URINARY FORMALDEHYDOGENIC CORTICOID AS DETERMINED AFTER ENZYMATIC HYDROLYSIS IN NORMAL SUBJECTS AND IN PATIENTS WITH ADRENAL AND HYPERTENSIVE DISEASE

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Corticosteroids appear in urine in free form and as conjugates. Most methods of assay of urinary corticoids involve acidification to pH 1 prior to extraction (1); increased yields thus obtained may be attributable to hydrolysis of steroid sulfates (2) since glucuronides are resistant to hydrolysis at pH 1 and room temperature. Treatment of urine with glucuronidase causes a further large increase in yield of both reducing (3) and formaldehydogenic (4, 5) corticoids because of hydrolysis of corticoid glucuronides. Measurement of urinary formaldehydogenic corticoids after enzymic hydrolysis should include all or nearly all the urinary content of C-21 steroids carrying α-ketol or α-glycol side chains.

Conditions of hydrolysis of urinary corticoid glucuronides have therefore been examined and procedures developed and applied to the assay of urines of normal subjects, of patients with essential hypertension, and of patients with diseases which affect the function of the adrenal glands. The two procedures used are similar in principle; they vary in detail and were developed and used independently; specimens were exchanged between the two laboratories and analyzed by both methods.

CONDITIONS OF HYDROLYSIS

Orienting experiments indicated that, using calf spleen β-glucuronidase (Viobin), maximal yields of formaldehydogenic corticoid (FC) were usually obtained after 48 hours' incubation at 37° C of a mixture of urine, buffer and enzyme to which chloroform was added as a preservative. Addition of penicillin and streptomycin did not enhance yields. In most of these experiments, the concentration of enzyme was 150 Fishman units per cubic centimeter of urine. The increments of FC with time did not exhibit the logarithmic progression characteristic of the action of the enzyme on a pure substrate (6), probably because of differences in rates of hydrolysis of the several corticoid glucuronide substrates which were ultimately measured together as FC.

The relatively slow release of FC in urine from glucuronide combination contrasts with the rapidity of the enzyme's action on pure steroid glucuronide substrates (7) and suggested to us that an enzyme inhibitor might be present in urine. After 1 hour, yields of phenolphthalein from incubation of phenolphthalein glucuronide, enzyme and urine were 40 to 70 per cent of those found in incubations of enzyme and substrate in water; the inhibition of the enzyme by the urine was roughly proportional to urinary specific gravity. It seemed that glucuronic acid, free or combined, might be the solute concerned, since the free acid is known to inhibit the action of β-glucuronidase (8), and noncorticoid glucuronides would compete for the enzyme. This assumption is supported by data which demonstrated the correlation between urinary inhibition of enzyme action and the concentration of free plus combined glucuronic acid in urine. However, the method used (9) does not discriminate between glucuronic acid and glucuronide.

Fortunately, the inhibition of enzyme action by urinary glucuronide or glucuronic acid does not interfere with complete hydrolysis of phenolphthalein glucuronide when the incubation is sufficiently prolonged.

The course of the hydrolysis can be speeded by increasing the temperature of incubation; maxi-

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mal yields of FC were obtained by incubation of urine, buffer and enzyme for 24 hours at 50° C (Procedure 2, below).

PROCEDURES

Principle. Hydrolysis of corticoid glucuronide with calf spleen β-glucuronidase, subsequent acidification and extraction of the mixture with chloroform yield what is assumed to be total FC (TFC); acidification and extraction of another appropriate aliquot permits determination of the FC fraction arbitrarily termed "free" (FFC), although it may include some FC derived from conjugates other than glucuronides; the difference between TFC and FFC in milligrams is the yield of corticoid derived from glucuronide (FCG). The values are calculated in terms of desoxycorticosterone.

Procedure 1

Reagents: 1. β-Glucuronidase (Viobin), 25,000 Fishman units per gram.
2. Acetate buffer 1.0 M, pH 4.5 (6).
3. Chloroform C.P., freshly redistilled over potassium carbonate.
4. Other reagents as described for determination of FFC (10).

Apparatus: 1. Detergent-free, 125 cc. Erlenmeyer flasks.
2. Incubator, 37° C.
3. Automatic flask shaker (Burrell and Co., Pittsburgh).
4. Pipet and distillation apparatus (11).

Procedure. A 25 or 50 cc. aliquot of a 12 or 24 hour urine specimen is selected, the choice depending on the volume of the specimen, and transferred to a 125 cc. flask. The pH is brought to about 4.5 with HCl and secured at this level by addition of 0.1 volume of acetate buffer. Chloroform (10 cc.) is added, and 300 mg. (7,500 U.) of enzyme preparation admixed; the flask is closed with a foil-covered stopper and incubated at 37° C for 72 hours. The mixture is then brought to pH 1 with HCl and at once extracted with chloroform. Extraction and further procedures are as described by Corcoran and Page (10) appropriately adjusted for the smaller quantities of urine extracted.

Blank determinations are run in parallel with the tests on reagents, including enzyme. The concentration of FC is estimated from the difference in color density (D) of test and blank samples. Large inaccuracies may arise from this subtraction and multiplication of the result when color density of the test sample is low and urine volume large. When D as measured in a Coleman spectrophotometer, model 6A, is less than 0.1, the determination is usually repeated on a larger aliquot of urine; only exceptionally, as in cases of Addison's disease, have D values of 0.05 or less been accepted. We have taken a D of 0.025 as representing the possible maximal accumulation of errors in instrumentation and procedure. This color density corresponds to 0.018 mg. of desoxycorticosterone in the sample. Values reported hereinafter have been evaluated in terms of this potential error.

Procedure 2

Reagents: 1. Chloroform and β-glucuronidase (see Procedure 1).
2. Absolute alcohol. Commercial absolute alcohol distilled from 2,4 dinitrophenylhydrazine.
3. Periodic acid, 0.05 M in 0.3 N H_2SO_4.
4. Stannous chloride, 1.5 g. $SnCl_2 \cdot 2H_2O$ in 25 cc. of water containing 4 or 5 drops of concentrated hydrochloric acid. Prepare fresh daily.
5. Chromotropic acid. One gram recrystallized [2] sodium 1,8-dihydroxy-3,6-disulfonate is dissolved in 5 cc. of water in a 500 cc. Pyrex volumetric flask, 416 cc. of concentrated sulfuric acid is added cautiously with cooling, and the solution finally is diluted to volume with water. When stored in a brown glass-stoppered bottle the reagent prepared from the recrystallized salt will keep for two weeks without an appreciable increase in the blank.

Procedure. An aliquot of 1 to 2 per cent of the urine collected during a 24 hour period, but not more than 25 cc., is placed in a 50 cc. beaker. The aliquot is diluted to 25 cc. if necessary, and with the aid of a glass electrode pH meter the pH is adjusted to 4.8 with a 10 per cent solution of acetic acid. After addition of 6,000 units (240 mg.) of β-glucuronidase (3 cc. of a solution of the Viobin preparation, 2,000 units per cc. in 0.45 per cent sodium chloride) and 10 cc. of chloroform, the beaker is covered with a watch glass and placed in an incubator at 50° C. At the end of 24 hours, the mixture is diluted with 50 cc. of water and the aqueous phase is extracted by hand shaking in a separatory funnel four times with 20 cc. portions of chloroform. The chloroform extract is washed with a 5 per cent solution of sodium carbonate and then with water until neutral. Each aqueous extract is backwashed with a little chloroform which is added to the main extract. The chloroform is removed in an all-glass vacuum still, keeping the temperature of the water bath below 50° C. The residue is dissolved in 0.5 cc. of purified absolute

[2] Dissolve 10 g. practical grade sodium 1,8-dihydroxynaphthalene-3,6-disulfonate (Eastman No. P230) in 25 cc. of water by warming. Add about 0.2 g. Na_2SO_3, 5 to 10 drops of concentrated HCl and 1 g. activated charcoal. Heat on the steam bath 10 to 15 minutes. Filter hot with suction through a pad of infusorial earth and wash the filter with a little hot water. Warm the filtrate on the steam bath while slowly adding with stirring 200 cc. of acetone. If the acetone is added too rapidly at first the substance may separate as an oil. Once crystallization has started the acetone may be added more rapidly. Cool, filter, and dry the product in a vacuum desiccator over sulfuric acid.

TABLE I

*Urinary formaldehydogenic corticoids in normal subjects**

a. Procedure 1

	Males			Females			Both sexes	
	No.	Mean	Range	No.	Mean	Range	No.	Mean
FFC, mg.	17	1.2	0.34– 2.5	11	1.22	0.43– 2.1	28	1.22
TFC, mg.	20	24	7.1 –62	15	16.6	4.9 –33	35	21
FCG/FFC	17	19	8.0 –41	11	14	5.0 –27	28	17

b. Procedure 2

FFC, mg.	15	0.66	0.40– 0.86	8	0.66	0.46– 1.06	23	0.66
TFC, mg.	30	16.2	5.5 –39.0	18	13.5	6.8 –32.4	48	15.1
FCG/FFC	15	27.2	9.7 –52.7	8	28.5	12.3 –71.4	23	27.7

* Values obtained by each procedure in normal subjects. FFC is "free" formaldehydogenic corticoids in milligrams per 24 hours; TFC is urinary formaldehydogenic corticoid output as determined after enzymatic hydrolysis; FCG is the portion of TFC taken to be present as glucuronide; the ratio FCG/FFC demonstrates the increment in urinary corticoid yield obtained by enzymatic hydrolysis.

alcohol and 4.5 cc. of water is added. To a 2 cc. aliquot in a 50 cc. round-bottom flask with standard taper joint are added 2 cc. 5 N sulfuric acid and 1.0 cc. periodic acid. After 60 minutes at room temperature, excess periodic acid is destroyed with 1.0 cc. of stannous chloride, the formaldehyde is distilled and color is developed essentially according to the procedure of Corcoran and Page (10). An unoxidized blank is prepared by addition of 2 cc. 5 N sulfuric acid and 1 cc. of stannous chloride to another aliquot of the extract, then 1 cc. of periodic acid, followed by distillation. The distillate (approximately 5 cc.) is collected in a 10 cc. volumetric flask and diluted to 10 cc. Aliquots of 2 cc. are transferred to glass-stoppered test tubes and 5 cc. of chromotropic acid reagent is added. The color is developed by heating 0.5 hr. at 100° C.

The same procedure was used for determination of FFC except that the urine was acidified to pH 1, allowed to stand overnight at room temperature, and then extracted with chloroform.

Procedure 2 differs from Procedure 1 considerably in some respects. The enzyme is dissolved in 0.45 per cent sodium chloride solution. No extra buffer is added, but the pH has been checked repeatedly after incubation and found to be unchanged. Hydrolysis is accelerated by incubation at 50° C., and the enzyme remains active at this temperature. Under these conditions, the enzyme shows maximal activity at pH 4.8. From 82 to 105 per cent of cortisone added to the urine before incubation has been recovered. The average recovery in 16 experiments was 91 per cent. The incubation mixture is extracted at pH 4.8. This procedure may result in loss of the small amount of extra FC that ordinarily can be extracted at pH 1. However, the amount is negligible in comparison with the large amounts of FC liberated by β-glucuronidase. Cleaner extracts and more consistent results were obtained by extracting at pH 4.8.

TABLE II

Urinary formaldehydogenic corticoids of patients having Cushing's syndrome

Patient	Sex	Age, years	FFC,* mg. per 24 hours	TFC,* mg. per 24 hours	Remarks
1	F	15	1.3	17.9	Tumor
2	F	25	3.4	27.8	Tumor
3	F	27	1.1	14.6	Hyperplasia
4	F	34	2.5	9.0	Hyperplasia
5	F	37	1.7	13.1	Hyperplasia
6	F	21		45.0†	Carcinoma
7	M	30	2.0	24.4	Hyperplasia
8	M	31	1.4	23.4	Hyperplasia
9	M	40	2.0	45.0	Hyperplasia
10	M	50		21.4†	Hyperplasia
11	M	38	15.3	140.0†‡	Hyperplasia

* FFC, "free" formaldehydogenic corticoids. TFC, corticoids after enzymic hydrolysis.
† Procedure 1.
‡ Average of 3 determinations.

RESULTS

Normal subjects. Procedure 1—Analyses of 12 hour night, 24 or 48 hour urine specimens from laboratory and staff personnel carrying on their usual activities are summarized in Table I, a. Values of FFC in this series correspond generally with those previously reported (12); the difference previously observed as between males and females is not confirmed, possibly because of the smaller group examined. TFC outputs average 15 to 20 times those of FFC; these outputs are greater in males than in females. The proportion of corticoid excreted as glucuronide (FCG) to that excreted as FFC is widely variable.

Procedure 2—The normal subjects were similar to those described under Procedure 1. It will be noted that values (Table I, b) for FFC are distinctly lower than those obtained by Procedure 1, and the range of values is smaller. The reason

for these differences is not apparent. Probably because of the smaller values for FFC, the average ratio FCG/FFC calculated from the results of Procedure 2 is greater than that derived from the results of Procedure 1, although the average values of TFC and FCG are somewhat less. In general, results with Procedure 2 are of the same order of magnitude as those of Procedure 1.

Adrenal diseases. Cushing's syndrome, whether associated with hyperplasia or tumor of the adrenal cortex, is now considered to be a manifestation of overproduction of adrenocortical hormones. It would be expected, therefore, that an increased excretion of urinary corticoids would accompany this syndrome; indeed, an increase of FFC above the normal level is observed often. In those cases without a significant increase in FFC it was hoped that TFC would be increased much above the normal level, thereby facilitating the diagnosis of cortical hyperfunction. The results given in Table II show that, in general, this hope was not realized. The values for FFC were elevated definitely in 6 of the 9 cases for which data are available (cases 2, 4, 5, 7, 9, and 11); TFC could be considered to be definitely increased above the normal level only in cases 6, 9, and 11. However, the mean value of TFC (21.9 mg.) in the 8 cases in which procedure 2 was used, was increased above the mean of the normal values (15.1 mg.) determined by this procedure. Thus, it appears that FFC more often than TFC is indicative of adrenocortical hyperfunction associated with Cushing's syndrome.

Our data on untreated Addison's disease are few. In 3 such cases the values for FFC were 0.26, 0.32 and undetermined, and for TFC 2.2, 2.8 (Procedure 2) and 1.6 mg. (Procedure 1) per 24 hours, respectively. In one other case in which the disease evidently was not severe since the patient had maintained normal activities and had passed through two pregnancies uneventfully after the onset of pigmentation, the output of FFC was 0.28 and of TFC 22.0 mg. per 24 hours.

Essential hypertension. Nearly all these specimens were obtained from patients hospitalized in the ward of the Research Division, Cleveland Clinical Hospital, because of severe essential or, in some cases, malignant hypertension. The 80 determinations (Procedure 1) summarized in Table III are from 24 hour urine samples of 35 patients, of whom 16 were female; only 5 of the 80 determinations were done on specimens from patients whose renal failure had advanced sufficiently to cause azotemia.

The levels of FFC, in accord with former observations (12) are often increased and widely variable. The view was then expressed that this abnormality might be due to disproportionate outputs of FFC, rather than to increment in total corticoid. That this is the case is confirmed by the finding that outputs of TFC are not increased; thus, the increased outputs of FFC sometimes observed are associated with decreases in the ratio FCG/FFC.

Reproducibility of analyses. Duplicate samples analyzed by procedure 1 were found to agree within ± 15 per cent and usually within ± 10 per cent of the mean of the values obtained. Agreements in cortisone recoveries by Procedure 2 as noted earlier in this paper were within about 10 per cent of the mean. While each procedure thus seemed relatively satisfactory, it seemed desirable to test reproducibility severely by an exchange of samples between laboratories.

Results of this comparison are listed in Table IV. Among 23 specimens exchanged, 8 agree within 15 per cent and 14 within 24 per cent. The wide percentile variability between some of the remaining samples does not seem to be systematic, since the distribution is random. Making allowance for inherent methodological error equivalent to a color density of 0.025 on the galvanometer

TABLE III

*Urinary formaldehydogenic corticoids in hypertensive patients**

	Males			Females			Both sexes	
	No.	Mean	Range	No.	Mean	Range	No.	Mean
FFC, mg.	11	1.67	0.55– 5.0	20	2.2	0.54– 7.5	31	2.0
TFC, mg.	49	15.7	3.3 –41	31	15.3	3.3 –39.0	80	15.5
FCG/FFC	11	17	2.9 –47	20	13	1.5 –47	31	14.0

* Analyses by procedure 1. Terms as in Table I.

TABLE IV

*Comparisons of analyses by each procedure**

Patient	Procedure 1				Procedure 2				
	1	2	M	E	1	2	3	M	E
1	6.7			0.7	6.4	5.8		6.1	
2	7.8	7.3	7.5	2.2	5.8	6.0	6.2	6.0	
3	7.1			1.2	6.3	7.0	6.7	6.7	
4	29			1.5	27.0				4.5
5	28			1.7	24.4				3.7
6	25.4			1.2	21.3				3.7
7	15			1.0	13.1				2.9
8	16.8	14.2	15.5	0.9	15.3	16.7		16.0	1.8
9	21			1.2	21.6	19.5		20.6	1.8
10	18.2			1.0	19.4	21.0		20.2	1.6
11	14.8			1.3	12.7	12.8		12.8	2.0
12	1.7			0.9	2.2				
13	8.3	8.6	8.4	1.7	10.8	9.1	12.4	10.8	2.7
14	16.5			1.0	11.7				
15	24.4			0.7	16.3				1.3
16	5.5			0.5	2.8				
17	47.4			1.2	27.8				1.7
18	8.2			1.0	14.8				3.1
19	11			1.6	12.7	14.7		13.7	2.3
20	34			1.7	25.2	25.2		25.2	1.7
21	21.1			1.7	29.4	27.2		28.3	1.6
22	8.4			1.3	21.0				4.1
23	27.3	26.4	26.8	1.8	16.4	13.5	18.4	16.1	2.0

* Results of independent analyses of urine for TFC by each procedure expressed in milligrams per 24 hours. Numerical headings indicate the number of replicates analyzed, and M indicates the mean of these or the result of a single estimate. E is the potential error of each analysis as defined in the text and expressed in milligrams per 24 hours.

scale, 14 of 21 samples (excluding Nos. 14 and 16) agree completely. The desirability of duplicate rather than single analyses is suggested by the fact that in 4 instances of wide variation, single analyses were done by each procedure and in 2, by Procedure 1, with duplicates available by Procedure 2. In 2 instances (samples 14 and 16) in which the potential errors of Procedure 2 were not calculated, the absolute differences in TFC yields are relatively small—respectively, 4.8 and 2.7 mg. per 24 hours; single analyses only were available from each laboratory. The disparities in the cases of samples 17, 22, and 23 may be systematic; this interpretation is suggested in the case of sample 23 by the close intrinsic agreement between duplicates by Procedure 1 and triplicates by Procedure 2 and the disparate mean values obtained.

COMMENT

FC increment. The procedures confirm the presence in urine of amounts of TFC greatly in excess of those of FFC. Yields of reducing corticoids (RC) are similarly augmented by either enzymatic (3) or acid (13) hydrolysis; enzymatic yields of RC increase only "3 to 5 times" and with acid "4 to 5 times"; enzymatic hydrolysis in our hands increased yields of FC about 17-fold (range 5 to 70) and, in the hands of Cox and Marrian (5) "about 10 times." Thus, it seems that increments of FC on hydrolysis are greater than those of RC; the discrepancy can be explained if it is assumed that much of the urinary corticoid glucuronide is excreted with an α-glycol rather than an α-ketol side chain.

The chemical properties of the FC fraction of urine correspond to those of adrenal corticosteroids or metabolites thereof; it may be provisionally assumed that the bulk of this material is of adrenal cortical origin and that the amounts of TFC found in urine represent some fraction of the hormonal output of the adrenal gland. Actually, the mean level of TFC output in the urine of normal subjects corresponds roughly to the minimal maintenance requirement of cortisone in adrenalectomized human beings (14); the lability of TFC output in normal subjects exposed to the stresses and strains of daily life agrees with the known labile responsiveness of the pituitary-adrenal axis.

An extra-adrenal origin of urinary TFC has been suggested from observations on two Addisonian patients by Daughaday, Farr and Houghton (15), since in both cases the outputs of TFC after bacterial enzyme hydrolysis were within the normal range. The values for TFC in 2 other cases of Addison's disease were 1.7 and 0 mg. We have mentioned one patient with Addison's disease who excreted 22 mg. of TFC. Ward, Parkin, and Howell (16) have reported a case of co-existing Addison's disease and diabetes mellitus in which the FFC excretion was 0.66 to 1.06 mg. per 24 hours. The Addison's disease had developed some years after the diagnosis of diabetes. Although patients with this combination of diseases usually are very sensitive to insulin, the requirement for insulin in this case had not changed after the development of Addison's disease. This latter circumstance indicates that the patient was supplied with a fairly normal amount of carbohydrate-active adrenal hormones in spite of clinical Addison's disease. The normal values for FFC are in accord with this presumption. These observations suggest that Addison's disease is not always associated with complete failure of adrenocortical function. On this assumption, it is not necessary to conclude that the normal amounts of urinary formaldehydogenic material excreted by these unusual patients must be of extra-adrenal origin.

It is possible that some formaldehydogenic material may be of extra-adrenal origin since the value of FFC seldom is zero even in cases of long-standing severe Addison's disease or panhypopituitarism with clinical evidence of adrenal failure. More disturbing is the finding of Wilson (17) that most of the formaldehydogenic material freed by β-glucuronidase could not be recovered in the $C_{21}O_3$, $C_{21}O_4$, and $C_{21}O_5$ steroids separated by paper chromatography whereas these fractions of FFC gave titers comparable to the material applied to the paper. These results imply that the formaldehydogenic material extracted at pH 5 and also at pH 1 is steroid in nature but that most of that freed by enzymic hydrolysis is not steroid in nature. If that is true, then there would be no reason to expect correlation of the amount of this fraction with adrenal function. On the other hand, Cohen (7) has summarized evidence which lends support to the belief that the formaldehydogenic substances released by glucuronidase hydrolysis are indeed corticosteroids. Also, the amount of tetrahydrocortisone isolated by Baggett, Glick and Kinsella (18) after enzymic hydrolysis accounts for a fair proportion of TFC. At the present time these conflicting bits of evidence cannot be reconciled. In our opinion, the weight of evidence favors the view that urinary TFC is related to the adrenal cortex.

Essential hypertension. Levels of TFC are not increased in essential hypertension; indeed, the mean output in male patients is lower than that found in the normotensive males by Procedure 1, possibly because most of the patients were under the uniform and restful conditions of a stay in the hospital. In contrast, levels of FFC were frequently increased in both male and female patients; this may result either from some defect in corticoid conjugation or from intrarenal or intraurinary hydrolysis of preformed corticoid glucuronide. The presence of β-glucuronidase in renal epithelium, the potentiation of its activity by serum albumin (19), and the association of hypertensive renal disease with albuminuria and desquamation of renal epithelium argue in favor of the latter possibility.

Adrenal cortical disease and dysfunction. Levels in 3 patients with untreated Addison's disease are consistent with the concept that TFC accounts for a large proportion of the daily hormonal output of the adrenal cortex. The levels found in Cushing's syndrome do not accord too well with this view, since they are only exceptionally increased above the normal range. However, it may be significant that the mean value of TFC in 8 cases (Procedure 2) was increased 45 per cent over the mean of the normal values. Exceptionally, in 1 of the cases of Cushing's syndrome studied by Procedure 1, there was hypercorticoiduria both free and combined. The paradox of manifest hypercorticoidism without hypercorticoiduria, like that of hypocorticoidism without hypocorticoiduria, may depend on the metabolism of hormonal steroids. Thus, it may be that the diseased mesenchymal tissues of most patients with Cushing's syndrome "utilize" and degrade disproportionately the side chain of the excess steroid.

TFC output and urine volume. Since a large proportion of urinary 17-ketosteroids, like corticoid, is excreted as a glucuronide (20, 21, 22) and since it has been suggested that this excretion varies in part as a function of urine volume, TFC outputs obtained by Procedure 1 were examined for this association. Some association may be demonstrable in urines from normal subjects ($r = + 0.43$, standard error ± 0.17); in hypertensive males, the respective values were $+ 0.43$, ± 0.15 and in hypertensive females $+ 0.19$, ± 0.18. Thus, the degree of association is not great. It may be methodological and due to the multiplication of small positive errors in analyses or urines of large volume.

SUMMARY

1. Determinations of urinary formaldehydogenic corticoid (FC) by two somewhat different procedures aimed at estimating total FC by treatment of urine with a preparation of spleen β-glucuronidase yield similar estimates in two groups of normal human subjects. The mean by Procedure 1 is 21 mg. and by Procedure 2 it is 15 mg. per 24 hours and the increments in FC effected by the enzyme preparation vary from 5- to 70-fold.

2. Low total FC (TFC) outputs were observed in 3 patients with untreated Addison's disease; normal outputs observed in some patients may depend on persistence of partial, albeit inadequate, cortical function. By Procedure 2, TFC outputs in 7 of 8 patients with Cushing's syndrome did not exceed the normal range, although the mean of the group was greater than the normal mean. In 1 of this group and in 2 of 3 analyzed by Procedure 1, TFC outputs were abnormally high.

3. Since outputs of TFC are within the normal range in hospitalized patients with severe essential hypertension, the increases in "free" FC sometimes observed in these patients are attributable to decreases in the proportion of FC excreted as glucuronide.

4. Independent analyses by each procedure were commonly in good agreement; the disparities sometimes found established the desirability of duplicate analyses.

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