

Pregnane-3 α ,17 α ,20 α -Triol and Pregnane-3 α ,17 α ,20 α -Triol-11-One Excretion by Patients with Adrenocortical Dysfunction

R. I. Cox, M. Finkelstein

J Clin Invest. 1957;**36**(12):1726-1735. <https://doi.org/10.1172/JCI103575>.

Research Article

Find the latest version:

<https://jci.me/103575/pdf>



PREGNANE-3 α ,17 α ,20 α -TRIOL AND PREGNANE-3 α ,17 α ,20 α -TRIOL-11-ONE EXCRETION BY PATIENTS WITH ADRENOCORTICAL DYSFUNCTION¹

BY R. I. COX AND M. FINKELSTEIN²

(From the Department of Veterinary Physiology, University of Sydney, Sydney, Australia)

(Submitted for publication July 9, 1957; accepted August 27, 1957)

The first evidence that adrenocortical hyperplasia involves a fault in steroid metabolism was provided by Butler and Marrian (1-3) with the isolation of large amounts of pregnane-3 α ,17 α ,20 α -triol from three cases of adrenocortical hyperplasia. This compound was later isolated by Mason and Kepler (4) from three patients with hyperplasia and also from a patient with an adrenocortical tumor, and by Miller and Dorfman (5) from a further case of adrenal hyperplasia. More recently Cox and Marrian (6) isolated it in small quantities from the urine of normal men. The closely related compound pregnane-3 α ,17 α ,20 α -triol-11-one was isolated from the urine of two patients with adrenocortical hyperplasia by Finkelstein, v. Euw, and Reichstein (7) and more recently by Fukushima, Meyer, Ashworth, and Gallagher (8). The demonstration of the effectiveness of cortisone administration in the relief of symptoms of adrenocortical hyperplasia by Wilkins, Lewis, Klein, and Rosemberg (9) stimulated research into steroid metabolism in this condition.

A method for the quantitative estimation of pregnane-3 α ,17 α ,20 α -triol and other 17,20-dihydroxy-20 methyl steroids ("acetaldehydogenic steroids") was developed by Cox (10). The acid and enzymic hydrolysis of conjugates of these steroids and their chromatographic separation were investigated by Cox and Marrian (11, 6). Bongiovanni, Clayton, and Eberlein (12, 13), using a reaction based on chromogen production with sulfuric acid, have estimated the excretion of a pregnanetriol-like fraction in adrenocortical hyperplasia, and Zondek and Finkelstein (14, 15) have used fluorometric estimation of a pregnanetriolone-

like fraction to distinguish congenital adrenocortical hyperplasia from true hermaphroditism and male pseudo-hermaphroditism. None of these methods is specific for any single compound.

More recently Finkelstein and Goldberg (16, 17) developed an analytical method including paper chromatographic separation for the estimation of pregnane-3 α ,17 α ,20 α -triol-11-one in human urine. They reported the presence of this compound in the urines of patients with adrenocortical hyperplasia, but not in detectable amounts in the urine of normal people or of patients with adrenal tumors. Because of this finding the latter workers suggested that the presence of pregnanetriolone is characteristic of adrenal hyperplasia and that its detection may be useful in the diagnosis of this condition. By contrast, since pregnanetriol appears to be excreted in greater than normal amounts by patients with tumors of adrenal origin as well as by patients with adrenal hyperplasia, its excretion at abnormally high levels cannot be considered characteristic of adrenal hyperplasia. However, the estimation of pregnanetriolone and pregnanetriol simultaneously is of obvious value in the diagnosis and differentiation of adrenal hyperplasia and adrenal tumors.

A procedure for the simultaneous estimation of pregnanetriol and pregnanetriolone was briefly described by Finkelstein and Cox (18). The present paper describes in detail a method for the identification and estimation of both pregnane-3 α ,17 α ,20 α -triol and pregnane-3 α ,17 α ,20 α -triol-11-one suitable for routine clinical use. The results of applying the method to clinical cases are reported and discussed.

METHODS

Reagents. Benzene (thiophene free), chloroform (B.P.), and ethylene dichloride were fractionally distilled before use. Ethanol (absolute) and methanol were refluxed 8 hours with 1 per cent by weight of sodium hy-

¹ This work was supported by a grant and a Senior Research Fellowship (R. I. Cox) from the New South Wales State Cancer Council.

² On leave from the Hebrew University. Present address: Hormone Research Laboratory, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

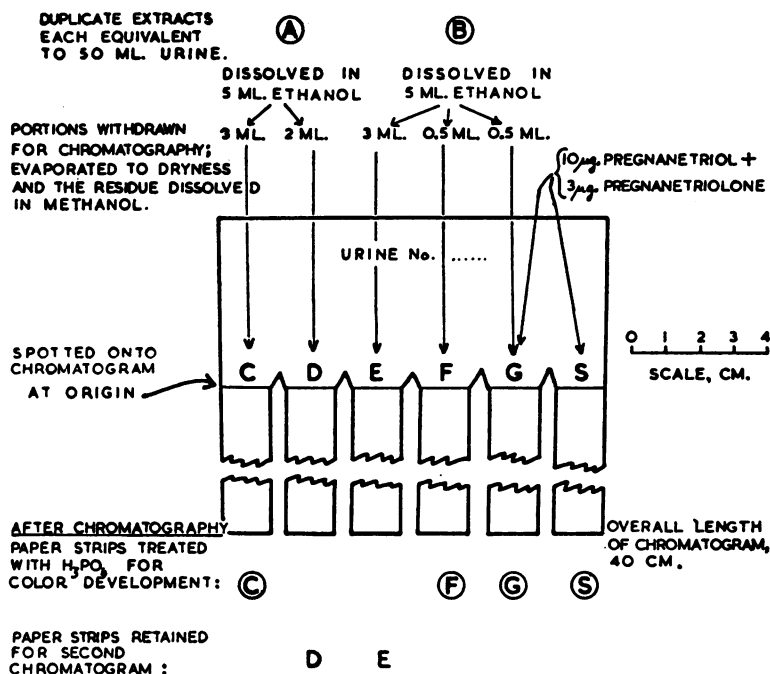


FIG. 1. CHROMATOGRAPHY OF URINE EXTRACTS

dioxide, and distilled twice. Formamide, laboratory grade (British Drug Houses Ltd.), was used without purification. Acetic acid, sodium acetate, sodium bicarbonate, sodium hydroxide, anhydrous sodium sulfate, and phosphoric acid were of analytical reagent grade. The β -glucuronidase used was derived from the mollusc *Celana tramoserica*, a limpet common on sea shores near Sydney (19). Other preparations of β -glucuronidase would be expected to be satisfactory. Pregnane-3 α ,17 α ,20 α -triol, m.p. 245 to 247° C.³ and pregnane-3 α ,17 α ,20 α -triol-11-one, double m.p. 189° C. and 210° C., were used as reference compounds.

Urine collection. A 24 hour collection of urine over 2 ml. of chloroform is made. The volume is measured.

Hydrolysis procedure. A 100 ml. portion of urine is withdrawn and the pH adjusted to 4.6 with glacial acetic acid. Two ml. of 2M acetate buffer of pH 4.6 are added and, after mixing, the sample is divided into two equal portions (A and B). These samples are placed in a 37° C. water bath and allowed to warm for half an hour. To each is added 5 ml. of chloroform and 1 ml. of the β -glucuronidase preparation containing 2,500 units of enzyme. The samples are allowed to stand at 37° C. for 24 hours. These conditions do not necessarily achieve maximum hydrolysis of the steroid conjugates, but have been found satisfactory for clinical use with this method. Other enzyme preparations may require use of different conditions for hydrolysis.

Extraction procedure. Each of the duplicate samples is extracted twice with 100 ml. of chloroform. The chloroform

extracts are washed with 20 ml. N sodium hydroxide and the alkali is drained off as thoroughly as possible. The emulsion at the interface is discarded. The chloroform is washed with 10 ml. of 8 per cent sodium bicarbonate solution, draining off the alkali thoroughly. The chloroform extract is run into a clean funnel and washed with 10 ml. of water twice. The washed chloroform is run into a dry flask containing 2 Gm. anhydrous sodium sulfate and the flask is shaken and allowed to stand for ten minutes. The chloroform is filtered through filter-paper into a 1-liter round bottom flask for evaporation. The evaporation is carried out under reduced pressure on a water bath, making sure that the flask is removed from the bath as soon as the last drop of chloroform has evaporated. The residue is dissolved with slight warming in 3 ml. of ethanol and the ethanol extract transferred with a pipette to a graduated centrifuge tube. The flask is rinsed again twice with 1 ml. portions of ethanol. The volume is made up to 5 ml. and stirred to mix the solutions. The portions shown in Figure 1 are withdrawn from the ethanol extract and evaporated to dryness in test tubes (about 1 cm. in diameter) under a gentle stream of nitrogen while heating on a water bath at about 80° C. The test tubes are withdrawn from the water bath as soon as the extracts are dry. These samples are now ready for chromatography.

Paper Chromatography

Apparatus

Whatman No. 1 paper "for chromatography" is used without any purification. Chromatograms are con-

³ We are indebted to Professor G. F. Marrian for the gift of a sample of this steroid.

veniently run horizontally (20) in shallow glass trays, the top edge of the tray being ground flat. A piece of three-eighths inch plate glass is placed on top of the tray to form a closed tank. A glass rack 1 to 2.5 cm. high supports the paper chromatogram inside the tray (Figure 2).⁴ A suitable sized tray is 50 cm. by 30 cm. by 5 cm., although smaller trays down to about 30 cm. by 20 cm. by 3 cm. are satisfactory. The solvent systems used are those, or similar to those, developed by Zaffaroni and Burton (21), so other chromatography apparatus suitable for the Zaffaroni-type chromatograms may be used. An incubator regulated at 37° C. is convenient for the horizontal chromatograms and can accommodate many trays of the above type. The chromatograms are also effective at lower temperatures such as 25° C.; the higher temperature was chosen for convenience in working in Sydney.

Procedure

(a) First chromatogram

For each urine sample a chromatogram strip is cut with six tongues 1.5 cm. wide, 35 cm. long, and separated by 0.5 cm. from one another. A headband of 5 cm. is used (see Figure 1). The paper is marked in pencil on the headband with the reference number and letter as shown in Figure 1.

For the first chromatogram the benzene-formamide system is used. Equal volumes (500 ml.) of benzene and formamide are equilibrated at 37° C., and the layers are separated in a funnel at 37° C.; these equilibrated sol-

⁴We are indebted to Dr. S. E. Wright for suggesting the use of this modification of the horizontal chromatographic tank.

vents may be stored at room temperature until required for use. The stock bottles are shaken before withdrawing samples of the solvents. The chromatogram trays are warmed for at least one hour in the 37° C. incubator used for chromatography before starting a run. The ground glass edge of the tray is moistened with formamide to provide a seal and 100 ml. of equilibrated benzene is run into the tray at the start of the warming period.

To impregnate the paper with formamide, 10 ml. of formamide (equilibrated with benzene at 37° C.) is freshly mixed with one volume of methanol. The paper is dipped through the mixture quickly and the excess liquid blotted off thoroughly between filter paper.

The paper is placed on a glass rack with a nitrogen distributor about 3 cm. from the paper surface. An electric lamp to provide gentle warmth and speed up evaporation of the solvent applied with the extracts is placed close to the paper. The extracts in the test tubes are dissolved by adding 0.05 ml. of methanol to each and warming slightly. Each extract is transferred to a strip on the chromatogram with a pipette and the paper is spotted along as narrow a line as possible. To the residue in the test tube is added 0.01 ml. of methanol and this wash is transferred to the paper. The wash is repeated with 0.01 ml. of methanol. To the strip with extract G is added 10 μ g. of triol and 3 μ g. of triolone in methanol, and to the sixth strip S is added these amounts of pure substances alone. When the methanol has dried off, the chromatogram is placed on the glass rack in the pre-warmed tank at 37° C. and the run is started by dipping the end of the paper in the solvent. The chromatogram is allowed to run for 2 to 3 hours when the solvent front should have travelled about 30 cm.

At the end of the run the chromatogram is removed

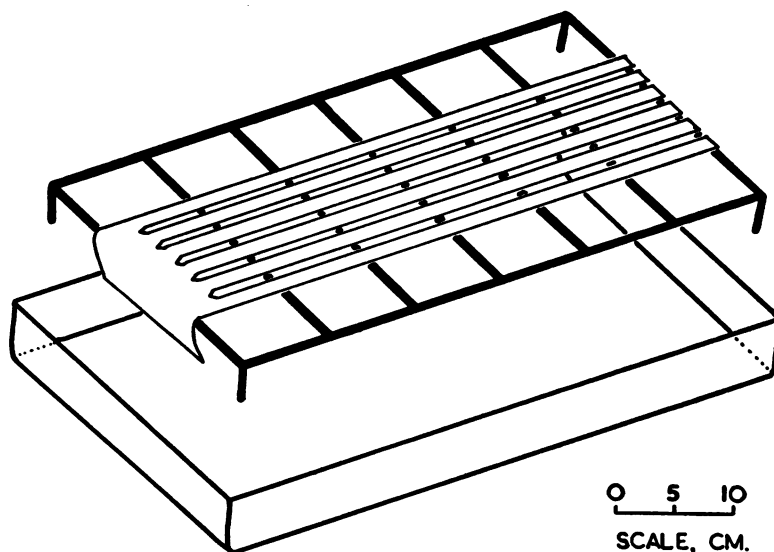


FIG. 2. APPARATUS FOR HORIZONTAL CHROMATOGRAPHY
Glass tray and rack; paper chromatogram in position on rack.

estimated by comparison of spot size and intensity of the standards. With a standard of 10 $\mu\text{g.}$ of pregnanetriol, less than 5 $\mu\text{g.}$, 5 $\mu\text{g.}$, 10 $\mu\text{g.}$, 20 $\mu\text{g.}$, and greater than 20 $\mu\text{g.}$ are estimated levels; with 3 $\mu\text{g.}$ of pregnanetriolone as a standard, less than 1½ $\mu\text{g.}$, 1½ $\mu\text{g.}$, 3 $\mu\text{g.}$, 6 $\mu\text{g.}$, and greater than 6 $\mu\text{g.}$ are estimated. Other substances besides these steroids may give rise to colors of this type, but normally are readily differentiated on the basis of R_f values by comparison with the positions of the standard substances. In some cases blue spots, distinctly different from the blue-violet given by pregnanetriolone, move close to the latter compound. In doubtful cases it is desirable to rechromatograph on a second solvent system.

Amounts of compounds estimated. Amounts equivalent to the standard, to double and to half the standard, and amounts outside this range can readily be estimated. Thus, using the above chromatograms with the equivalent of 5 and 30 ml. of urine extract on a strip, the ranges of steroids estimated are approximately 0.2 to 4.0 mg. per L. triol, and 0.05 to 1.2 mg. per L. triolone each in six steps. In addition, higher and lower levels than these are indicated. The range may be extended by running the second chromatogram using smaller portions of extracts for higher values of steroids, or running on 0.5 cm. wide strips rather than 1.5 cm. wide strips for low values of steroids. The 30 ml. (range 1) and 5 ml. (range 2) urine extract volumes have been chosen in such a way that normal people show pregnanetriol, and very occasionally a spot similar to pregnanetriolone, on range 1, but not on range 2; the abnormally high excretion shown by patients with adrenal hyperplasia result in these steroids being visible on range 2 as well as range 1.

(b) *Second chromatogram*

The strips D and E which have not been dipped in phosphoric acid are cut according to the positions of the steroids found in the strips C, F, G, and S. Normally the solvent fronts should have moved almost equal distances on each strip and a direct comparison of D and E with C, F, G, and S may be made. If the solvent fronts differ from one strip to another, the expected positions of the spots are calculated relative to the movement of the solvent front. An extra margin of 0.5 cm. is allowed at each edge of the spot. Each piece of paper is cut into small pieces about 1 to 2 mm. by 3 to 5 mm. The triol and triolone spots from any one strip are combined. The small pieces of paper are then extracted by soaking and warming in 2 ml. of methanol for a few minutes, decanting off the methanol into a graduated centrifuge tube, and repeating the extraction with 2 ml. of warm methanol twice more. The volume of the extracts is made up to 6 ml. with methanol, and the solutions mixed with a stirring rod. Suitable portions of these extracts, as indicated from the results of the first chromatogram, are evaporated to dryness. The portions are chromatographed on a four-strip paper, the solvent system ethylene dichloride-formamide is used. The paper is impregnated with formamide-methanol 1:1 using formamide equili-

brated with ethylene dichloride. The solvent placed in the tank for this chromatogram is ethylene dichloride pre-equilibrated with formamide; otherwise, conditions and techniques are as described for the first chromatogram. The strips are treated with phosphoric acid after running the chromatogram. As an alternative to the ethylene dichloride-formamide system, the chloroform-formamide system may be used but in this case the chloroform should be freshly distilled as required.

The distances moved by pregnanetriol and pregnanetriolone relative to the solvent fronts are 0.3, 0.08 (benzene-formamide); 0.7, 0.4 (ethylene dichloride-formamide); 0.75, 0.5 (chloroform-formamide), respectively. These values apply to the apparatus and conditions used above and may vary somewhat, but are given as a guide (see Figure 3).

RESULTS

Using the method described, the urinary levels of pregnanetriol and pregnanetriolone shown in Table 1 were found for 24 normal adults and children, 6 patients with adrenocortical hyperplasia before treatment, 10 patients with adrenocortical hyperplasia receiving cortisone, prednisolone or similar therapy, and a patient with a tumor of adrenal origin. The observed values for pregnanetriol and pregnanetriolone on the first and second chromatograms are given. Between the normal groups and the patients with adrenocortical hyperplasia there is a clear difference. In the latter, pregnanetriolone is excreted in relatively large amounts and there is an associated increase in the pregnanetriol levels. The absence of pregnanetriolone with tumors of adrenal origin is suggested by the case reported here and by the two cases of women, one with a virilizing adrenal tumor and the other with an adrenal adenoma, noted by Finkelstein and Goldberg (17). Differentiation of patients with tumors of adrenal origin from adrenocortical hyperplasia on the basis of the pregnanetriolone excretion seems possible. Confirmation of this supposition awaits study of further cases, but it does not appear unreasonable in view of the very definite excretion of pregnanetriolone by every one of twelve patients with adrenocortical hyperplasia (present paper, six cases), its excretion by six hyperplasia patients maintained on cortisone or similar therapy, and its absence in the three tumor cases.

Cortisone treatment is known to decrease the pregnanetriol values (22) and pregnanetriolone values (15). In the above series of results such

TABLE I

Excretion of pregnanetriol and pregnanetriolone by normal subjects and by patients with adrenocortical hyperplasia or tumors

	Urine sample	Subject	Age (yrs.)	Amount of steroid found (mg./24 hrs.)			
				Pregnanetriol		Pregnanetriolone	
				1st chromatogram	2nd chromatogram	1st chromatogram	2nd chromatogram
Normal males	162*	K. W.	5	<0.1	<0.1	†	††
	181	L. E.	5	0.2	0.1	‡	‡‡
	163*	R. G.	6	<0.1	<0.1	0.07	†††
	164*	R. O. G.	6	0.2	<0.1	‡	‡‡
	160*	J. D.	11		<0.1	‡	‡‡
	158	J. T.	23	0.2	0.3	‡	‡‡
	157	D. L.	28	0.6	0.8	‡	‡‡
	174	R. C.	28	0.3	0.1	‡	‡‡
	175	R. P.	28		0.3	‡	‡‡
	178	H. S.	36	0.3	<0.2	†	‡‡
Normal females	188	J. M.	2.3	<0.04	<0.04	‡	‡‡
	165*	C. S.	5	<0.1	<0.1	‡	‡‡
	167*	N. C.	7	0.3	<0.1	0.08	‡‡
	169*	M. H.	8	0.2	0.1	†	‡‡
	161*	R. K.	13	0.1	<0.1	‡	‡‡
	182	H. E.	13		<0.1	‡	‡‡
	180	J. E.	15	0.2	0.2	‡	‡‡
	186	D. G.	18	<0.2	<0.2	‡	‡‡
	184	T.	19	0.6	0.3	‡	‡‡
	183	F. B.	27	0.5	0.3	‡	‡‡
	176	E. C.	27	0.5	0.2	‡	‡‡
	185	R. P.	30	0.1	0.1	‡	‡‡
	177	H. S.	32		<0.2	‡	‡‡
179	C. E.	44	0.4	0.2	†	‡‡	
Adrenal hyperplasia	190	A.	2 wks. ♀	>0.3	0.4	>0.7	0.9
	194	R. P.	7 wks. ♂	0.6	1.0	0.6	0.6
	149	S. T.	8 wks. ♀	0.2	0.3	0.3	0.5
	101	J. T.	6 ♀	3	4	0.2	0.4
	173	C. J.	13 ♀	3	3	0.5	0.4
	166	R. M.	16.5 ♂	>4	7	1.2	1.0
Adrenal hyperplasia on steroid therapy††	200¶	R. P.	10 wks. ♂	0.05		0.05	
	152	G. S.	1.5 ♀	0.3	0.4	0.2	0.2
	156	L. R.	3 ♀	0.3	0.3	0.1	0.2
	153	S. S.	4 ♀	0.8	0.8	0.4	0.5
	148	B. H.	4 ♂	0.4	0.1	‡	‡‡
	171**	J. T.	6 ♀	0.3	0.3	‡	‡‡
	172	M. T.	8 ♂	0.1	0.4	‡	‡‡
	154	R. Mi.	11 ♀		0.3	‡	0.07
	197	H. P.	12 ♀	1.0	1.3	0.3	0.2
	145††	K. F.	14 ♀	>3	4	2.5	1.4
Tumor	23††	S. Ta.	9 ♀	3	3	‡	‡
	189††	S. Ta.			<0.1	‡	‡

(6 mos. post-operative)

* Hospitalized patients towards end of convalescence.

† Doubtful spot.

‡ Faint spot, not pregnanetriolone (see text).

§ Levels below 50 µg. per 24 hours.

|| Spot partly obscured by other material or atypical color.

¶ Compare with 194 above, same subject.

** Compare with 101 above, same subject.

†† 17-ketosteroid levels estimated by Institute of Child Health, Sydney, were within normal range except for sample 145; the latter patient showed very variable excretion which here was high at 29 mg. per 24 hours.

‡‡ "Adrenal adenoma" of ovary.

a decrease in indicated in samples 101, 171, 194, and 200, being samples of urine from the same patients before and after cortisone treatment, respectively. It is noteworthy that in samples 152, 156, and 153 from patients with adrenal hyperplasia but on steroid therapy, the 17-ketosteroid excretion was within the normal range, whereas marked amounts of pregnanetriolone were still being excreted.

Presence of other compounds besides pregnanetriol and pregnanetriolone

In some of the chromatograms of the 41 urine extracts reported in this paper, there appeared a number of other spots of a variety of colors apart from the spots of pregnanetriol and pregnanetriolone. Most of the blue colors which have been observed were notably different from the blue-violet color given by pregnanetriolone in the color test. With high levels of pregnanetriol and pregnanetriolone, the presence of other spots did not interfere with the detection and estimation of these steroids on the first chromatogram. However, in extracts from some of the normal subjects, interference was found in the first chromatogram because of the relatively low levels or absence of the steroids. No interference was noted in the second chromatogram even if it occurred on the first.

It should be noted that the spots which are estimated as pregnanetriol and pregnanetriolone are assumed to be these compounds from the colors developed in the phosphoric acid reaction and from their R_F values. However, it is possible that closely related compounds may not be distinguished, especially at low levels of pregnanetriol and pregnanetriolone. Thus, samples 162, 163, and 167 in the normal group showed faint spots corresponding to pregnanetriolone; however, the higher resolution of a 27 hour descending chromatogram showed that these trace substances were not pregnanetriolone. With all the adrenocortical hyperplasia patients, pregnanetriolone showed up very clearly; usually the spot was so intense as to require dilution of fractions for the second chromatogram. Two liters of urine from an adrenocortical hyperplasia patient, R. M., sample 166, were processed by this method except for the use of a sheet of Whatman No. 3 MM paper for chromatography. From the area on the paper

corresponding to pregnanetriolone a substance was isolated directly after the second chromatogram. This substance was identified as pregnanetriolone from its infra-red absorption spectrum. The compounds recently isolated by Fukushima, Meyer, Ashworth, and Gallagher (8), allopregnane-3 α , 17 α , 20 α -triol and pregnane-3 α , 11 β , 17 α , 20 α -tetrol,⁵ are completely resolved from pregnanetriol and pregnanetriolone in the benzene-formamide system as used in the method described in this paper (23).

DISCUSSION

The method presented is simple and effective for the detection of pregnane-3 α , 17 α , 20-triol and pregnane-3 α , 17 α , 20 α -triol-11-one in urine, and can be carried out routinely by a technician. No elaborate precautions are required and, in particular, purification of filter paper is not necessary. In tests of the urine of patients with adrenal hyperplasia or adrenal tumors, a single chromatogram is usually sufficient to show the presence and indicate the levels of these steroids. In some cases where there is uncertainty, a second chromatographic separation is required. The minimum amounts detectable are about 100 μ g. per 24 hours for pregnanetriol and 50 μ g. per 24 hours for pregnanetriolone. The lower levels of pregnanetriol associated with normal adults and especially with normal children sometimes may not be detectable. The tests may be made more sensitive, or larger amounts of urine may be extracted; however, the method as presented is designed primarily to detect abnormally high levels.

The methods which have been in use for estimation of pregnanetriol (10, 12) and pregnanetriolone (15) are not specific for these steroids and demand their prior separation from related or interfering substances. Thus, estimation by means of acetaldehyde produced on periodate oxidation (10) is a general reaction for C21 steroids of the 17,20-dihydroxy-20-methyl series. Substances, in addition to pregnanetriol or pregnanetriolone, giving this reaction have been extracted from urine (6, 8). The method for pregnanetriol based on chromogens produced on reaction with concentrated sulfuric acid (12) requires adequate purification of extracts due to the poor specificity of

⁵ Samples of these steroids were generously donated by Dr. T. F. Gallagher.

the reagent. Pregnanetriolone produces an intensely fluorescent compound on heating with concentrated phosphoric acid, but fluorogenic substances other than this compound in neutral urine extracts are excreted by normal people and in increased amounts by patients with adrenal tumors and in late pregnancy (14, 17).

Recently, De Courcy (24) elaborated a method for the estimation of pregnane-3 α ,17 α ,20-triols, and Finkelstein and Goldberg (16, 17) for pregnane-3 α ,17 α ,20 α -triol-11-one. Both methods utilize paper chromatography for the separation of the respective compounds, which are detected by means of fluorescence. It is worth mentioning, however, that in the method of De Courcy only pregnanetriol, but not pregnanetriolone, is visualized by the reagent employed. On the other hand, in the method of Finkelstein and Goldberg, pregnanetriol is destroyed by the hot acid hydrolysis as used for the liberation of pregnanetriolone from its conjugate (25).

Since pregnanetriol produces fluorescence and can be easily detected on paper with the 70 per cent phosphoric acid reagent used for estimation of pregnanetriolone, substitution of the hot acid treatment by enzymic hydrolysis, which liberates both compounds without destroying them, allows for their simultaneous estimation. Moreover, in enzymic hydrolysis, less chloroform extractable impurities are formed than in hot acid hydrolysis, thus making unnecessary the rigorous purification of the extracts described previously for the detection of pregnanetriolone (17). The present method described primarily for clinical use, and obviating the use of precise optical apparatus such as spectrophotometer or fluorimeter, is semi-quantitative. Used in conjunction with estimation of acetaldehydogenic (10) or fluorogenic substances (14), it can give precise quantitative measurements of individual compounds such as pregnanetriol or pregnanetriolone and may be used to detect unknown steroids on the chromatograms. Results of such studies will be published elsewhere.

The differentiation between adrenal hyperplasia and adrenal tumor is a problem of clinical importance. Jailer, Gold, and Wallace (26) suggested that the decrease in excretion of 17-ketosteroids on cortisone administration would indicate adrenal hyperplasia. However, in some cases of adrenal hyperplasia of the adreno-genital type

the 17-ketosteroids do not readily respond to cortisone therapy (27) (case No. 145, this paper). Moreover, in Cushing's syndrome the differentiation between adrenal hyperplasia and adrenal tumor is hardly possible on the basis of 17-ketosteroid suppression on cortisone administration [*cf.*, Wilkins (28) and Thorn (29)]. Bongiovanni (22) associated high excretion of the pregnanetriol-like fraction with adrenal hyperplasia and observed that on cortisone administration this fraction decreased. In addition to the original isolation of pregnanetriol from the urine of patients with the adreno-genital syndrome by Butler and Marrian (1-3), it has also been isolated from the urine of patients with adrenal tumors (4), and from the urine of normal men (6). High levels of pregnanetriol appear to be associated with adrenal tumors as well as with adrenal hyperplasia, and thus do not allow for differentiation between these two conditions. As to the decrease of pregnanetriol on cortisone treatment, it remains to be proved that such a decrease is more consistent than the corresponding decrease of the 17-ketosteroids. On the other hand, the consistent presence of pregnanetriolone in the urine of patients with adrenal hyperplasia and its apparent absence in detectable amounts from the urine of three patients with adrenal tumor can make it a valuable differential diagnostic aid between these conditions. It has been reported that one patient with the hypertensive form of adrenal hyperplasia (13, 30) excreted no detectable amounts of expected 11-oxy steroids in the urine; although pregnanetriolone was not specifically searched for in this work, it is desirable that data be obtained on the excretion of this steroid by such patients. It is fully realized that a general conclusion on the absence of pregnanetriolone from urine of patients with adrenal tumors is not permissible on the basis of the results obtained on only three cases of tumors examined so far. However, in view of the rarity of this disease and thus of a small chance that our laboratories (located in countries with relatively small populations) will be able to compile a large series of adrenal tumors within a reasonable time, it seems to us that by reporting on the difference in steroid excretion by patients with adrenal hyperplasia and adrenal tumors, research on larger series may be stimulated.

It was suggested in a previous paper (17) that pregnanetriolone is characteristic of adrenal hyper-

plasia. Only six cases of adrenal hyperplasia were investigated. The presence of pregnanetriolone in an additional six patients with adrenal hyperplasia not receiving cortisone therapy and in six patients maintained on cortisone, reported in the present paper, greatly substantiates this supposition. On the other hand, analysis of urine of 24 "normal" people described here, of 37 reported previously (17), and of an additional 25 "normal" persons (31) shows that pregnanetriolone is not detectable in "normal" persons' urine, and if present, it must be in relatively small quantities (less than 50 μ g. per 24 hours). High levels are associated with adrenal hyperplasia (see Table I). Children with adrenal hyperplasia of the virilizing type show definite presence of pregnanetriolone, and milligram quantities are excreted by adults with the adreno-genital syndrome due to adrenal hyperplasia.

SUMMARY

A method for the detection and estimation of urinary pregnane-3 α ,17 α ,20 α -triol and pregnane-3 α ,17 α ,20 α -triol-11-one suitable for routine clinical use is described.

The results of estimation of the excretion of these steroids by 41 people, including "normal" persons and patients with adrenocortical hyperplasia or tumors of adrenal origin, are presented. Normally, pregnanetriolone is not excreted in detectable amounts but this steroid appears in relatively large amounts in the urine of patients with adrenocortical hyperplasia; pregnanetriolone has not been found in the urine of three patients with tumors of adrenal origin. On the other hand, high pregnanetriol levels appear to be associated with both adrenocortical hyperplasia and with tumors of adrenal origin.

The characteristic finding of pregnanetriolone in cases of adrenocortical hyperplasia should be a useful diagnostic aid.

ACKNOWLEDGMENTS

The authors wish to thank Professor C. W. Emmens for his interest and encouragement in this work and for the facilities which enabled the work to be carried out. We are grateful to Professor B. Zondek for his helpful advice and interest in the preparation of this paper. We are indebted to Drs. R. H. Vines, K. Harrison, A. W. Steinbeck and Miss Stobo for obtaining urine specimens, and to Miss Helen D'Arcy for skilled technical assistance.

REFERENCES

1. Butler, G. C., and Marrian, G. F., The isolation of pregnane-3,17,20-triol from the urine of women showing the adrenogenital syndrome. *J. Biol. Chem.*, 1937, 119, 565.
2. Butler, G. C., and Marrian, G. F., Chemical studies on the adreno-genital syndrome. (1) The isolation of 3(α)-hydroxyetiocholane-17-one, 3(β)-hydroxyetioallocholane-17-one (isoandrosterone), and a new triol from the urine of a woman with an adrenal tumor. *J. Biol. Chem.*, 1938, 124, 237.
3. Marrian, G. F., and Butler, G. C., Chemical studies on the adreno-genital syndrome. *Nature*, London, 1938, 142, 400.
4. Mason, H. L., and Kepler, E. J., Isolation of steroids from the urine of patients with adrenal cortical tumors and adrenal cortical hyperplasia: a new 17-ketosteroid, androstane-3(α),11-diol-17-one. *J. Biol. Chem.*, 1945, 161, 235.
5. Miller, A. M., and Dorfman, R. I., Metabolism of the steroid hormones: isolation of 13 steroid metabolites from a patient with (probable) adrenal hyperplasia. *Endocrinology*, 1950, 46, 514.
6. Cox, R. I., and Marrian, G. F., The isolation of pregnane-3 α ;17 α ;20 α -triol from the urine of normal men. *Biochem. J.*, 1953, 54, 353.
7. Finkelstein, M., v. Euw, J., and Reichstein, T., Isolierung von 3 α ,17,20 α -Trioxy-pregnanon-(11) aus pathologischem menschlichem Harn. *Helvet. chim. acta*, 1953, 36, 1266.
8. Fukushima, D. K., Meyer, E. D., Ashworth, E., and Gallagher, T. F., Isolation and characterization of two new urinary steroids. *Federation Proc.*, 1956, 15, 257.
9. Wilkins, L., Lewis, R. A., Klein, R., and Rosemberg, E., The suppression of androgen secretion by cortisone in a case of congenital adrenal hyperplasia. *Bull. Johns Hopkins Hosp.*, 1950, 86, 249.
10. Cox, R. I., A method for the quantitative determination in urinary extracts of C21 17:20-dihydroxy-20-methylsteroids. *Biochem. J.*, 1952, 52, 339.
11. Cox, R. I., and Marrian, G. F., A note on the hydrolysis of the urinary conjugated adrenocortical steroids by β -glucuronidase preparations. *Biochem. J.*, 1953, 54, 45.
12. Bongiovanni, A. M., and Clayton, G. W., Jr., A simplified method for the routine determination of pregnanediol and pregnanetriol in urine. *Bull. Johns Hopkins Hosp.*, 1954, 94, 180.
13. Eberlein, W. R., and Bongiovanni, A. M., Congenital adrenal hyperplasia with hypertension: unusual steroid pattern in blood and urine. *J. Clin. Endocrinol. & Metab.*, 1955, 15, 1531.
14. Zondek, B., and Finkelstein, M., A neutral fluorogenic substance in human urine and its increased excretion in pregnancy and pseudohermaphroditism. *Acta endocrinol.*, 1952, 11, 297.
15. Zondek, B., and Finkelstein, M., Quantitative estimation of 3 α ,17,20 α -pregnanetriol-11-one in urine as

- an aid in the diagnosis of pseudohermaphroditic conditions. *Acta endocrinol.*, 1954, 17, 452.
16. Goldberg, S., and Finkelstein, M., Studies in steroid excretion in patients with a virilizing syndrome. *Bull. Research Council of Israel*, 1956, 5A, 310.
 17. Finkelstein, M., and Goldberg, S., A test for qualitative and quantitative estimation of pregnane-3 α , 17 α , 20 α -triol-11-one in urine and its significance in adrenal disturbances. *J. Clin. Endocrinol. & Metab.*, In press.
 18. Finkelstein, M., and Cox, R. I., A method for simultaneous estimation of pregnane-3 α , 17 α , 20 α -triol and pregnane-3 α , 17 α , 20 α -triol-11-one in urine. *Proc. Soc. Exper. Biol. & Med.*, 1957, 95, 297.
 19. Cox, R. I., β -glucuronidase and a sulphatase from an Australian mollusc. *Australian J. Sc.*, 1957, 19, 202.
 20. Meredith, P., and Sammons, H. G., Horizontal paper chromatography. *Analyst*, 1952, 77, 416.
 21. Zaffaroni, A., and Burton, R. B., Identification of corticosteroids of beef adrenal extract by paper chromatography. *J. Biol. Chem.*, 1951, 193, 749.
 22. Bongiovanni, A. M., The detection of pregnandiol and pregnantriol in the urine of patients with adrenal hyperplasia. Suppression with cortisone. A preliminary report. *Bull. Johns Hopkins Hosp.*, 1953, 92, 244.
 23. Cox, R. I., Unpublished data.
 24. De Courcy, C., A trichloroacetic acid reagent for the detection of pregnane-3 α ; 17 α ; 20-triols on paper chromatograms. *J. Endocrinol.*, 1956, 14, 164.
 25. Marrian, G. F., and Atherden, S. M., Urinary adrenocortical steroids: II. Formaldehydogenic and acetaldehydogenic substances in extracts of hot acid-hydrolysed urine. *Acta endocrinol.*, 1953, 13, 265.
 26. Jailer, J. W., Gold, J. J., and Wallace, E. Z., Evaluation of the "cortisone test" as a diagnostic aid in differentiating adrenal hyperplasia from adrenal neoplasia. *Am. J. Med.*, 1954, 16, 340.
 27. Hampson, J. G., Money, J., and Hampson, J. L., Hermaphroditism: Recommendations concerning case management. *J. Clin. Endocrinol. & Metab.*, 1956, 16, 547.
 28. Wilkins, L., General Discussion of Cushing's Syndrome in *Ciba Foundation Colloquia on Endocrinology*, Vol. VIII. *The Human Adrenal Cortex*. London, J. & A. Churchill Ltd., 1955, p. 523.
 29. Thorn, G. W., Discussion of Cushing's Syndrome in *Ciba Foundation Colloquia on Endocrinology*, Vol. VIII. *The Adrenal Cortex*. J. & A. Churchill Ltd., 1955, p. 523.
 30. Eberlein, W. R., and Bongiovanni, A. M., Plasma and urinary corticosteroids in the hypertensive form of congenital adrenal hyperplasia. *J. Biol. Chem.*, 1956, 223, 85.
 31. Goldberg, S., Unpublished data.