the most important questions—for example, the risk of meningitis at different ages—and that I do not think children with asymptomatic primary infection should be treated in sanatoria unless home circumstances are unfavourable, or by antibiotics except under very special circumstances. These children should nevertheless remain under regular supervision and a close watch be kept for signs of disseminated disease. Knowledge of the primary complex is, moreover, of great assistance in detecting the early case of tuberculous meningitis or miliary tuberculosis, in which the prognosis depends almost entirely upon the time interval between onset and the beginning of treatment.

The routine use of streptomycin and P.A.S. cannot be recommended, but in cases of prolonged fever of onset, or when the infant is infected in the first year and is febrile or losing weight, or in certain cases of tuberculous cervical lymphadenitis, these compounds are of very real value.

Finally, I would repeat my plea that, wherever home care is competent and conditions are good, children with primary tuberculosis who do require treatment should be kept at home or be allowed to go home as soon as possible after confirmation of the diagnosis in hospital. This applies particularly to the child under the age of 5 years, who should be admitted to a "long-stay" hospital only when no other course is possible. We must strike a careful balance between the chance of diminishing the risk of disseminated disease and improving the child's physical state and the chance of interfering with or distorting his emotional development by long separation from home and parents.

THE CHEMICAL ESTIMATION OF CORTISONE-LIKE HORMONES IN URINE

BY

C. L. COPE, D.M., F.R.C.P.

AND

B. HURLOCK, B.A.

(From the Department of Medicine, Postgraduate Medical School of London)

Now that the presence of adrenal cortical hormones in both blood and urine has been detected with certainty, the problem of quantitative assay of these hormones becomes a matter of increasing importance.

In blood the presence of 17-hydroxycorticosterone and also of corticosterone has been demonstrated by several workers (Reich, Nelson, and Zaffaroni, 1950; Pincus, Hechter, and Zaffaroni, 1951), but cortisone has not so far been detected in appreciable quantity. In urine both cortisone and 17-hydroxycorticosterone (compound F) have been identified (Burton, Zaffaroni, and Keutmann, 1951b) and actually isolated by Schneider (1950, 1952). They occur in urine in association with a number of other steroid compounds, most of which are probably devoid of biological activity.

The problem of assay of these hormones in biological fluids is made difficult by the small quantities which are involved, for present estimates indicate from 5–10 µg. per 100 ml. in blood and about the same concentration in urine.

The most direct estimation is achieved by use of a biological method, since the specificity of biological responses is high. Venning, Kazmin, and Bell (1946) have developed biological estimations based on the power of these hormones to encourage glycogen deposition in the liver of adrenalectomized mice. Similarly, Cope (1951) has developed, and Cope, Boysen, and McCrae (1951) have used, a method based on the ability of these hormones to depress the count of eosinophil cells in the blood of adrenalectomized mice. Although the latter method is appreciably less laborious than the former, yet both suffer from disadvantages inherent in their biological nature. They are technically difficult to perform, they require relatively large quantities of hormone-containing extract for assay, and they are inaccurate unless large numbers of animals are used for each assay. When accuracy is increased in this way still more labour is involved and still more material is needed. The reality of these deterrent factors is well indicated by the great scarcity of published work during the past six years on adrenal hormone assays in clinical material using the liver glycogen methods. The outstanding lead given by Venning (1945) has not been followed.

Although good approximations to the hormone content of urine extracts can be obtained with much less effort by the mouse-eosinophil technique, the need for a still simpler, and chemical, method of estimation remains. The observations reported here will, it is hoped, provide the basis for such a method. The difficulties to be overcome may be divided into three main groups: (a) quantitative extraction, (b) purification from interfering contaminants, and (c) quantitative estimation by a suitable chemical reaction. Each of these may be considered separately.

Quantitative Extraction

The state of the adrenal cortical hormones in urine is such that they cannot be extracted completely by the usual lipid solvents such as chloroform. A preliminary hydrolysis is necessary to convert them into a chloroform-soluble form. The steroids probably exist in urine largely as sulphates and glucuronides (Cohen, 1951b). If the urine is acidified to pH 1 the former but not the latter are hydrolysed at room temperature. With stronger conditions of hydrolysis, such as warm acid, destruction of hydrolysed hormone may occur. Proper extraction of the hormone thus requires hydrolysis of the esters without destruction of the free hormone. Extensive studies have been made by several workers to determine optimum conditions of extraction for the formaldehydogenic steroids. The yield of these latter extracted with chloroform is increased 10 to 20 times if the urine is first hydrolysed with the enzyme β -glucuronidase (Cohen, 1951a; Daughaday, Farr, and Houghton, 1951). No studies have yet been reported on the effects of enzymatic hydrolysis on the recovery of biologically active hormones from urine, although their behaviour is likely to be similar.

Bayliss (1952), as a result of his studies on the extraction of the formaldehydogenic steroids from urine, has suggested an extraction procedure designed to recover the free hormone, the glucuronide, and the sulphate with the minimum of destruction by exposure to acid. This extraction involves three stages: (a) chloroform extraction after 48 hours' hydrolysis with β -glucuronidase, (b) immediate re-extraction after acidifying to pH 1, and (c) acid hydrolysis at pH 1 for 24 hours at room temperature and a third extraction with chloroform. In the belief that such a triple extraction represents the most complete extraction procedure at present available we have compared the yield of biologically active hormone obtained by its use with the

yield obtained by the more conventional single extraction with chloroform after 24 hours' acid hydrolysis at room temperature and pH 1.

Methods

Samples of urine from selected subjects were collected for three or four days. Each day's collection was halved. One-half was acidified to pH 1 and stood for 24 hours at room temperature before extraction four times with 15 volumes % of chloroform. This extract was prepared for bio-assay as described by Cope, Boysen, and McCrae (1951). The remaining half of each day's urine was adjusted to pH 4.5 with acetate buffer and treated with 50 units of β -glucuronidase per ml. for 48 hours at 37° C. The Bayliss (1952) type of triple extraction was then carried out, each extraction using three successive lots of 15 volumes % of chloroform. These extracts were combined and prepared for bio-assay in the usual manner. The final extracts from the four half-days were pooled. In this manner each day's urine could be extracted while fresh, bacterial action was minimized, and the separate extracts from acid hydrolysis and from the triple extraction procedure represented comparable samples of urine.

β -glucuronidase was prepared by the rapid method of Fishman (Talalay, Fishman, and Huggins, 1946) from rabbits' livers, spleens, and kidneys. The activity of the final product was 7,000 units per ml. when measured by the phenolphthalein-glucuronide method of Talalay, Fishman, and Huggins (1946). Bio-assay of the adrenal cortical hormones was performed by the method developed by Cope, Boysen, and McCrae (1951) using the eosinophil drop in adrenalectomized mice. Only three animals were used for each assay, since our mice were relatively insensitive and only the stronger extracts contained enough hormonal activity for a greater number of mice. To eliminate errors due to variations in individual mice the same three animals were invariably used for testing the glucuronidase triple extract as were used for the corresponding pH 1 extract from the same urine sample.

Results

Fourteen urines have been used for comparison of the yields by the two methods. The specimens have been chosen to cover the range from low to high content of active adrenal hormone. The results are shown in Table I, the samples being arranged in order of activity of the pH 1 extracts. The increase in yield by the triple-extraction method varied

TABLE I.—Effect of Glucuronidase on Cortisone Extracted from Urine (μ g. daily)

Extract	pH 1 Hydrolysis (a)	Glucuronidase Triple Extraction (b)	% Rise in Yield on Glucuronidase Treatment $\frac{(b-a)}{a} \times 100$
1	0	0	—
2	50	190	280
3	50	207	315
4	120	158	30
5	140	250	78
6	170	185	8
7	180	315	75
8	192	270	40
9	195	240	23
10	240	520	117
11	300	480	60
12	320	600	87
13	342	696	104
14	360	636	76
		Mean percentage increase	99

from 8 to 315%. The mean increase for the series is 99%. Since the accuracy of assay by this method is low with the weaker extracts, the apparent greater percentage increases in yield from some of these may be fallacious. It may be concluded, however, that the triple extraction after glucuronidase increases the recovery of active adrenal hormone by an average of 100%, and that this applies to both weak

and strong urines. It is possible that a further increase in yield may result from greater concentrations of β -glucuronidase, and this point is being investigated.

Purification from Contaminants

Among the reactions available for a chemical assay of adrenal cortical hormone the Porter-Silber reaction (1950) appears to hold the highest place for sensitivity and suitability. In this reaction a yellow colour with maximum absorption at $410\text{ m}\mu$ is produced when a solution containing the hormone is heated with phenylhydrazine in sulphuric acid solution. The colour is given by both cortisone and 17-hydroxycorticosterone, and its intensity is proportional to the concentration present over a considerable range. Nevertheless the reaction cannot be safely applied direct to the usual urine extracts because of the development of interfering pigments which also absorb light at $410\text{ m}\mu$. Satisfactory reagent blanks cannot be prepared to compensate for this, because the pigments often give more colour with sulphuric acid alone than they do with the sulphuric acid and phenylhydrazine reagent. As a result erroneous figures are often given by the reaction on unpurified urine extracts, and apparent negative readings may be obtained on the less potent urines. This interference is due to substances other than the hormones themselves, and the colour of the hormones develops independently of the contaminating chromogens. Consequently the intensity of colour of the final solution, after allowing for reagent blanks, is the algebraic sum of the colour due to hormone with that due to contaminant. Because of this, the ability to detect quantitatively extra hormone added to a urine or urine extract is no proof that the method can reliably estimate the hormone content of the original urine or extract, since an unknown amount of contaminant colour is included in the latter.

Experience with two typical urine extracts may be used to make this point clear (Table II). The first extract, from a patient with Addison's disease, gave no detectable corti-

TABLE II.—*Recovery of Cortisone Added to Urine Extracts*

	Cortisone Added ($\mu\text{g.}$)	" Apparent Cortisone " Porter- Silber ($\mu\text{g.}$)	Recovery of Added Cortisone ($\mu\text{g.}$)
Extract A. Bio-assay: zero:			
3-hour aliquot	Nil	-10	—
" " " " " " "	10	-1	9
" " " " " " "	20	+11	21
" " " " " " "	50	+42	52
Extract B. Bio-assay: 190 $\mu\text{g.}$ daily			
1-hour aliquot	Nil	-11	—
" " " " " " "	10	-1	10
" " " " " " "	20	+10.5	21.5
" " " " " " "	50	+40	51

sone activity in the mouse-eosinophil test. The Porter-Silber reaction applied direct to this extract gave a negative reading equivalent to $-80 \mu\text{g. a day}$. The second extract, from a patient with haemochromatosis, was deeply pigmented and had, according to mouse-eosinophil assay, a cortisone content of $190 \mu\text{g. a day}$. In a one-hour aliquot of this extract the Porter-Silber reaction gave an apparent cortisone content of $-11 \mu\text{g.}$, equivalent to $-264 \mu\text{g. a day}$. In both these extracts added cortisone, as acetate, could be quantitatively detected by the reaction in spite of the erroneous estimation on the original extracts themselves.

Carroll, McAlpine, and Noble (1951) have claimed that the Porter-Silber reaction can be used direct on urine extracts without further purification, yet they admit that negative values have often been obtained by them. They have not compared their results with analyses by an alternative method. For the reasons stated above, their ability to recover added cortisone is no proof of the reliability of the method for urine extracts, since no proper account is taken of the interfering chromogens. Although the Porter-Silber reaction can quantitatively detect cortisone added to urine

extracts in spite of the interfering chromogens, the estimation of the cortisone present in the original urine extract will be possible only if these interfering pigments can be removed or effectively neutralized. The problem is similar to that encountered in the chemical estimation of oestrogens.

We have attempted to remove those pigments by paper chromatography, using the system of Burton, Zaffaroni, and Keutmann (1951a). Both cortisone and the active hormone in urine extracts can readily be eluted from such paper chromatograms, using the toluene/propylene glycol system. Amounts may be recovered which are sufficient for assay by the mouse-eosinophil method. So far as the limited accuracy of this mouse method enables us to determine, the recovery by elution with methanol is almost complete. It was readily shown that all the biological activity of urine extracts remained in that part of the paper chromatogram representing a flow of less than $R_f=0.1$, and that an eluate of all the paper from $R_f=0.1$ to $R_f=1.0$ showed no detectable activity.

When chemical analysis of the eluate is being attempted it is preferable to use the benzene/formamide solvent system, because small traces of propylene glycol persisting in the eluate produce appreciable yellow colour at 410 m. μ , whereas traces of formamide produce no interfering colour. When cortisone is run for 24 hours in the benzene/formamide system and then subsequently eluted, the recovery varies between 75 and 95%. The mean of six trials, using amounts ranging from 35 to 300 μ g. of cortisone, was 86% recovery.

When urine extracts are submitted to paper chromatography with the benzene/formamide system most, but not all, of the interfering pigment is fast-moving and can be removed. A small amount, however, has a slow rate of flow approximating to that of cortisone, and appears in the final methanol eluate containing the adrenal hormones. Considerable improvement is nevertheless obtained in this way, and the loss of quantitative accuracy entailed by the paper chromatography is more than counterbalanced by the advantages resulting from removal of the interfering pigment.

Quantitative Chemical Estimation

On urine extracts purified by such chromatography the Porter-Silber reaction seems to give a much more reliable index of the true adrenal hormone content. To show that this is so we have compared chemical analyses obtained in this way with biological assay of the same extracts by the mouse-eosinophil method. The degree of agreement between the two methods is such as to suggest that the differences in result still persisting are due mainly to the experimental errors of the two methods, which are considerable.

Methods

Urine extracts were prepared in the usual manner after either pH 1 hydrolysis at room temperature or after β -glucuronidase treatment with subsequent triple extraction as suggested by Bayliss (1952). Part of the extract was assayed by the mouse-eosinophil method, using three mice. Another part of the same extract, representing a known fraction in methanol solution, was applied to the starting line of paper chromatograms. At least 1 cm. width of paper was allowed for each one-hour aliquot of extract applied. The benzene/formamide system was used, and was allowed to run 24 hours at room temperature. The paper was then removed and dried free of formamide at about 50° C. overnight. The top 10 cm. containing all the active hormones was then cut out, divided into small pieces, and eluted with three successive lots of 20 ml. of methanol. Smaller volumes of methanol resulted in incomplete recovery. The 60-ml. eluate was evaporated to dryness *in vacuo* and was redissolved in exactly 2.5 ml. of methanol. One millilitre of this solution was used for analysis by the Porter-Silber reaction, and 1 ml. was used for the sulphuric acid blank. In some earlier experiments another area of paper from lower in the chromatogram was also eluted similarly to serve as a paper blank. Since the blank value

from this was small, and as it was not strictly representative of the active area which still retained some non-specific pigment, these paper blanks were discontinued. A recovery of 85% was assumed and the appropriate correction was applied.

Results

The results obtained in 23 such direct comparisons are shown in Table III. With the exception of extract 6, estimates of cortisone content by both methods are of the

TABLE III.—Comparison of Chemical and Biological Assay

Urine Extract No.	"Cortisone," μ g. Daily		Percentage Deviation of Chemical from Bio-assay
	Bio-assay	Chromatography Chemical	
1	0	0	0
2	0	7	—
3	30	42	+40
4	146	106	-27
5	150	98	-35
6	158	(395*)	(+150*)
7	185	255	+38
8	190	224	+18
9	192	127	-34
10	240	188	-22
11	300	306	+2
12	300	203	-32
13	320	262	-18
14	320	353	+10
15	328	262	-20
16	342	344	0
17	360	330	-8
18	480	678	+41
19	520	474	-8
20	600	618	+3
21	636	659	+4
22	700	941	+34
23	1,116	1,706	+53
Mean deviation % ..			+2
Standard deviation from mean ..			27

* This anomalous result has been excluded in calculating the mean and S.D.

same order of magnitude. In only two other extracts does the difference between the two estimates exceed 40%, and in 14 of the 23 the agreement is within 30%. The average difference (excluding extract 6) is +2%. The standard deviation from the mean is 27%. Since an error of at least 30% is to be anticipated in bio-assays carried out in this way, the agreement must be regarded as good.

Discussion

The even scatter of the differences between the two methods of estimation suggests that they are due mainly to experimental errors, which are at present large in both methods. In urine extracts many other steroids occur, and some of these are probably present in the final methanol eluates from the paper chromatograms in greater quantity than the cortisone itself. Yet these inert steroids, of which tetrahydrocortisone is probably the most important, seem to produce no systematic error in the estimation. The amount of material producing yellow colour with the Porter-Silber reagent is apparently the same as that producing depression of eosinophil cells in the mouse. Only cortisone and compound F are known to cause such eosinophil depression in significant degree. It may be concluded that the Porter-Silber reaction can be satisfactorily applied to urine extracts purified by paper chromatography to give an approximate estimate of the content of cortisone-like hormones.

Schneider (1952) has been able to recover large amounts of a tetrahydrocortisone from urine extracts treated with β -glucuronidase. This and allied compounds must be expected to give a yellow colour with the Porter-Silber reagent, and these tetrahydrocortisone-like compounds, having a slow rate of flow in the chromatogram, will be present in the final eluates. When present in large amounts these compounds must be expected to interfere with the estimation of the cortisone and compound F in the chromatogram eluates. It is improbable that they are biologically active. We must presume from the agreement between bio-assay and chemical analysis that interference

from this cause has not been serious in our own urine extracts. But if greater specificity in analysis is needed then recourse must still be had to the biological methods of assay.

It seems likely that similar considerations will apply to the comparable method for the estimation of compound F in blood published by Nelson and Samuels (1952), whilst our own work was in progress, and that their method will be similarly capable of giving a reasonable approximation to the true compound F content of the blood extracts used.

Summary

The yield of cortisone-like hormone recoverable from human urine has been estimated after enzymatic hydrolysis with β -glucuronidase and chloroform extraction. It has been compared with the amount recovered after the more usual acid hydrolysis at room temperature.

The enzyme treatment increases the yield of cortisone-like hormone by from 8 to 315%, the mean increase being 99%.

The Porter-Silber colour reaction can detect quantitatively cortisone added to urine extracts, but often gives quite erroneous values for the cortisone-like hormone content of the extracts themselves.

Much of the interfering chromogen can be removed by paper chromatography.

On extracts purified by paper chromatography the Porter-Silber reaction can give estimates of cortisone-like hormone content which agree with the results of bio-assay of the same urine extracts.

Acknowledgments are due to the Medical Research Council for a grant for Miss B. Hurlock; to Smith, Kline, and French Laboratories for a generous gift of phenolphthalein glucuronide; to Dr. D. Marrack for assistance with the Beckman spectrophotometer readings; and to Dr. R. I. S. Bayliss for help in the preparation and assay of the glucuronidase. We are grateful to Miss E. Burton for much technical assistance.

REFERENCES

- Bayliss, R. I. S. (1952). *Biochem. J.*, **52**, 63.
 Burton, R. B., Zaffaroni, A., and Keutmann, E. H. (1951a). *J. biol. Chem.*, **188**, 763.
 — (1951b). *Ibid.*, **193**, 769.
 Carroli, K. K., McAlpine, H. T., and Noble, R. L. (1951). *Canad. med. Ass. J.*, **65**, 363.
 Cohen, S. L. (1951a). *Fed. Proc.*, **10**, 173.
 — (1951b). *J. biol. Chem.*, **192**, 147.
 Cope, C. L. (1951). *British Medical Journal*, **1**, 271.
 — Boysen, X., and McCrae, S. (1951). *Ibid.*, **2**, 762.
 Daughaday, W. H., Farr, A. L., and Houghton, E. (1951). *Endocrinology*, **49**, 146.
 Nelson, D. H., and Samuels, L. T. (1952). *J. clin. Endocr.*, **12**, 519.
 Pincus, G., Hechter, O., and Zaffaroni, A. (1951). *Proceedings Second Clinical A.C.T.H. Conference*, edited by J. R. Mote, **1**, 40. Churchill, London.
 Porter, C. C., and Silber, R. H. (1950). *J. biol. Chem.*, **185**, 201.
 Reich, H., Nelson, D. H., and Zaffaroni, A. (1950). *Ibid.*, **187**, 411.
 Schneider, J. J. (1950). *Ibid.*, **183**, 365.
 — (1952). *Ibid.*, **194**, 337.
 Talalay, P., Fishman, W. H., and Huggins, C. (1946). *Ibid.*, **166**, 757.
 Venning, E. H. (1945). *Transactions of Tenth Conference on Metabolic Aspects of Convalescence*, p. 177. Josiah Macy Jr. Foundation, New York.
 — Kazmin, V. E., and Bell, J. C. (1946). *Endocrinology*, **38**, 79.

In a paper which he read to the New York Health Conference last year Dr. I. Galdston discussed some of the public health problems in that city. He mentioned that there was a corp of police in the New York subway to hand out summonses for carrying lighted cigarettes or cigars, though the staff was insufficient to provide adequate or decent toilet facilities. For that matter, he added, there were inadequate public toilet facilities through the whole city of New York, despite the ageing population. Again, in a city which stretches for more than 10 miles, there were "few facilities whereby the over-taxed heart, the aching bones, or the fatigued muscles might be given respite on a public bench or park seat."

17-KETOSTEROID EXCRETION IN GOUT

BY

W. R. BUTT, B.Sc., A.R.I.C.

AND

F. GRAHAM W. MARSON, M.D., M.R.C.P.

(From the Department of Clinical Endocrinology, United Birmingham Hospitals, and the Department of Therapeutics, University of Birmingham)

Wolfson *et al.* (1949) reported that urinary 17-ketosteroid (17-K.S.) excretion was low in gout. In a group of 11 gouty patients, representing all stages of the disease, the average excretion was 3 mg. per 24 hours, with a range of 0.67–6.6 mg. per 24 hours. They stated that biological evidence of androgen activity was normal in 9 of the 10 men in this group, and explained this seemingly anomalous finding by postulating the presence of an abnormal androgen in gout which, when metabolized, does not materially contribute to the urinary 17-K.S.

Later, Robinson *et al.* (1949) reported findings in which 26 determinations of 17-K.S. in 10 gouty patients gave values consistently lower than normal, both during attacks and in interval periods.

Material and Methods

Our findings were obtained from a study of 33 gouty patients (see Table).

Basic Data on 33 Gouty Patients, Showing Serum Urate and 17-ketosteroid Levels

Case	Sex	Age (Years)	Average Serum Urate*	17-K.S. (mg./24 hours)	Acute Gout Witnessed	Satisfactory Colchicine Response
Recurring Acute Gouty Arthritis						
1	M	43	9.8 (5)	4.4		
2	M	53	6.6 (23)	7.4	+	+
3	F	68	6.7 (15)	5.4	+	+
4	M	55	5.6 (8)	10.9		
5	M	37	9.2 (21)	9.5	+	+
6	M	42	8.3 (18)	11.9	+	+
7	M	48	8.2 (6)	9.8	+	+
8	M	49	6.3 (5)	18.0		
9	M	57	5.1 (12)	8.3	+	+
10	M	36	7.8 (5)	16.4		
11	M	52	7.2 (7)	5.0		
12	M	69	7.5 (5)	6.7		
Chronic Gouty Arthritis						
13	M	53	5.0 (22)	7.0	+	+
14	M	65	5.5 (19)	10.8	+	+
15	M	56	4.8 (17)	6.8	+	+
16	M	59	7.2 (8)	14.0	+	+
17	M	60	10.8 (16)	11.0	+	+
18	M	40	8.7 (11)	8.6		
19	M	70	6.8 (9)	10.0		+
20	M	64	9.9 (7)	11.9		
21	M	56	8.4 (13)	13.1	+	+
22	M	59	9.2 (3)	9.3	+	+
23	M	54	9.2 (12)	13.9	+	+
24	F	61	7.4 (11)	6.0	+	+
25	M	45	7.2 (4)	11.2	+	+
26	F	67	5.0 (9)	7.3	+	+
27	M	57	5.8 (9)	11.7		
28	M	62	9.1 (3)	12.3		
29	M	45	8.5 (3)	12.4	+	+
30	M	58	8.6 (3)	5.7	+	+
31	F	68	7.4 (20)	1.4	+	+
32	M	49	8.6 (14)	10.7	+	+
33	M	37	12.7 (13)	5.0	+	+
Average all patients		54.4	7.7	9.5		

* The numbers of serum urate estimations are given in parentheses.

The neutral 17-K.S. values were obtained by a polarographic method (Barnett *et al.*, 1946a; Butt, 1950). Normal ranges for males are 10–20 mg. per 24 hours (age group 20–38 years), and for females 6–18 mg. per 24 hours (age