. Lupus erythematosus diagnosed in 1938. Treated by sympathectomy in 1940, at Mass. General Hospital. Childbirth in August 1945. Examination shows extensive scleroderma and dark pigmentation of face and scalp. Blood pressure 110/70. No symptoms of adrenal insufficiency. Received stilboestrol 1 mg. daily from June to August 1946, when urine collection made as an out-patient. At least two previous bioassays in 1945-46, showed less than 10 g.u.

No. 53 - C. V. (female). Royal Victoria Hospital, ward No. 46-4531. Admitted April 1946.

Diagnosis: Hypertension. Age 56 years; height 64 inches; weight 210 lbs. Gradual weight gain for 25 years, and increased blood pressure for 9 years. Dyspnoea and substernal pain 4 years. Blood pressure after rest 180/110, but fell to 135/78, with sod. amytal. Blood NPN normal but some evidence of renal functional impairment. Discharged on NH_4Cl therapy. Blood pressure 234/124 in June 1946. Urine (a) collected on wards and (b) through O. P. D.

No. 54 - A. B. (female). Montreal General Hospital No. 6673-46. Admitted October 1946.

Diagnosis: Nephritis with hypertension. Renal calculi. Diabetes. Hirsutism. Age 50 years; height 54 inches; weight 139 lbs. Blood pressure 180/100. Blood sugar 300. Born near Naples, Italy. Facial Hirsutism began at 17 years. Had six children. Amenorrhoea at age 40 years. Lost 35 lbs. in past 3 years when weakness and dyspnoea began. Very poor renal function.

Cataracts. Mild diabetes controlled by diet alone. Hair distribution normal on body but excessive on face. No temporal or apical baldness No clittoral hypertrophy. No striae. Urine collection October 26-27, made while ward patient.

No. 55 - C. W. D. (male) Admitted wards of Royal Victoria Hospital, March 1946 - No. 46-4080).

Diagnosis: Hypertensive heart disease. Anxiety State. Slight dyspnoea. Blood pressure 263/130. Did not fluctuate much with sodium amytal. Urine f.tr. albumin. Blood NPN 23.8 mg. Sodium 324 mg%. Rx. ammonium chloride 6 G. daily begun on April 4th. Recently Blood Pressure 248/130. Urine (a) collected on wards: (b) collected through O. P. D.

No. 56 - L.C.Mc.(male) Royal Victoria Hospital - Ward No. 46-5582. Admitted April 1946.

Diagnosis: Buerger's Disease. Age 50 years; height 68 inches; weight 187 lbs. intermittent claudication left claf 1½ years. No dyspnoea. Blood pressure 160/85, falling to 130/80 in hospital. Urinalysis negative. Urine collection (a) made as an out-patient. (b) and (c) collected as up-patient in hospital.

No. 57 - V. R. (female). Royal Victoria Hospital ward No. 46-4734. Admitted April 1946.

Diagnosis: Arthritis, ?chronic rheumatoid or ? acute rheumatic. Age 24 years. Intermittent migrating point pains and fever for 4 years. Blood pressure 108/70. No cardiac signs. Temperature 99^{6} for 2 days. Pulse 90. Sed. rate accelerated. Received salicylates with soda from April 5 to May 1. Urines (a) and (b) collected as bed-patient in hospital. Other daily volumes were about 500 cc. at this period.

No. 58 - E. D. (female) Patient was admitted on June 6, 1946, to the Notre Dame Hospital No. 5245-46, under the care of Dr. C. E. Grignon. Clinical Diagnosis: Probably Addison's Disease.
Age 38 years; height 62 inches; weight 103 lbs. Skin pigmentation began 5 years ago and asthenia and dyspnoea 3 years ago. Her last pregnancy was in 1942, and last menses on May 27, 1946. On June 6, 1946, she was admitted in shock with unobtainable blood pressure. She received 36 cc. of cortical extract and 8000 cc. of intravenous fluids during the first urine collection (a and b). She made a good clinical response. During a second collection (c and d) one week later she was receiving only desoxycorticosterone acetate by injection and salt. There was no mucosal pigmentation. The glucose tolerance curve was normal. It was found that she could be maintained on salt alone in hospital without further collapse. She did not report to the hospital during the five months after discharge.

No. 59 - Y. H.(female) Royal Victoria Hospital O. P. D. No.C-6690. Diagnosis. Intermittent hydrarthrosis and pregnancy ($5\frac{1}{2}$ mos.)
Age 30 years. Height 63 inches; weight 163 lbs. Several months bed-confinement for rheumatism at ages 7 and 17 years. In past year fleeting pains and swellings of small joints following beer ingestion, but no symptoms followed this drink under experimental observation. No definite allergic history otherwise. Blood pressure 118/70 and routine urinanalysis normal prior to assay, which was collected as out patient. Normal delivery October 22, 1946.

No. 60 - E. M. (female). Royal Victoria Hospital

O. P. D. No. B-6775.

Diagnosis: Acromegaly. Age 49 years; height 65 inches; weight 160 lbs. Bitemporal headaches began at age of 16 years, and thickening of face, hands feet at 38 years. Amenorrhoea began at 39 years. High glucose curves. Blood pressure 160/80. Urinanalysis negative. In 1943, X-ray of sella was grossly enlarged to 1.8 x 2.1 cm. and X-ray of fingers showed tufting. No treatment accepted. Symptoms unchanged in 1946. Fields of vision normal. Sella large. No endocrine therapy prior to urine collection in June 1946 as out-patient.

No. 61 - S. (female) Patient of Dr. J. E. Howard in Johns Hopkins Hospital.

Diagnosis: Cushing's Syndrome. Also intermittent claudication.

Age 39 years; Height 62 inches; Weight 150 lbs. Blood pressure 150/95. Onset at 36 years of age with developement of typical habitus, obesity, amenorrhoea, bruisability, and striae. However, chief complaint was intermittent claudication associated with marked sclerosis in legs. Physical examination as above with mild hirsutism, no clittoral enlargement and normal pelvic organs of post-menopausal type. Pyelogram showed that left kidney was lower than right. Marked generalized osteoporosis including the skull. Blood sugar curve: fasting = 94, 1/2 hour = 212; 1 hour = 252; 2 hours = 176; 3 hours = 94. On July 16, 1946, the 17-ketosteroids = 10.8 mg. The 48-hour urine collection assay was considered complete. An aliquot was shipped to Montreal with 5 cc. chloroform as preservative, but was 2 weeks in transit without refrigeration. No exploratory operations had been made and no hormonal therapy instituted.

No. 62 - R. M. (female) Patient in Johns Hopkins Hospital under the care of Dr. J. E. Luetscher, Jr.

Diagnosis: Addison's Disease.

Patient had had this disease for many years. Treatment was desoxycorticosterone acetate by injection at the time of urine collection which was shipped to Montreal in same way as No. 61.

No. 63 - A. (female) Patient in Johns Hopkins Hospital under the care of Dr. J. E. Luetscher, Jr.

Diagnosis: Addison's Disease.

Her symptoms were considered classical, but it is known that her glucose tolerance curve "was not strikingly abnormal". Treatment desoxycorticosterone acetate only at the time of study. The urine collection was shipped to Montreal under the same conditions as No. 61.

No. 64 - F.G. (female) Patient in Johns Hopkins Hospital under the care of Dr. J. E. Luetscher, Jr.

Diagnosis: Addison's Disease.

This disease was of long standing, and she was only being treated by desoxycorticosterone acetate by injection at the time of study. The urine collection was shipped to Montreal under the same conditions as No. 61.

No. 66 - D.G. Children's Memorial Hospital Ward 46-1206. Admitted April, 1946.

Diagnosis: Still's Disease (rheumatoid arthritis).

Age 3-4/12 years; Weight 31 lbs. Patient had had bouts of fever and generalized arthritis for 1 year. Heart normal. Blood pressure 100/72. Elevated sedimentation rate. Afebrile at time of urine collection in hospital but collection was known to be incomplete.

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