of vitamin B_{12} recovered appeared to be greater than the amount given. The liver of this patient contained appreciable amounts of both pteroylglutamic acid and citrovorum factor.

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Some Observations on the Hydrolysis and Extraction of Formaldehydogenic Corticosteroids

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Of the methods recently reviewed by Dorfman (1950) and Callow (1950) for estimating urinary corticosteroids only a few have been generally adopted. In the bioassay procedure of Venning, Kazmin & Bell (1946) use is made of the ability of certain 11-oxysteroids to promote the deposition of glycogen in the liver of adrenalectomized mice. The chemical procedures are of two main types. The first depends on the reducing properties of steroids with a primary or secondary α-ketol group, or an αβ-unsaturated 3-keto group or both. Talbot, Saltzman, Wixom & Wolfe (1945) used this property to reduce cupric ions, and Heard & Sobel (1946) phosphomolybdic acid. Since urine may contain a wide variety of reducing agents, the specificity of the assay depends upon the efficiency of the extraction and the purification of the extract. The second chemical method depends upon the liberation of formaldehyde when steroids with a free terminal vicinal glycol or hydroxyketone group are oxidized with periodic acid (Lowenstein, Corcoran & Page, 1946; Daughaday, Jaffe & Williams, 1948; Corcoran, Page & Dustan, 1950). The liberated formaldehyde reacts with chromotropic acid to give a lavender colour.

More recently, Zaffaroni, Burton & Keutman (1950) and Burton, Zaffaroni & Keutman (1951) have evolved a paper partition chromatographic procedure which permits separation of urinary corticoids into individual components. Such fractionation is an advance on the less specific methods, but unfortunately permits only semi-quantitative determinations.

All these methods require the extraction of corticosteroids from urine. Hormones which are excreted in the free form as neutral lipid-soluble substances are readily extracted by organic solvents. Others, however, are excreted in water-soluble conjugated form as sulphates and glucuronides (Klyne, 1946; Klyne & Marrian, 1945; Lieberman & Dobriner, 1948) and these must be hydrolysed before extraction. In the method of Talbot *et al.* (1945) the urine is extracted without adjusting the pH, and the steroids obtained are

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probably only those excreted in the free form. Heard & Sobel (1946) have found that the pH at which urine is extracted markedly influences the yields of both glycogen-depositing and total reducing substances. On acidification to pH 1 and immediate extraction the biological titre by Venning's method was approximately twice that obtained on extracting a sample of the same urine at pH 7. Likewise the reducing power was greater with increasing hydrogen-ion concentration during extraction, the value at pH 2 being two to three times that at pH 7. Lieberman & Dobriner (1948) found that the yield of compounds possessing glycogen-depositing activity could be increased by hydrolysis at pH 1 at room temperature for 24 hr. By model experiments they showed that when sulphates of certain 17-ketosteroids were continuously extracted at room temperature for 24–48 hr. at pH 1, the conjugate was completely hydrolysed and the free steroid recovered quantitatively.

That the optimum conditions for hydrolysis have not been fully considered is shown by the fact that Talbot *et al.* (1945) use extracts of unacidified urine; Lowenstein *et al.* (1946), Heard & Sobel (1946) and Daughaday *et al.* (1948) extract the urine immediately after acidification, and Mason & Sprague (1948) allow the urine to stand at pH 1 for 1–3 days before extraction. None of these procedures, with the possible exception of the last, would be likely to hydrolyse sulphate or glucuronide conjugates.

The hydrolysis of glucuronide conjugates has been achieved with β-glucuronidase prepared from either animal viscera (Fishman & Talalay, 1947) or *Escherichia coli* (Kinsella, Doisy & Glick, 1950). By this procedure the yield of formaldehydogenic steroids has been 5- to 20-fold greater than following acidification of urine (Corcoran *et al.* 1950; Corcoran, Dustan & Page, 1951; Kinsella *et al.* 1950; Cox & Marrian, 1951).

Marrian and his associates have recently reported their investigations on the conjugated adrenocortical steroids in urine (Paterson, Cox & Marrian, 1950; Paterson & Marrian, 1951; Cox & Marrian, 1951). They have found two types. The first conjugate was readily extractable with chloroform from acidified but not from neutral urine, and was rapidly hydrolysed at pH 1 to yield acid-labile steroid. The unhydrolysed conjugated compound could be recovered from the chloroform phase by extracting with aqueous sodium bicarbonate and could then be hydrolysed to the free compound in 1–3 hr. at room temperature and pH 3–4. The free steroid was stable at this pH for at least 6 hr. but was slowly destroyed at pH 1. This conjugate was not a glucuronide because it could not be hydrolysed with β-glucuronidase. The second conjugate was not extracted by chloroform even from acidified urine but was slowly hydrolysed at pH 1. It was obtained by extracting with *n*-butanol acidified urine which had previously been extracted with chloroform to remove free steroids and chloroform-soluble conjugates. The butanol phase was found to contain a glucuronide conjugate of corticosteroids which could be removed with aqueous sodium hydroxide and hydrolysed with β-glucuronidase to yield formaldehydogenic steroid in amounts about ten times greater than could be obtained by hydrolysis for 24 hr. at pH 1 and 37°.

In view of the present uncertainty as to the best method of hydrolysing urinary corticosteroids, studies were undertaken to define in what different conjugated forms the steroids were excreted, and to devise procedures which would ensure their hydrolysis and permit extraction of the free compounds liberated. Unless such methods are used, it may be argued that the quantity of steroid obtained by extracting acidified or unacidified urine only represents a proportion of the total excretion, and may not be a true reflexion of adrenocortical activity.

METHODS

Material. 24 hr. Urine specimens were collected without preservative from healthy males aged 25–35. In experiment (*j*) urine was obtained from three males suffering from rheumatic fever.

Apparatus. Glassware was cleaned by soaking in a hot solution of detergent ('Calgonite' or 'Haemosol'), washed in tap water and rinsed in distilled water. It was dried by draining at room temperature. The same apparatus was used throughout, and the dimensions of the vessels were the same at each stage of the procedure.

Hydrolysis. Urine for acid hydrolysis was adjusted to pH 1 (thymol blue) with conc. HCl; that for extraction at neutral pH was adjusted to pH 7·4 (neutral red) with N-HCl or N-NaOH; that for glucuronidase hydrolysis was adjusted to pH 4·5 (bromocresol green) with N-HCl.

β-Glucuronidase was prepared from rabbit livers and spleens after the method of Fishman & Talalay (1947). The enzyme obtained after the second precipitation with $(NH_4)_2SO_4$ was dissolved in the smallest possible volume of water and had an activity of 19000 Fishman units/ml. (1 unit liberates 1 μg. phenolphthalein in 1 hr. at 38° from phenolphthalein mono-β-glucuronide at pH 4·5 in 0·1 M-acetate buffer, Talalay, Fishman & Huggins, 1946). The batch of enzyme concentrate was stored at below −15°, and by repeated assay was found not to have lost any of its activity in 9 months. Urine for glucuronidase hydrolysis was adjusted to pH 4·5 and 5 ml. of 1·0 M-acetate buffer added per 100 ml. urine. 5000 units of enzyme were added to each 100 ml. urine. It was found that doubling or trebling the amount of glucuronidase did not increase the yield. The effectiveness of smaller amounts of enzyme was not investigated. The urine was incubated at 37–38° for 48 hr.

Extraction. Throughout, 200 ml. samples of urine were assayed. The samples were extracted by hand three times with 50 ml. portions of redistilled A.R. $CHCl_3$ or ether. The solvent phase was washed twice with 50 ml. portions of 5% (w/v) Na_2CO_3 and with 50 ml. portions of water until neutral. The carbonate and water washings were each back

extracted with 25 ml. solvent. The solvent phase was dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure at 40°.

Choice of solvent. Talbot *et al.* (1945) recommended the use of $CHCl_3$ for extracting neutral lipid-soluble steroids from urine. They found that $CHCl_3$ effected almost quantitative recovery, but ether proved less efficient and only 51% of 11-dehydro-17-hydroxycorticosterone and 65% of 17-hydroxycorticosterone were recovered when 0·2 mg. of either of these steroids was dissolved in 50 ml. water and shaken for 3 min. with an equal volume of solvent. Heard & Sobel (1946), by trial extractions of 11-deoxycorticosterone from water and urine, found that three extractions with ether alone recovered 80%, whereas an ether-$CHCl_3$ mixture (4:1) gave almost quantitative recovery. There seems little doubt that these steroids are more soluble in $CHCl_3$ than in ether, but the difference is not so great as to preclude the use of ether if it has other advantages. Emulsion formation is often excessive when extracting glucuronidase-hydrolysed urine with chloroform owing to the proteinous nature of the enzyme. This difficulty is considerably reduced with ether, which was mainly used in these experiments. As shown in Table 1, the yield of formaldehydogenic substances from two samples of the same urine does not differ significantly when the extraction is made with chloroform or with ether.

Table 1. *Comparison between the yield of FS when samples of urine are extracted with ether and with chloroform*

Urine no.	Hydrolysis	*FS* (mg. compound E/24 hr.) Extracted with ether	Extracted with $CHCl_3$
I	pH 1 and immediate extraction	1·6	1·5
II	Extracted at pH 7·4	0·7	0·8
III	pH 1 and immediate extraction	1·9	1·9
IV	β-Glucuronidase	4·5	4·9
V	β-Glucuronidase	19·4	18·5
VI	β-Glucuronidase	12·0	8·6*

* Bad emulsion formation.

Chemical method. The urinary extracts were dissolved in 10 ml. of 10% (v/v) ethanol, and 6 ml. transferred to a 30 ml. distilling flask. (When amounts of formaldehydogenic steroid greater than 4 mg./24 hr. were encountered, the extracts were diluted with larger volumes of ethanol. Smaller volumes were used when less than 1 mg./24 hr. was expected.) A reagent blank of 6 ml. 10% ethanol and standards of 11-dehydro-17-hydroxycorticosterone (compound E) in 6 ml. 10% ethanol were run concurrently in distilling flasks of the same dimensions. Oxidation with periodic acid, removal of excess oxidant with $SnCl_2$, the distillation of formaldehyde and the colour reaction with chromotropic acid were carried out as described by Daughaday *et al.* (1948). With the reagent blank set at 100% transmission, the unknowns and standards were read in a Coleman 'Universal' spectrophotometer at 550 mμ. The formaldehyde liberated from 100 μg. compound E gave an optical density of 0·241 with minor variations from day to day. All results are expressed as mg. of compound E excreted in 24 hr.

Controls obtained by extracting 200 ml. water with $CHCl_3$ and with ether gave a colour equivalent to 11 and 9 μg. respectively of compound E. These would have affected the values for a 24 hr. urine specimen of 1400 ml. by 0·08 and 0·06 mg. When 200 ml. water, buffered with 10 ml. 1·0 M-acetate buffer and containing 10 000 units of glucuronidase concentrate, was incubated for 48 hr. and then extracted with $CHCl_3$ or ether, values of 27 and 20 μg. compound E were obtained. These would have affected the values for a 24 hr. urine specimen by 0·19 and 0·14 mg. No corrections have been made for these controls.

RESULTS

(a) *Formaldehydogenic substances extracted at* pH 7·4 *compared with those extracted immediately after acidification to* pH 1

One sample of each urine specimen was extracted at pH 7·4, and another sample after acidification to pH 1. In most instances acidification increased the yield of formaldehydogenic substances (*FS*) (Table 2).

Table 2. *Comparison between the yield of FS obtained after extraction at* pH 7·4 *and after extraction immediately following acidification to* pH 1

Urine no.	*FS* (mg. compound E/24 hr.) Extracted at pH 7·4	Extracted immediately after acidification to pH 1
I	0·5	1·5
II	0·8	1·5
III	1·3	1·9
IV	2·7	2·8
V	1·2	5·4
VI	0·3	0·8

Comment. Similar observations have been made by Heard & Sobel (1946) and by Paterson *et al.* (1950). Acidification may increase the yield by (*a*) causing more complete extraction of the free steroids, (*b*) permitting extraction of a conjugated steroid, or (*c*) by hydrolysing a steroid conjugate and then allowing extraction of the free compound liberated. The observations of Paterson & Marrian (1951) suggest that some, if not all, of the increase is due to the rapid hydrolysis at pH 1 of a conjugated compound, which, however, in the conjugated form is also extracted by chloroform from acidified urine (see Exp. *c*).

(b) *FS extracted first at* pH 7·4 *and then at* pH 1 *compared with FS extracted at* pH 1 *only*

The quantity of *FS* obtained from a urinary sample first extracted at pH 7·4 and then extracted again immediately after acidification was compared with the yield when another sample was extracted only after acidification (Table 3). A greater yield

was not obtained by the double extraction procedure. In the first two specimens (urines III and IV) the increment of *FS* obtained after acidification was not large relative to the amount extracted at neutral pH, whereas in the third specimen (urine VI)

Table 3. *FS obtained from urinary samples first extracted at* pH 7·4 *and then re-extracted at* pH 1 *compared with FS obtained from samples extracted only at* pH 1

	FS (mg. compound E/24 hr.)		
	Urine III	Urine IV	Urine VI
Extracted at pH 7·4	1·3	2·7	0·3
Re-extracted immediately after acidification to pH 1	0·4	0·3	0·4
Total	1·7	3·0	0·7
Extracted only at pH 1	1·9	2·8	0·8

0·3 mg. was obtained at pH 7·4 and 0·4 mg. after acid hydrolysis. This experiment also shows that no destruction of the free steroids present in neutral urine is detectable when the urine is acidified to pH 1 and extracted immediately.

(*c*) *The role of chloroform-soluble conjugates in increasing the yield of FS from acidified urine*

In Exp. (*a*) it was found that acidification of urine followed by immediate extraction increased the yield of *FS*. It was suggested that this increase may be due to the rapid hydrolysis of a conjugated compound such as the chloroform-soluble conjugate described by Paterson & Marrian (1951). However Heard & Sobel (1946) and Paterson *et al.* (1950) have shown that in some instances no increase is obtained if the acidified urine is neutralized before it is extracted. The latter authors suggested that the explanation for this was that the chloroform-soluble conjugate could not be extracted from neutral urine. It was only extractable from acidified urine and the conjugate could be recovered from the chloroform phase by extracting with aqueous sodium bicarbonate. It could then be hydrolysed to the free compound in 1–3 hr. at room temperature and pH 3–4.

The following experiment was done to determine how much of the chloroform-soluble conjugate, after it had been subjected to acid hydrolysis, could be recovered in the conjugated form from the chloroform phase. A sample of urine was extracted with chloroform at pH 7·4 and then acidified to pH 1 and extracted with chloroform again. The alkali washings from the second extraction procedure were freed of organic solvent by bubbling nitrogen through, adjusted to pH 4·5 and buffered with 1·0M-acetate buffer. One half of the washings was incubated with β-glucuronidase; the other half was incubated without enzyme. After 24 hr. the alkali washings were extracted with chloroform and the amount of *FS* determined. Another sample of the same urine was extracted immediately after acidification to pH 1. The results (Table 4) indicate that acidification increased the yield of *FS* 0·4 mg. above the amount obtained from urine extracted at pH 7·4. Despite this increase the alkali washings contained 0·3 mg. when hydrolysed without β-glucuronidase and 0·4 mg. when hydrolysed with the enzyme.

Table 4. *Effect of acid on the hydrolysis of chloroform-soluble conjugates, and their subsequent recovery from alkali washings*

	FS (mg. compound E/24 hr.)
First sample:	
(*a*) Extracted at pH 7·4	1·3
(*b*) Extracted again immediately after acidification to pH 1	0·4
Total	1·7
Extraction of the alkali washings obtained during procedure (*b*)	
Hydrolysed with β-glucuronidase	0·4
Hydrolysed without enzyme	0·3
Second sample:	
Extracted immediately after acidification to pH 1	1·9

Comment. If the increased yield after acidification is due to the hydrolysis of a chloroform-soluble conjugate or conjugates, this hydrolysis only affected a proportion of the conjugate, the remainder passing into the alkali washings. The amount of *FS* in the alkali washings was approximately the same whether the washings were hydrolysed with or without β-glucuronidase. It is not possible to say, therefore, whether the conjugate was a glucuronide or not.

(*d*) *The solubility of the chloroform-soluble conjugate in ether*

Two samples of urine were acidified to pH 1. One was extracted immediately with ether, the other with chloroform. The alkali washings of the ether and chloroform extracts were separately adjusted to pH 4·5, buffered with 1·0M-acetate buffer and incubated for 24 hr. at 37°. The alkali washings were then extracted with chloroform. The ether and chloroform extracts each contained 1·9 mg. of *FS*. The alkali washings of the ether extract contained 0·2 mg. and those of the chloroform extract 0·3 mg. (Table 5). This suggests that the chloroform-soluble conjugate is also soluble in ether.

(*e*) *The effect of acid on FS*

To determine whether *FS* are acid-labile a sample of urine was extracted with ether immediately after acidification to pH 1. The ether phase

was washed with sodium carbonate and water and then divided into two halves. One half was taken to dryness under reduced pressure and the residue dissolved in 20 ml. 10 % (v/v) ethanol. The ethanolic solution was adjusted to pH 1 with hydrochloric acid and allowed to stand at room temperature for 24 hr. before being extracted with chloroform. The other half was treated similarly except that the ethanolic solution was not acidified. The amount of *FS* in the acid-treated sample was 0·4 mg. and in the unacidified sample 1·3 mg. A repetition of this experiment with another urine specimen gave 0·2 mg. for the acidified solution and 0·6 mg. for the unacidified solution. These observations indicate that certain *FS* extractable from urine are acid-labile.

Table 5. *Comparison between the solubility of the chloroform-soluble conjugate in chloroform and in ether*

	FS (mg. compound E/24 hr.)	
	Ether extract	$CHCl_3$ extract
Urine extracted immediately after acidification to pH 1	1·9	1·9
Alkali washings incubated at pH 4·5 and then extracted with $CHCl_3$	0·2	0·3

(f) The effect of prolonged standing in acid on the yield of FS

The destructive action of acid on *FS* was studied by acidifying two specimens of urine to pH 1 and then extracting with chloroform samples of each immediately, and 4, 12 and 24 hr. after acidification (Table 6). The decreasing yield which occurs as the period of standing in acid is lengthened confirms that prolonged standing at pH 1 destroys certain *FS*.

Table 6. *Effect of prolonging the acid treatment on the yield of FS*

Time after acidification (hr.)	*FS* (mg. compound E/24 hr.)	
	Urine II	Urine III
Immediate	1·5	1·9
4	1·4	1·7
12	0·8	1·0
24	0·4	0·3

Comment. Genest (1951) has also found that prolonged standing in acid caused a decreased yield. On the other hand, Paterson *et al.* (1950) observed that although the yield of *FS* was reduced when urine was extracted 4 hr. after acidification, it rose again when the extraction was performed 12 or 24 hr. later. This increase was attributed to the slow hydrolysis of a conjugated compound.

The following experiments provide further data on the effect of prolonged acidification which, in this work, had reduced the yield of *FS* and in the hands of Cox & Marrian (1951), had increased it. Samples of urine were acidified to pH 1. One was allowed to stand at room temperature for 24 hr. before extraction; the other was extracted immediately after acidification and again 24 hr. later (Table 7). When the urine was extracted immediately after acidification, a further increment was obtained when the extraction was repeated 24 hr. later. This suggests that prolonged acidification causes slow hydrolysis of a conjugated compound. On the other hand, the yield of *FS* was less when the urine was extracted after 24 hr. hydrolysis at pH 1 than when it was extracted immediately after acid fication and again 24 hr. later.

Table 7. *Action of acid in destroying free compounds and slowly hydrolysing a conjugated compound*

	FS (mg. compound E/24 hr.)			
	Urine I	Urine III	Urine VII	Urine IX
First sample:				
Extracted immediately after acidification	1·5	1·9	4·6	2·7
Re-extracted after 24 hr.	0·5	0·3	0·6	1·3
Total	2·0	2·3	5·2	4·0
Second sample:				
Extracted 24 hr. after acidification	0·4	0·3	2·7	2·3

Comment. The yield of *FS* after acidification and extraction 24 hr. later represents the balance between those free compounds remaining after partial destruction and those which have been liberated from a conjugated form. The proportion destroyed varies from one urine to another. In urine I, for example, immediate extraction after acidification gave 1·5 mg. Repeated extraction 24 hr. later gave 0·5 mg. which does not differ from the value (0·4 mg.) obtained when another sample was allowed to stand for 24 hr. at pH 1 before being extracted. In this instance it would seem that most of the free substances and those liberated from the chloroform-soluble conjugate, which amounted to 1·5 mg., were acid-labile and destroyed by prolonged acidification. The same holds for urine III. On the other hand, in urines VII and IX, there was less destruction of the free substances because the values obtained when the urine was extracted after standing at pH 1 for 24 hr. (2·7 and 2·3 mg. respectively) were greater than those when other samples were extracted for the second time (0·6 and 1·3 mg. respectively). It will also be seen that the proportion of free compounds, excreted as such or hydrolysed from the chloroform-soluble conjugate, to that conjugated compound, which is slowly hydrolysed at pH 1, varies from one urine to another.

(g) Further observations on the action of acid in hydrolysing conjugates

A sample of urine was acidified and extracted immediately, yielding 1·5 mg. *FS*. The same sample was allowed to stand at room temperature and pH 1 for 24 hr. when it yielded another 0·5 mg. After a further 24 hr. it was extracted again and then gave 0·3 mg. Finally the same sample was hydrolysed with β-glucuronidase and 4·1 mg. obtained.

In another experiment a sample of urine was extracted at pH 7·4 (2·0 mg.) and again immediately after acidification (0·4 mg.). 4 hr. later, after standing at room temperature and pH 1, it was extracted again (0·5 mg.). 24 hr. after acidification, extraction of the same sample yielded 1·2 mg. Finally, the sample was hydrolysed with β-glucuronidase and yielded 18·5 mg.

Comment. These experiments provide further evidence that prolonged treatment with acid causes slow hydrolysis of a conjugated compound—in the first instance yielding 0·8 mg. and in the second 1·7 mg. Even then the urines contained 4·1 and 18·5 mg. respectively of *FS* conjugated with glucuronide.

(h) Action of β-glucuronidase on the yield of FS

To confirm that hydrolysis with β-glucuronidase increases the yield of *FS*, samples of urine were adjusted to pH 4·5 and buffered. The enzyme preparation was added to one sample; another sample acted as a control. After incubation at 38° for 48 hr. both were extracted without altering the hydrogen-ion concentration. The results (Table 8) indicate that β-glucuronidase hydrolysis causes a considerable increase in the yield of *FS*.

Table 8. *Effect of β-glucuronidase hydrolysis on the yield of FS*

	FS (mg. compound E/24 hr.)	
Urine no.	β-Glucuronidase hydrolysis at pH 4·5	Control hydrolysis at pH 4·5
X	4·1	1·4
XI	12·6	1·5
XII	9·3	1·8

(i) The effect of acid on FS liberated from glucuronide conjugation

The following experiments were designed to determine whether the free substances liberated from glucuronide conjugation were acid-labile. Two samples of urine were hydrolysed with β-glucuronidase. After incubation one was extracted at pH 4·5; the other was acidified to pH 1 before extraction (Table 9). In two instances acidification caused a slight reduction in the yield of *FS* and in a third instance the reduction was more marked. Thus acidification appears to cause some destruction of the liberated compound. No observations were made to determine whether prolonged acidification caused a greater degree of destruction.

Table 9. *Effect of acid on free compounds liberated from glucuronide conjugation*

	FS (mg. compound E/24 hr.) Urine hydrolysed with β-glucuronidase	
Urine no.	Extracted at pH 4·5	Extracted at pH 1
X	4·1	1·7
XI	12·6	10·4
XII	9·3	8·1

(j) Comparison between the yields of FS after acid and β-glucuronidase hydrolysis

One sample of urine was hydrolysed at pH 1 for 24 hr. Another was hydrolysed with β-glucuronidase, and after incubation the urine was made pH 1 immediately before extraction. The yield of *FS* after enzymic hydrolysis was always greater than that obtained after acid hydrolysis (Table 10).

Table 10. *Comparison between the yield of FS obtained after β-glucuronidase and* pH 1 *hydrolysis*

(Samples (*c*), (*d*), (*e*), etc., are different 24 hr. samples from the same individual.)

	FS (mg. compound E/24 hr.)		
Urines	pH 1 hydrolysis for 24 hr. (*a*)	β-Glucuronidase hydrolysis (*b*)	Ratio (*b*/*a*)
J.H. (*c*)	3·1	12·6	4
(*d*)	1·0	3·9	3·9
(*e*)	2·6	15·0	5·8
(*f*)	2·4	19·5	8·1
(*g*)	1·9	9·3	4·9
(*i*)	1·2	15·4	12·8
V.C. (*a*)	0·8	2·1	2·5
(*b*)	0·5	13·6	27·2
(*c*)	0·8	9·7	12·1
(*d*)	2·4	15·7	6·5
E.E. (*a*)	2·5	19·5	7·8
(*b*)	0·8	11·5	14·4
(*c*)	1·0	9·1	9·1
(*h*)	0·7	5·8	8·3
(*i*)	1·6	3·1	1·9

There were wide fluctuations in the quantities of *FS* liberated by β-glucuronidase, and, although in general they tended to rise and fall in parallel with the values obtained after acid hydrolysis, there does not appear to be any relationship between the amount of steroid excreted as glucuronide and the amount obtained after acid hydrolysis.

DISCUSSION

Assuming that the formaldehydogenic substances measured by the method used are in fact formaldehydogenic steroids, the findings reported here and the observations of Heard & Sobel (1946) and of Marrian and his colleagues emphasize that corticosteroids are excreted in the urine in the following forms: (1) As free compounds which can be extracted from urine at pH 7·4. Some of these free compounds may be destroyed on prolonged contact with acid, but acidification to pH 1 followed by immediate extraction does not cause any significant loss. (2) As chloroform- or ether-soluble conjugates, some of which appear to be rapidly hydrolysed by acid to liberate a free acid-labile compound. Others, however, are not so rapidly hydrolysed by acid; they pass from acidified urine into the chloroform or ether phase whence they are extracted by alkali and can be hydrolysed at room temperature or 37° at pH 3–4·5. (3) As a conjugate which is slowly hydrolysed at room temperature and pH 1. (4) As a conjugate with glucuronide, which can be hydrolysed with β-glucuronidase. The free compounds liberated appear to be acid-labile. The proportion of these different forms appears to vary from person to person and in the same person from day to day.

The current methods of hydrolysis are clearly inadequate. If the urine is extracted immediately after acidification to pH 1, only free steroids and those rapidly hydrolysed from the chloroform-soluble conjugate will be obtained. If the urine is extracted 24 hr. after acidification free steroid will be obtained from that conjugate which is slowly hydrolysed at pH 1 and room temperature, but there will be a greater or lesser destruction of those steroids which are extractable immediately after acidification but are acid-labile. In neither case will there be effective hydrolysis of the glucuronide conjugates. A true index of adrenocortical function may not be given unless all the different excretory products are extracted and the total steroid output determined. Acidification is an essential step in hydrolysing those conjugates which rapidly yield a free compound. Since these free compounds, and also those initially excreted as such, are acid-labile, acidification must be followed by immediate extraction. Other conjugates are hydrolysed more slowly and so the urine must be extracted again after prolonged acidification. Hydrolysis with β-glucuronidase also appears essential, and since the enzyme may be inactivated by small amounts of organic solvent it is more practical to carry out the enzymic hydrolysis before the acid hydrolysis. It is tentatively suggested that the following procedure may extract the corticosteroids and their conjugated compounds which have so far been recognized as being present in urine:

(*a*) A suitable sample of urine is brought to pH 4·5 and buffered with 1·0M-acetate buffer. β-Glucuronidase is added and the urine incubated for 48 hr. The enzyme will hydrolyse the glucuronide conjugates, and in addition the pH and temperature may also hydrolyse some of the chloroform-soluble conjugates.

(*b*) The urine is then extracted without altering the pH from 4·5. This will remove steroids excreted initially in the free form and those liberated from glucuronide conjugation. It will also extract any free steroid liberated from the chloroform-soluble conjugates.

(*c*) The urine is adjusted to pH 1 and extracted immediately. This will remove that moiety which it has been shown is extractable immediately after acidification and which may arise from the rapid hydrolysis of any remaining chloroform-soluble conjugates.

(*d*) The urine is left at room temperature and pH 1 for 24 hr. before it is finally extracted. This will liberate free compounds from conjugates which are slowly hydrolysed by acid.

Preliminary trials using this technique have yielded 15–30 mg. formaldehydogenic steroid/24 hr., the largest moiety always being liberated from glucuronide conjugation. All the steroid obtained by β-glucuronidase hydrolysis would not necessarily be expected to be biologically active, and Venning (1951) has found that whereas glucuronidase hydrolysis may increase the yield of formaldehydogenic substances tenfold, the yield of glycogen-depositing steroids is only increased fivefold.

SUMMARY

1. Experimental data are presented which suggest that formaldehydogenic substances (*FS*, assumed to be corticosteroids) are excreted in urine as (*a*) free compounds, (*b*) in conjugation with glucuronic acid and (*c*) as at least two other conjugates.

2. Methods for hydrolysing the conjugates and extracting the free compounds are described.

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The Content of Glycogenolytic Factor in some Insulin Samples

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Sutherland & Cori (1948) have reported that a sample of insulin manufactured by the Eli Lilly Research Laboratories contained a glycogenolytic factor, whereas a sample prepared by the Novo Terapeutisk Laboratorium A/S contained none. Later work (Sutherland & Duve, 1948) has shown that the factor is secreted by the pancreas, probably by the α cells, and Sutherland & Cori have suggested that the factor is responsible for the initial hyperglycaemia which may follow an intravenous injection of insulin. The variations in this initial hyperglycaemia reported by different authors, e.g. Burger & Kramer (1930), suggest that the amount of factor associated with different insulin samples may vary. An assay of glycogenolytic-factor content has been carried out on some insulin samples available in this laboratory and has shown that the amount does vary considerably, both in samples prepared by different manufacturers and in samples from the same firm.

The test system was based on that described by Sutherland & Cori (1948) and Sutherland & Duve (1948), where activity was estimated from the increased rate of production of glucose by liver slices, incubated in buffered saline, caused by the addition of the factor. The experimental procedure is described somewhat fully, as satisfactory results were only obtained when it was followed in detail. Two methods of assay were used. The first, or single-dose, method gives an approximate estimate of activity after testing at one concentration; the second, or multiple-dose, method requires testing of samples at several concentrations and gives a more accurate assessment of relative potency.

EXPERIMENTAL

The test system

Reference standard. A sample of insulin containing the factor (Eli Lilly, no. 987267) was used as the reference standard for comparison of glycogenolytic-factor content.

Insulin samples. Samples, prepared by a number of manufacturers from the pancreas of ox, calf, sheep and pig, were given to us for assay by Prof. F. G. Young and Dr A. C. Chibnall.

Choice of animal. In preliminary experiments, liver slices from several animals were tested with the reference standard used at a concentration of 53 μg./ml., which was sufficient to give a maximum response with rabbit and rat liver (Table 1). While it would be an advantage to select an animal such as the cat, whose liver gives both a large basic output of glucose and a large response to the factor (especially when testing at concentrations of factor giving a sub-