THE METABOLISM OF PROGESTERONE.

A Thesis presented
for the Degree of

DOCTOR OF PHILOSOPHY.

by

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GENERAL INTRODUCTION.

The presence of a hormone-like principle in the corpus luteum was suspected following the observations made by Fraenkel in 1903 upon the effect of ablation of the corpus luteum of pregnancy. A correlation between the activity of the corpus luteum and histolo-:gical changes in the uterus had been suggested by Bouin and Ancel (1909, 1911) and Meyer (1911), but it was not until 1928 that the active principle of the corpus luteum was clearly demonstrated by Corner and Allen and the term "progestational" applied to its effect upon the endometrium.

In the following year, a saturated dihydroxy alcohol was isolated from human pregnancy urine by Marrian (1929) and although progesterone itself was not to be isolated and identified until 1934 and the biochemical significance of this new compound could not be foreseen, its discovery marked the beginning of a new phase in the study of the corpus luteum hormone. The structural relationship of this substance to the bile acids was established by Butenandt (1931) and the new steroid was named "pregnanediol".

In 1934 the isolation of progesterone was reported almost simultaneously by Butenandt, Westphal and Hohlweg; Slotta, Ruschig and Fels; Allen and Wintersteiner; and Hartmann and Wettstein, and following the elucidation of its structure by Butenandt et al. (1934)

the possibility that pregnanediol might be a metabolic reduction product of progesterone in the body became apparent.

The isolation of allopregnanediol frum human pregnancy urine by Hartman and Locher (1935) appeared to lend support to this hypothesis although it may be stated in parenthesis that conclusive evidence that allopregnanediol is a metabolite of progesterone is still awaited.

In 1936, Venning and Browne demonstrated the conjugation of pregnanediol as a sodium salt of glucuronic acid in pregnancy urine and in the following year Venning (1937) developed a method for the determination of pregnanediol glucuronide in urine. Using this method, Venning and Browne (1937, 1938, 1940) studied the excretion of urinary pregnanediol in normal pregnancy, in the menstrual cycle, and in a series of investigations in which progesterone was administered to human subjects.

These important studies which established pregnanediol as a metabolic product of progesterone, form the essential basis for further investigations such as are envisaged in the present work. These workers suggested that the uterine endometrium was involved in the metabolic reduction of progesterone to pregnanediol, that the pregnanediol so formed was conjugated with glucuronic acid in the liver, and that the glucuronide was excreted by the kidney. A study of the literature, however, reveals the paucity of evidence referable to

the intermediary metabolism of progesterone and the conflicting nature of existing observations.

It must be admitted therefore that, at the present time, very little is known concerning the biochemical processes involved in the metabolic reduction of progesterone to pregnanediol, concerning the part played by any organ or tissue in that conversion and concerning the factors which determine the metabolism of progesterone under physiological conditions.

The isolation of pregnanolone and allopregnanolone by Marker et al (1937) and the establishment of pregnanolone glucuronide as a metabolic product of progesterone by Dorfman, Ross & Shipley (1948) have raised new problems - technical and theoretical - which complicate the interpretation of the results of previous studies upon the metabolism of progesterone.

The rationale of an attempt to develop an improved method for pregnanediol determination and of the necessity to reinvestigate many of the observations of Venning and Browne and others, are discussed, along with details of previous work on the metabolism of progesterone, in the relevant introductory sections throughout the thesis.

The main objectives of the present work were as follows:-

(1) The development of a method for the quantitative determination of small amounts of pregnanediol in human urine.

Specificity was considered as essential as accuracy. As it was intended to carry out determinations daily and in duplicate, and coincident determinations of other urinary steroids when desired, it was necessary that the method should be rapid and convenient and that not more than one quarter or one fifth of a twenty-four hour specimen of urine should be employed in a single determination.

(2) The application of such a method to the excretion of urinary pregnanediol in human subjects. It was considered that priority should be given to studies which might throw some light upon the intermediary metabolism of progesterone rather than to clinical studies of a less fundamental nature.

SECTION II.

SECTION II.

QUANTITATIVE DETERMINATION of PREGNANEDIOL in HUMAN URINE.

Introduction.

(a) Methods for the determination of pregnanediol as its glucuronide.

It may be stated at the outset that no method has been evolved for the quantitative determination of sodium pregnanediol glucuronidate, but several methods have been described for the determination of the glucuronides of pregnanediol-like substances. The most widely employed of such methods is that of Venning (1937, 1938), and it is to this method that we are indebted for almost all the observations on the excretion of urinary pregnanediol which have been made during the last decade. This method is however open to criticism on the grounds of lack of specificity. It has been shown by Marrian and Gough (1946) that the final product by this method contains only about 80% of the pure sodium pregnanediol glucuronidate and that the remaining 20% is largely composed of such ketonic glucuronides as sodium pregnanolone glucuronidate (Sutherland and Marrian (1946,1947). Apart from lack of specificity, the Venning method is time-consuming and laborious and a reasonable degree of accurary is not achieved when the method is applied to urines containing amounts of pregnanediol less than about 10 - 15 mg./24 hr. specimen. At these low levels of excretion the identity of the final product becomes

even less certain as larger volumes of urine must be extracted to yield a measurable amount of glucuronide.

A serious disadvantage which applies to all methods for the determination of pregnanediol as its glucuronide (Allen and Viergiver, 1941, Jayle, Crepy and Wolf, 1943, Bisset, Brooksbank and Haslewood, 1947) is the fact that hydrolysis of the glucuronide and liberation of the free pregnanediol readily occur as a result of bacterial action in the urine. Venning (1938) stresses the importance of refrigeration of specimens and states that hydrolysis will occur at room temperature even in the presence of such preservatives as tricresol, toluene, or chloroform. Bisset, Brooksbank and Haslewood (1947) who describe an entrainment procedure for the determination of a pregnanediol-like glucuronide (PLG), advocate refrigeration at 0 - 5° during collection followed by immediate analysis.

This disadvantage creates yet another potential source of error and justifies a somewhat sceptical approach to reports of low or irregular excretions of urinary pregnanediol as determined by these methods. On the other hand the labour involved in obtaining specimens under these conditions might well be justified by results, should a more specific method for the determination of pregnanediol as its glucuronide be evolved.

(2) Methods for the determination of pregnanediol after acid hydrolysis of its glucuronide.

In 1941, Astwood and Jones described a method for the determination of the free form of pregnanediol which consisted essentially of the following steps:-

(i) liberation of free pregnanediol by acid hydrolysis.

(ii) extraction of the free pregnanediol with toluene.

(iii), separation of impurities from the toluene extract by precipitation with methanolic sodium hydroxide solution.

(iv) purification of the pregnanediol in the toluene extract by precipitation from ethanolic solution with four volumes of dilute sodium hydroxide solution of water.

In a series of recovery experiments in which "sodium pregnanediol glucuronidate" was added to male urine, Astwood and Jones obtained an average yield of 68% with amounts of pregnanediol greater than about 2mg./litre.

Talbot, Berman, MacLachlan and Wolfe (1941) increased the sensitivity of this method by estimating the purified pregnanediol colorimetrically in terms of the yellow colour which it yields with concentrated sulphuric acid. These workers obtained recoveries similar to those of Astwood and Jones in a short series of experiments. One litre of urine was required for a single determination by either method.

As has been stated above, the "sodium pregnanediol glucuronidate" used by these workers

contained only about 80% of pure sodium pregnanediol glucuronidate and their results must therefore have been rather higher than they themselves reported but in our experience the combined Astwood-Talbot procedure did not give accurate or reproducible results when applied to urines containing less than 10mg./24 hr. urine volume.

It seemed possible however that by certain modifications in this procedure and by rigid standard-:isation of technique, the degree of accuracy might be increased to include these important lower levels of pregnanediol excretion. (Guterman (1944) described a qualitative method for the detection of impure pregnanediol, based on the procedure of Atwood and Jones as modified by Talbot et al. This and other semi-quantitative simplifications of the original procedures will be discussed in Section 3.)

A colorimetric method for the estimation of pregnanediol based upon interaction with acetyl chloride and zonc chloride in glacial acetic acid solution has been described recently by Goldzieher (1948). Such a reaction has been used previously for the determination of the blood cholesterol (Rose, Schattner and Exton, 1941). The procedure prior to colorimetry is more troublesome and time-consuming than that described by Talbot et al and although data regarding the degree of specificity is not submitted, the fact that the reaction can be utilised for the

determination of cholesterol is not encouraging.

After very numerous preliminary experiments the following modifications were made in the Astwood-Talbot procedure by Dr Nancy Gough and Prof. G.F. Marrian:-

(i) Washing the toluene extract with aqueous sodium hydroxide was found to be a simpler method of removing acidic and phenolic substance than the boiling and precipitation by methanolic sodium hydroxide advocated by Astwood and Jones (1941).

(ii) In recovery experiments in which pure pregnane-3(α),20α-diol was added to the neutral toluene-soluble fraction of acid-hydrolysed male urine, satisfactory male urine blanks and improved recoveries of pregnanediol were obtained when the precipitation was carried out by dissolving the neutral fraction from 1/5 of a 24hr. specimen in 4ml. ethanol and precipitating with 16ml.N/10 sodium hydroxide on one occasion and with water on two subsequent occasions.

(iii) To facilitate the quantitative collection of the pregnanediol precipitated and to avoid as far as possible transference of material from vessel to vessel, the precipitation was carried out in centrifuge tubes and the precipitate collected by centrifugation instead of filtration.

(iv) It was found that a blue pigment probably of dietary origin which occasionally contaminated the final product could be removed without loss of pregnanediol by warming in ethanolic solution with charcoal. This treatment with charcoal was adopted as a routine to maintain uniformity of procedure and formed an essential part of the procedure in later work. (Section 111).

In experiments carried out after these modifications had been adopted, recoveries of 80% or higher were invariably obtained when more than about 4 mg. of pregnanediol were present in 1/5 of a 24 hr.

specimen of urine. With smaller amounts of pregnanediol the recoveries were lower and often very irregular.

This marked irregularity suggested that some variable in the purification process was not being properly controlled.

1. Standardisation of conditions during the precipitation of the pregnanediol from hot ethanolic solution.

The temperature conditions at the time of precipitation of the pregnanediol from ethanolic solution are not accurately defined in the original procedure of Astwood and Jones, and have found rather differing interpretations by subsequent workers.

(i) Astwood and Jones (1941): The neutral residue is completely dissolved in 10ml. of 95% ethanol by heating and while the heating is continued, 40ml of hot aqueous 0.1N, sodium hydroxide solution is added slowly. The flask is allowed to stand at room temperature until cold and then placed in a refriger-:ator overnight.

(ii) Talbot, Berman, MacLachlan and Wolfe (1941): The neutral residue is dissolved in 10ml. absolute ethanol by heating on a water bath and while heating is continued, 40ml. aqueous 0.1 N. sodium hydroxide is added slowly and the flask placed in a refrigerator overnight.

(iii). Guterman (1944,1945): 5 c.c. acetone (the change of solvent will be discussed later)is added to

the residue and the mixture is warmed over a hot plate until solution is complete, 20 c.c. 0.1 N. sodium hydroxide is added while the mixture is still on the hot plate. The flask is then placed in an ice-water bath for 30 minutes or in a rerrigerator (5°C) for one hour.

Other workers have favoured conditions selected from the above, for example Wheatley and Maclagan (1948) precipitate from acetone and cool to room temperature.

It seemed probable that recoveries would be improved by the mere replacement of such terms as "hot" and "room temperature" by standardised conditions.

(i) Temperature during addition of N/10 sodium hydroxide solution or water.

It was apparent that a temperature of the order of 75°C was necessary to ensure complete solution of the crude pregnanediol in the toluene-soluble residue. This temperature could be maintained at the time of adding the N/10 sodium hydroxide solution in the following manner: the centrifuge tube containing 4.0ml. othanol was placed in a beaker containing water which was maintained for four minutes at 75°C. After one minute, the 16.0 ml. of N/10 sodium hydroxide were added drop by drop from a burette during 3 minutes with stirring, the last 1 ml. being used to wash down the stirring rod into the tube. Beaker and contents were then maintained at 75°C for a further minute.

(ii) Rate of cooling of the hot precipitation mixture:

The question now arose whether the precipitation mixture should be allowed to cool rapidly or slowly. To allow it to cool to room temperature would not be consistent with a desire for a uniform technique since this would constitute fairly rapid cooling in a Scottish laboratory in winter-time but might approximate to slow cooling at other times and in other laboratories.

In addition it seemed possible that the size of the crystals or precipitated aggregates might be determined by the rate of cooling so that by slow cooling, larger particles might be produced, centrifug-:ation made more efficient, and loss of pregnanediol in the supernatant solution considerably reduced.

A preliminary investigation of this possibility was carried out as follows:

(a) Pure pregnane-3(α),20α-diol was prepared from human pregnancy urine and purified by Prof. Marrian via its diacetate (m.p. 236 - 237° corr.).

(b) The toluene-soluble neutral fraction of acid-hydrolysed pooled male urine, which was employed in this and other preliminary experiments, was prepared by the following procedure: the pooled urine was heated to boiling together with one-fifth of its volume of toluene (B.P. sulphur-free, redistilled) acidified with one-tenth of its volume of concentrated hydrochloric acid (A.R.) and boiling continued for 10 minutes. After cooling, the mixture was extracted three times with one-fifth volumes of toluene, emulsions being broken by filtration through a Buchner funnel with gentle suction. The combined toluene extracts were washed twice with one-fifth volume of N. sodium hydroxide, three times with one-fifth volumes of water, evaporated to a small volume (ca. 30 c.c.) and finally taken to dryness under reduced

pressure in the water-bath. The dark reddish-brown gummy material will be referred to as "neutral fraction male urine" (N.F.M.U.).

In the recovery experiments to be described, an aliqot portion of this N.F.M.U. representing 500 c.c. of pooled male urine was added to each centrifuge tube, pregnane-3(α),20α-diol in ethanol added to a variable number of tubes while the remaining tubes containing only N.F.M.U. acted as "male urine blanks". It was absolutely essential that these "blanks" should, after the triple precipitation procedure, yield a very small amount of final product with a very low degree of chromogenicity in the sulphuric acid reaction.

In this experiment two series of tubes were compared in both of which the precipitation mixture was at a temperature of 75°C after addition of the N/10 sodium hydroxide solution by the procedure described above. In one series the mixture was cooled rapidly by immersion of the tube in an ice-bath and transfer-:ence to a refrigerator where it was left overnight before centrifugation. In a second series the tubes were allowed to stand in the beakers at the laboratory temperature (15°C), until (after 2½ hours) the temper-:ature of the mixture had fallen from 75°C to 18°C when the beakers were transferred to the refrigerator and left overnight before centrifugation.

These latter conditions did not constitute a very slow rate of cooling of the precipitation mixture but none the less the result upon the recovery of pregnanediol was dramatic.

TABLE 1.

Tube.	Pregnanediol added (mg.)	Pregnanediol recovered (mg.)	% Recovery.	
Rapid Cooling:				
a	0.00	0.05	10.0	
a_1	0.00	0.07	14.0	
b	0.50	0.09	17.0	
b_1	0.50	0.13	25.1	
b_2	0.50	0.04	8.0	
b_3	0.50	0.08	16.0	av. 16.5%
Slow Cooling:				
c	0.00	0.03	6.0	
c_1	0.00	0.03	5.5	
d	0.50	0.35	70.2	
d_1	0.50	0.26	55.5	
d_2	0.50	0.33	66.1	av. 63.9%

CONCLUSIONS:-

If the apparent pregnanediol recovery be corrected for the average male urine blank for each series the corrected recovery by Rapid Cooling is 4.5% and by Slow Cooling, 58.2%. This difference of over 50% in the recovery of pregnanediol at this level can only be accounted for by the difference in the rate of cooling of the precipitation mixture. Various methods of slow cooling were investigated and the following proved a satisfactory and simple procedure independent of laboratory temperature. The tube containing the mixture and surrounded by water in a 250 c.c. beaker at a temperature of 75°C was transferr-:ed immediately to an incubator at 37°C. The temperature of the mixture fell to 37°C in ca. two hours and the beaker was then transferred to a refrigerator when further cooling to 0° - 5° occurred overnight.

Until this time, overnight refrigeration had been repeated after the 2nd and 3rd precipitations and it now seemed possible that this time-consuming procedure might be eliminated by substituting two shorter periods of slow cooling in the incubator.

A comparison was therefore made between two series of tubes:

Procedure A : $2\frac{1}{2}$ hours slow cooling after the 2nd and 3rd precipitations in incubator followed by $\frac{1}{2}$ hour in refrigerator.

Procedure B: 4 hours slow cooling after the 2nd and 3rd precipitations.

(Centrifugation did not cause a detectable rise in the temperature of the mixture). The results are shown in Table 2.

TABLE 2.

Procedure.	Pregnanediol Added.mg.	Spekker	Pregnanediol Recovered mg. Apparent	Corr.	% Recovery (corr.)
A. $2\frac{1}{2}$ hours	0.00	0.017	0.021	-	-
	0.00	0.009	0.012	-	-
	0.51	0.352	0.453	0.436	86.2
	0.51	0.351	0.452	0.435	86.2
B. 4 hours	0.00	0.021	0.028	-	-
	0.00	0.020	0.027	-	-
	0.51	0.311	0.400	0.372	73.6
	0.51	0.306	0.398	0.370	73.5
Standards	0.25	0.195			
	0.25	0.196			
	0.51	0.392			
	0.51	0.403			

CONCLUSION: $2\frac{1}{2}$ hours slow cooling after the 2nd and 3rd precipitations was not detrimental to the recovery of pregnanediol. Because of this fact it became possible to shorten the time taken for each determination from four days to two days. The second day however was inconveniently long (10 hours).

If the time of centrifugation could be reduced to one hour on each occasion then a more convenient eight hour working day would be possible and accordingly attempts were made to improve the conditions of sedimentation

Attempts to improve sedimentation by filter aids:

Despite centrifugation for two hours at a speed of 1500 r.p.m. (centrifuge head of radius 15 cm.) difficulty was experienced in obtaining adequate packing down of the relatively light pregnanediol crystals and although a fine bore glass suction tube with fine terminal hook was used to syphon off the supernatant solution, some loss was suspected by this route. In addition loss was suspected by crystals held on the surface of the mixture by surface tension.

(a) Charcoal.

It was thought that some heavy absorbent such as charcoal might improve sedimentation and at the same time absorb some of the urinary pigments responsible for the male urine blank. This in fact occurred but on the addition of ethanol prior to filtering off the charcoal, this pigment - normally eliminated by the triple pptn. process, - was eluted out of the charcoal giving a wine colouration to the ethanol solution.

(b) "Cetavlon".

This detergent was investigated with a view to reducing surface tension and preliminary results were encouraging but as is shown in Table 3 it was not as

efficient as artificial filter aids and the fact that the latter gave better results when used alone than when used along with "Cetavlon" suggested that the detergent might in fact have a slightly detrimental effect by dissolving small amounts of pregnanediol which would otherwise have been dragged down by the filter aid.

(c) "Hyflo-Super Cel".

A fine spatula-point of this inert diatomaceous earth was added to the mixture immediately prior to centrifugation. As is shown in Table 3 and again in Table 4, this modification was probably an important step towards the elimination of that occasional low recovery which had been a feature of the method up to this time. In tubes b, b1, and b2 in Table 3 the recovery depended largely on how much of the precipitate adhered to the sides of the centrifuge tubes and this obviously could not be relied upon. The fact that greatly improved recoveries at low levels of pregnane-:diol resulting from slow cooling of the precipitation mixture in two of the tubes in the first series in Table 3 could still be obtained in the absence of filter aid makes it seem unlikely that the cooling effect could be explained on the basis that these inert particles acting as nuclei, favour the formation of larger aggregates of the precipitated material.

A filter aid of finer particle size was tried but this caused sedimentation of chromogenic

TABLE 3.

Procedure.	Pregnanediol added mg.	Spekker.	Pregnanediol recovered mg. Apparent:	Corr.	% Recovery (corr.)
No filter aid	0.00	0.008	0.010	-	-
	0.00	0.011	0.011	-	-
	0.51 (b)	0.336	0.415	0.404	80.6
	0.51 (b1)	0.363	0.445	0.434	86.5
	0.51 (b2)	0.102	0.127	0.116	24.5
Hyflo Super Cel.	0.00	0.060	0.075	-	-
	0.00	0.052	0.065	-	-
	0.51	0.340	0.422	0.352	68.6
	0.51	0.471	0.582	0.512	99.8
	0.51	0.438	0.545	0.475	92.5

TABLE 4.

Procedure.	Pregnanediol added mg.	Spekker.	Pregnanediol recovered mg. Apparent:	Corr.	% Recovery (corr.)
Hyflo Super Cel.	0.00	0.045	0.055	-	-
	0.00	0.027	0.035	-	-
	0.52	0.426	0.502	0.457	88.5
	0.52	0.350	0.415	0.370	71.2
Hyflo Super Cel plus Cetavlon	0.00	0.032	0.040	-	-
	0.00	0.030	0.036	-	-
	0.52	0.311	0.365	0.327	62.9
	0.52	0.421	0.449	0.411	79.0

material of the N.F.M.U. other than pregnanediol and defeated the purpose of the repeated precipitations.

(iv) Attempt to shorten time of centrifugation:

With improved sedimentation it now seemed possible that centrifugation for one hour at 1500 r.p.m. might suffice. In a series of experiments in which 0.504 mg. of pregnane-3(α),20α-diol was added to N.F.M.U. (Table 5), it was found that the second hour which had previously been essential was now redundant, the time taken for one determination was therefore reduced by three hours and the second day of the method became a convenient length (eight hours).

(v) Comparison of Rapid Cooling and Slow Cooling when varying amounts of pregnanediol are added to N.F.M.U.

At this stage in the evolution of the method, a series of experiments were carried out with the object of confirming the importance of the rate of cooling of the precipitation mixture upon the recovery of pregnanediol and of investigating this effect at various levels of pregnanediol.

The N.F.M.U. was prepared in the manner already described but pooled 24hr. urine specimens from five healthy males were used in place of pooled male urine. Two batches of urine, (A & B) were worked up in this way comprising a total of forty five lots of N.F.M.U. each of which was equivalent to one-fifth of a single 24 hr. specimen. Two solutions of pure pregnane-3(α),20α-diol were prepared.

Table 5.

Procedure.	Pregnanediol added. mg.	Spekker.	Pregnanediol recovered. mg. Apparent:	Corr.	% Recovery (corr.)
Centrifug 2 hour x 3.	0.00	0.018	0.021	-	-
	0.00	0.010	0.012	-	-
	0.00	0.012	0.015	-	-
	0.50	0.395	0.483	0.467	92.9
	0.50	0.435	0.557	0.541	107.0
	0.50	0.430	0.555	0.539	106.9
Centrifug 1 hr. x 3	0.50	0.426	0.520	0.504	100.0
	0.50	0.415	0.508	0.492	97.7
	0.50	0.431	0.556	0.540	106.9

(i) 6.648 mg. in 25 ml. ethanol.

(ii) 40.010 mg. in 50 ml. ethanol.

The results of this series of recovery experiments are given in Table 6.

CONCLUSION: Rapid cooling of the precipitation mixture gave lower and much more irregular results than were obtained by the slow-cooling technique. This difference in recovery due to the rate of cooling was most marked at levels below 0.53mg. Above this level rapid cooling tended to result in the production of occasional low recoveries. Between 0.40 mg. and 0.27 mg. there appeared to be a critical level below which recoveries by either procedure would be poor. Attempts were therefore made to lower this level with the possibility in mind that this might be achieved by still lower rate of cooling. Since the procedures of Talbot et al. and Guterman undoubtedly involved rapid cooling it may be concluded that such procedures would be liable to give low and erratic results with urines containing less than ca. 20 mg. of pregnanediol per 24 hr.

(vi) Attempt to eliminate refrigeration even at a low pregnanediol level.

An attempt was made to investigate the effect of slow cooling on a level intermediate between 0.4 mg - where recovery in the previous experiments was good - and 0.25 mg. where recovery was very bad. Accordingly 0.322 mg. of pure pregnane- 3(α),20α-diol was added to

Table 6.

The effect of varying the rate of cooling after precipitation on the recovery of pregnanediol added to "male urine neutral fraction".

Male Urine neutral fraction.	Pregnanediol added (mg.)	Pregnanediol recovered (mg.)		% pregnanediol recovered corrected for male urine blank.	
		Rapid cooling.	Slow cooling.	Rapid cooling.	Slow cooling
A	0.00	0.056)	0.056)	-	-
	0.00	0.012) 0.031	0.055) 0.050	-	-
	0.00	0.026)	0.040)	-	-
B	0.00	0.017)	0.040)	-	-
	0.00	0.015) 0.014	0.042) 0.040	-	-
	0.00	0.010)	0.037)	-	-
A	0.27	0.032)	0.123)	)0	)17
	0.27	0.012) 0.023	0.092) 0.096	)	)
	0.27	0.025)	0.072)	)	)
B.	0.40	0.016)	0.33)	)	)
	0.40	0.021) 0.017	0.38) 0.34	)1	)75
	0.40	0.015)	0.31)	)	)
A.	0.53	0.43)	0.45)	)66	)84
	0.53	0.42) 0.41	0.46) 0.49	)	)
	0.53	0.37)	0.57)	)	)
B.	0.80	0.63)	0.86)		
	0.80	0.82) 0.75	0.82) 0.84	89	99
	0.80	0.80)	0.83)		
A.	4.0	4.0)	4.2)		
	4.0	3.9)3.5	4.1)4.1	86	101
	4.0	2.5)	4.0)		

tubes containing N.F.M.U. prepared from pooled 24 hr. specimens from four healthy males. At the same time an attempt was made to eliminate the use of the refrigerator in the procedure.

Procedure 1 : as in previous experiment, with overnight refrigeration after 1st pptn. and ½ hr. refrig. after incubation following 2nd and 3rd pptns.

Procedure 2 : incubation overnight after 1st and 3rd pptns and incubation for two hours after 2nd pptn.

Results:

TABLE 7.

Method	Pregnanediol added (mg.)	Spekker.	Pregnanediol recovered (mg.) Apparent:	Corr.	% Recovery (corr)	
Procedure 1.	0.00	0.015	0.020	-	-	
	0.00	0.020	0.028	-	-	
	0.32	0.220	0.280	0.256	79.6	
	0.32	0.245	0.318	0.294	91.4	av.
	0.32	0.201	0.258	0.234	72.7	81.2
Procedure 2.	0.32	0.262	0.340	0.316	98.2	
	0.32	0.260	0.338	0.314	97.5	av. 97.9

CONCLUSION: It was possible to eliminate refrigeration and achieve a satisfactory recovery at this low level by the procedure described. Recoveries by the

same procedure were very poor at the slightly lower level of 0.25 mg. and could not be improved by slower cooling in the temperature range 75° to 55°C. This confirmed the impression that a critical level exists apparently between 0.25 and 0.35 mg. per one-fifth of a 24 hr. specimen. This may constitute a critical ratio between N.F.M.U. ethanolic solution and the pregnanediol ethanolic solution used in the recovery experiments. Below this level the pregnanediol may be held in solution due to a preponderance of some factor in the pigmented gummy neutral residue. If this is so it might be assumed that the quantitative recovery of pregnanediol in pure solution would be possible below this level.

(vii) Recovery of pure pregnane-3(α), 20(α)-diol in pure solution.

Pregnanediol solution: 9.810 mg. in 50 ml. ethanol.

Procedure: Procedure 2 of previous experiment.

Results are given in Table 8. Reagent "blanks" were not included in this experiment, but 20 ml. of the purified ethanol used was evaporated to dryness and found to have no chromogenic power when tested in the sulphuric acid reaction.

CONCLUSION: With careful technique a very small and relatively constant loss (0.01 mg.) occurs in precipitating from pure solution. There is no critical level.

Table 8.

Pregnane-diol added mg.	Spekker.	Pregnane-diol recovd. mg.	% Recovery.	Loss mg.
0.098	0.062	0.080	81.5	0.021
0.098	0.075	0.093	95.0	0.005
0.196	0.152	0.180	92.0	0.016
0.196	0.160	0.190	97.0	0.006
0.294	0.252	0.295	100.0	-
0.294	0.245	0.287	97.6	0.007
0.392	0.327	0.381	97.5	0.011
0.392	0.330	0.385	98.2	0.007
STANDARDS				
0.098	0.078			
0.098	0.082			
0.196	0.171			
0.196	0.168			
0.294	0.250			
0.294	0.253			
0.392	0.341			
0.392	0.335			

(viii) Further attempts to shorten the method:

(a) Centrifugation at 1500 r.p.m. for 30 minutes as shown in Table 9. This gave quite satisfactory results in this type of experiment but the results were less satisfactory in urines containing large amounts of pregnanediol as in late pregnancy.

(b) Filtration by porcelaid Filter Stick. This appeared to offer a possible method of filtration of the precipitation mixture which would not involve transference from vessel to vessel. The precipitation was carried out in short 1" diameter boiling tubes. A filter stick was chosen of a porosity which appeared to resist the passage of pregnanediol precipitated out of pure solution. Very low recoveries were obtained, however, when this was applied to the precipitate in the presence of N.F.M.U. This effect appeared to depend to some extent upon the alkalinity of the precipitation mixture and the success or failure of the procedure appeared to depend too much upon the nature of the neutral residue of the urine investigated.

(c) A one-day method was possible if each pptn. was followed by 2 hrs. incubation and 30 minutes centrifug-:ation. Recoveries were 10% lower by this method than by the previous one, but the results were encouraging for future work on a short method suitable for clinical use. On the other hand, as shown in Table 9, no detrimental effect was apparent after prolonged

incubation of the pptn. mixture - in this case at least 16 hours at 37°after each precipitation. This series of experiments again illustrates the excellent recovery at levels just above the critical level.

(ix) Recovery experiments to illustrate the impor-:tance of three pptns. in the purification process.

One precipitation only is employed in the Guterman modification of the Astwood-Talbot procedures. After centrifugation of the precipitation mixture in the present series of experiments, the precipitate was usually a pale brown colour due to the sedimenta-:tion and adsorption of highly chromogenic material of the N.F.M.U. Two series of tubes were compared as shown in Table 10.

CONCLUSION: At least two precipitations are essential to ensure reasonable blank readings and very high blank readings make it impossible to estimate the per centage recovery of added pregnanediol. The effect on the male urine blanks may be summarised as follows:-

Male Urine Blanks.

3 pptns. (1xN/10 NaOH; 2 x Water)	1 pptn (1xN/10 OH)
0.02 mg.	0.33 mg.
0.01 mg.	0.27 mg.
0.02 mg.	0.37 mg.
av. gives an apparent pregnanediol content of:-	
0.08 mg./24 hr.	1.6 mg./24 hr.

Table 9.

Procedure.	Pregnanediol added. mg.	Spekker.	Pregnanediol Recovered mg. Apparent:	Corr.	% Recovery (corr.)	
	0.00	0.026	0.034	-	-	
Present technique	0.39	0.352	0.410	0.376	95.5	av. 94.5
	0.39	0.336	0.390	0.356	90.5	
	0.39	0.360	0.418	0.354	97.4	
Centrifug 30 min x 3.	0.39	0.330	0.385	0.351	89.6	av. 91.5
	0.39	0.339	0.395	0.361	92.2	
	0.39	0.342	0.397	0.363	92.6	
Incubation 2 hr after 1st pptn.	0.39	0.302	0.352	0.318	81.2	av. 80.4
	0.39	0.296	0.345	0.311	79.5	
Prolonged incubation.	0.39	0.350	0.408	0.374	96.0	av. 93.3
	0.39	0.345	0.400	0.366	93.5	
	0.39	0.335	0.389	0.355	90.5	

Table 10.

Headings as above.

Present technique:	0.00	0.018	0.021	-	-	
	0.00	0.010	0.012	-	-	
3 pptns.	0.00	0.012	0.015	-	-	
	0.50	0.395	0.483	0.467	92.6	
	0.50	0.435	0.557	0.541	107.0	
	0.50	0.430	0.555	0.539	106.6	
Present technique:	0.00	0.272	0.332	-	-	
	0.00	0.220	0.272	-	-	
1 pptn.	0.00	0.302	0.368	-	-	
					uncorr.	corr.
	0.50	0.680	0.825	0.501	165.8	99.4
	0.50	0.900	1.092	0.768	216.0	151.6
	0.50	0.600	0.725	0.401	143.8	79.4
Standards.	0.25	0.205				
	0.25	0.199				
	0.50	0.405				
	0.50	0.412				

This finding would afford a reasonable explanation for the many false positive results which have been obtained when the Guterman test was used for the diagnosis of pregnancy. It is only fair to add however that filtration and not centrifugation is employed in the Guterman procedure and by choice of a filter of suitable porosity quite low blanks might be obtained although this would incur a proportionately higher loss of pregnanediol, (unless large crystals be produced by a slow cooling of the precipitation mixture - see Section III).

A more important disadvantage of the omission of the 2nd and 3rd pptns would almost certain-:ly be lack of specificity due to incomplete elimina-:tion of steroids other than pregnanediol which give a yellow colour with sulphuric acid. The specificity of the method was investigated in some detail (q.v.)

(x) Choice of solvent for pregnanediol in the precipitation process.

Although Astwood and Jones and Talbot et al. used ethanol in the precipitation process, Guterman and others who have followed him with qualitative or semi-quantitative simplifications of the Astwood-Jones procedures substituted acetone for this purpose. (Mack and Parks, 1947; Wheatley & Maclagan, 1948). In view of the marked difference in the solubility of pregnanediol in ethanol and acetone, it could not be assumed that the precipitation procedure described by

Astwood and Jones would be satisfactory when acetone was substituted for ethanol. Furthermore, after a paper had been read to the Society for Endocrinology upon the effect of slow cooling of the precipitation mixture upon the recovery of pregnanediol (Somerville, Gough and Marrian, 1947) several workers suggested the application of this technique to the Guterman method, or to one of its modifications.

It by no means followed that the temperature difference which has been proved in the series of recovery experiments reported above (Table 6), where ethanol was used as the solvent, would be found to the same extent in the case of acetone.

Three series of tubes with appropriate "blanks" were compared as shown in Table 11. The rapid cooling technique consisted of immediate transference of the tube containing the precipitation mixture to an ice-bath as in the original experiments. Precipitation from acetone was carried out at 65° (the acetone was not dried).

CONCLUSION: At this level of pregnanediol - 0.4 mg./ one-fifth of a 24 hr specimen, the slow cooling technique gave excellent recoveries using ethanol. Either rate of cooling gave very poor recoveries with acetone.

The acetone was purified by refluxing with potassium permanganate and then twice redistilled over a long column (24 inch).

Table 11.

Procedure.	Pregnane-diol added mg.	Spekker.	Pregnane-diol recovered. mg. Apparent:	Corr.	% Recovery (corr.)
Acetone	0.00	0.023	0.023	-	-
Rapid	0.00	0.025	0.025	-	-
Cooling.	0.40	0.022	0.022	-	-
	0.40	0.056	0.060	0.036	9.0
	0.40	0.041	0.045	0.021	5.3
Acetone	0.00	0.012	0.012	-	-
Slow	0.00	0.010	0.010	-	-
Cooling	0.40	0.155	0.160	0.149	37.3
	0.40	0.130	0.135	0.124	31.0
	0.40	0.195	0.200	0.189	47.2
Ethanol	0.00	0.016	0.016	-	-
Slow	0.00	0.011	0.011	-	-
Cooling.	0.40	0.384	0.384	0.375	93.8
	0.40	0.398	0.398	0.389	97.3
	0.40	0.367	0.367	0.358	89.5

To exclude the possibility that the purified acetone contained permanganate or other contaminant destructive to pregnanediol, two additional tubes were carried through the method using unpurified Acetonum B.P. and similar low recoveries were obtained.

A more satisfactory investigation of the effect of the choice of solvent in the Guterman and allied procedures, will be obtained by using the same volume ratios in the precipitating mixture, but it will be impossible to duplicate the less well defined temperature conditions.

Method finally adopted for the determination of Pregnanediol, in Urine.

Quickfit and Quartz glassware, with interchangeable standard glass joints, was used throughout in order to avoid contamination of the urinary extracts with coloured or chromogenic material that might be dissolved out of rubber or cork stoppers. The toluene was B.P. "sulphur free" and was redistilled before use. Ethanol was purified by refluxing for six hours with solid sodium hydroxide and then twice redistilled over a glass column containing glass beads. A 24 hr. specimen of urine collected with 5 ml. of toluene as preservative is made up to 2500 ml. and duplicate 500 ml. samples removed. Each sample is treated as follows: It is placed in a 1000 ml. flask and after the addition of 100 ml. of toluene brought to boiling point under a reflux condenser. To the

boiling mixture is added down the condenser 50 ml. of concentrated hydrochloric acid (A.R.) and the boiling continued for exactly 10 min. The flask is then rapidly cooled in cold water and the contents transferred to a separating funnel of 750 ml. capacity. After shaking and allowing the urine layer to separate, the latter is run off into the original flask and the layer of toluene and emulsion filtered with gentle suction through a Whatman No. 1 paper on a Buchner filter funnel. The urine layer is then returned to the separating funnel and extracted twice more with 100 ml. portions of toluene, each toluene and emulsion layer being filtered in succession through the same filter funnel. The combined filtrates are then transferred to a clean separating funnel, and after running off the small urine layer that separates, the toluene extract is washed twice with 100 ml. portions of N sodium hydroxide and twice with 100 ml. portions of water. The washed toluene extract is run into a 500 ml. round-bottomed flask and is evaporated nearly to dryness on an electric hot plate and then taken completely to dryness under reduced pressure on a boiling-water bath.

The dry residue is transferred quantitatively with warm ethanol to a 20 ml. conical centrifuge tube and the ethanolic solution evaporated to dryness in a water-bath under a stream of air. To the residue in the tube are added exactly 4.0 ml. of ethanol and the

tube is placed in a beaker of water maintained at 75°. After stirring with a glass rod for 1 min. to obtain complete solution, 16.0 ml. of N/10 sodium hydroxide are added drop-wise from a burette during 3 min. with stirring, the last 1 ml. being used to wash down the stirring rod into the tube. After a further 1 min. at 75°, the beaker of water containing the tube is transferred to an incubator at 37° and left overnight. Approximately 8-10 mg of 'Hyflo-Super Cel' (Johns-Manville Co. Ltd.) are added and the mixture stirred with a glass rod. The rod is washed down into the tube with 1 ml. of a 1 : 4 (v/v) ethanol-water mixture and the tube is then centrifuged for 1 hr. (1500 r.p.m; radius of centrifuge head: 15 cm.). The supernatant solution is finally sucked from the precipitate with the aid of a fine glass tube attached to a slowly running water-pump.

The second and third precipitations are carried out as described above, except that water instead of sodium hydroxide solution is used, and the incubation periods are reduced for convenience to 2 hr. No additional filter-aid is added before the centrifuga-:tions following the second and third precipitations.

To the final precipitate are added 5 ml. of ethanol and the pregnanediol dissolved by warming with stirring at about 75°. 'Norite' charcoal (c. 1-2 mg.) is then added and the warming continued for 2 min. The mixture is filtered through a small filter

(Whatman No. 1 paper) into a test-tube of 1 in. diameter, the centrifuge tube and filter being washed three times with 2 ml. portions of warm ethanol. The filtrate and washings in the tube are evaporated in a water-bath under a stream of air and the residue finally dried by leaving the tube in a vacuum desiccator over calcium chloride for several hours.

The colour reaction is carried out with not more than ca. 0.5 mg. of the finally purified product. If, therefore, the amount of the latter appears on inspection to be in excess of 0.5 mg. a suitable aliquot portion is removed after solution in a known volume of ethanol. To the dry pregnanediol 10.0 ml. of concentrated sulphuric acid (A.R.) are added from a burette, and the tube is left in a water-bath at 25° for 20 min. with occasional shaking. The intensity of the yellow colour produced is measured in a 'Spekker' photoelectric absorptiometer using a 'spectrum violet' No. 601 light filter.

The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-3(α), 20α-diol varying from 0.1 to 0.5 mg. It is advisable to construct a fresh calibration curve for each batch of unknowns.

(xii) Investigation of accuracy of the method finally adopted.

The accuracy of the method was tested in a series of experiments in which pure sodium pregnanediol

glucuronidate was added in varying amounts to the urine of healthy male subjects. The validity of such tests depends on two assumptions: (a) that all the pregnanediol in human urine is excreted as the glucur-:onide, and (b) that women's urine contains no substances which are not present in men's urine which would interfere with the determination of pregnanediol. No evidence contrary to these assumptions exists at the present time.

Sodium pregnane-3(α), 20 α-diol glucuronidate was prepared from human glucuronides by the method of Venning and Browne (1936) and freed from ketonic glucuronides by the method of Sutherland & Marrian (1946, 1947). The preparation used in the recovery experiments melted at 282-283° (corr.) with decomposi-:tion and evolution of gas. Samples of the glucuroni-:date were weighed out after exposure to moist air. As shown by Sutherland & Marrian (1947) material so treated is the <u>trihydrate</u> having the composition $C_{27}H_{43}O_8Na.\ 3H_2O$.

Twelve 24 hr. specimens of urine were collected from four normal men. Each specimen was made up to 2500 ml. and four 500 ml. samples removed. To each of two of these samples was added an identical amount of sodium pregnanediol glucuronidate dissolved in 80% ethanol, the other two samples being retained for working up as male urine "blanks". All four samples from each specimen were then treated as described in

Section xi. The results are summarised in Table 12.

CONCLUSION: High regular recoveries of the order of 95% are obtained above levels corresponding to 1.0 mg./500 ml. At the 0.4 mg. level, the critical effect due to the presence of pigments or gummy fractions of the N.F.M.U. is just apparent. In view of the small loss entailed in hydrolysis and extraction and the results of the previous recovery series Table 6, it seems justifiable to assume that the range of high regular recoveries extends down to a level of ca. 0.5 mg./500 ml. and of satisfactory duplicate determination to ca. 0.4 mg./500 ml. The concentration of the inimicable factor in the N.F.M.U. in each specimen under consideration will determine the % recovery below this level.

Table 12.

Recovery of pregnanediol after the addition of sodium pregnanediol glucuronidate to human male urine.

Male urine specimen.	Male urine blank as apparent pregnanediol in 1/5 of 24 hr specimen (mg.) (av. of duplicates).	Pregnanediol added as glucuronidate to 1/5 of 24 hr urine specimen (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovery (corrected). %
			Apparent.	Corr. for blank.	
C4	0.016	0.2	0.017	0.001	0
		0.2	0.012	-	0
A3	0.008	0.2	0.021	0.013	7
		0.2	0.047	0.039	20
B2		0.2	0.045	0.021	11
D4	0.035	0.4	0.32	0.29	72
		0.4	0.33	0.29	74
B3	0.015	0.4	0.28	0.27	67
		0.4	0.29	0.28	69
A2	0.018	0.4	0.35	0.33	82
		0.4	0.35	0.33	82
A4	0.044	1.0	0.99	0.95	95
		1.0	0.98	0.93	93
C3	0.019	1.0	0.94	0.92	92
		1.0	0.96	0.94	94
D2	0.077	1.0	1.0	0.92	92
		1.0	0.98	0.90	90
B4	0.030	2.0	1.9	1.9	95
		2.0	2.0	2.0	100
D3	0.017	2.0	2.0	2.0	100
		2.0	1.9	1.9	95
C2	0.026	2.0	1.9	1.9	95
		2.0	1.9	1.9	95

SPECIFICITY of the METHOD FINALLY ADOPTED.

Introduction.

The importance of attempting to assess the specificity of any method in which the final product is determined by the sulphuric acid reaction lies in the fact that many other steroids, many of which are present in human urine give similar colours to pregnanediol in this reaction. The specificity of the method described above must therefore depend upon the completeness with which other chromogenic steroids originally present in the urine are eliminated in the extraction and purification process.

Previous work:

(a) Astwood and Jones (1941): Using a double precipitation process, these workers showed that cholesterol and androsterone were completely elininated when present in pure solution in amounts not exceeding 16 mg. and 8 mg. respect-:ively.

(b) Talbot, Berman, MacLachlan and Wolfe (1941): The results obtained by these workers who also used a double precipitation process suggested that no interference resulted from amounts of dehydroisoandrosterone in amounts up to ca. 10 mg. per litre. This was an important observation in view of the intense chromogenicity of this compound in the sulphuric acid reaction.

(c) Dr Nancy Gough, in this laboratory, has investigated the behaviour of an ethanolic solution of pregnane-3(α), 17, 20-triol in the purification process. This steroid was readily eliminated and in any case, it would be largely decomposed during the initial hydrolysis of the urine with acid which thus affords an additional safeguard against interference.

In the present work, the behaviour of the following steriods in the triple precipitation process has been investigated using ethanolic solutions of the pure compounds.

(1) ANDROSTERONE: Androstan-3(α)-ol-17-one.

Amounts of androsterone corresponding to 1.0, 2.0, 4.0, and 8.0 mg/500 c.c. were added to duplicate centrifuge tubes and the three precipitations and charcoal filtration carried out as in the method.

Standard solutions:

(a) Pure androstan-3(α)-ol-17-one.
40.062 mg. in 20 ml. ethanol.
m.p.: 181° - 182° (corr).

(b) Pure pregnane-3(α), 20α-diol.
2.496 mg. in 10 ml. ethanol.
m.p.: 236° - 237° (corr).

Androsterone added. (mg.)	tube.	Spekker.	Mg. Recov. as pregnanediol /500 ml.	Mg. Recov. as androsterone /500 ml.
1.0	a.	0.024	0.237	0.25
1.0 (5.0/24hr.)	a.	0.032	0.035	0.35
2.0	b	0.039	0.045	0.40
2.0(10.0/24hr)	b	0.028	0.032	0.30
4.0	c	0.056	0.066	0.62
4.0(20.0/24hr)	c_1	0.094	0.115	0.98
8.0	d	0.250	0.624	5.24
8.0(40.0/24hr)	d_1	0.292 (1:2)	0.730	6.16

CONCLUSION: Satisfactory elimination of amounts of androsterone less than ca. 20 mg/24 hr. is obtained. Amounts of more than 20 - 30 mg. are unlikely to be found even in pathological conditions in man. A comparison of the calibration curves indicates that pregnanediol is ten times more chromogenic than androsterone in the sulphuric acid reaction. This fact contributes to the specificity of the method and constitutes an advantage of colorometric methods over gravimetric methods.

(2) ISOANDROSTERONE: Androstan-3(β)-ol-17-one.

The procedure was the same as in the previous experiment.

Standard solution: Pure androstan-3(β)-ol-17-one.

m.p. 172° - 174° (corr.)

40.051 mg. in 20 ml. Ethanol.

Isoandrosterone added (mg).	Tube.	Spekker.	Mg. Recovd. as pregnanediol.	Mg. Recovd. as Isoandrosterone.
1.0	e	0.033	0.037	0.22
1.0	e_1	0.019	0.021	0.10
2.0	f	0.027	0.031	0.15
2.0	f_1	0.029	0.035	0.16
4.0	g	0.048	0.057	0.30
4.0	g_1	0.021	0.023	0.12
8.0	h	0.301	0.750	2.01
8.0	h_1	0.278	0.696	1.90
		1:2		

CONCLUSION: Precipitation during incubation was slightly less complete than in the case of androsterone and occurred at temperatures below 37° so that crystals were visible in the supernatant even after centrifugation. The results were however very similar to those obtained with androsterone and this steroid is unlikely to interfere with the specificity of the method. Pregnanediol is approximately eight times more chromogenic than isoandrosterone.

(3) Dehydroisoandrosterone:

Procedure as in the previous experiment.

Standard solution of the pure compound (ex acetate and recryst. from aqueous methanol). m.p. 146° - 147° (corr).

Dehydroisoandrosterone added (mg.)	Tube.	Spekker.	Mg. Recovd. as pregnanediol.	Mg. Recovd. as dehydroisoandrosterone. (D.H.A.)
1.0	j	0.020	0.022	0.045
1.0	j_1	0.032	0.035	0.080
2.0	k	0.026	0.030	0.070
2.0	k_1	0.035	0.040	0.085
4.0	l	0.036	0.040	0.085
4.0	l_1	0.038	0.045	0.098.
8.0	m	0.030	0.036	0.082
8.0	m_1	0.056	0.065	0.145

CONCLUSION: Dehydroisoandrosterone is more powerfully chromogenic than the two previous compounds having approximately one-half of the chromogenicity of pregnanediol and it is providential that it is so completely eliminated in the precipitation process. The presence of dehydroisoandrosterone alone in the urine should not lead to an appreciable apparent pregnanediol recovery.

(4) Pregnanolone : Pregnan-3(α)-ol-20-one.

Pure pregnan-3(α)-ol-20-one prepared by the method of Sutherland and Marrian (1946,1947) from the ketonic fraction of impure "sodium pregnanediol glucuronidate". m.p.:- 147° - 149° (corr.)

Standard solution: 20.021 mg. in 20 ml: ethanol.

Pregnanolone added (mg.)	Tube.	Spekker.	Mg. Recovd. as pregnane-diol.	Mg. Recovd as Pregnanolone.
0.5	0	0.007	0.005	0.20
0.5	0_1	0.011	0.010	0.24
1.0	p	0.013	0.012	0.36
1.0	p_1	0.025	0.026	0.82
2.0	r_1	0.031	0.034	1.0
2.0	r_1	0.042	0.050	1.5
4.0	s	0.040	0.047	1.4

CONCLUSION: Although precipitation in the range of temperature 75° to 37° did not appear to be complete none the less relatively large amounts of this substance appeared in the final product after purification, and the fact that only traces of apparent pregnanediol were recovered when large amounts of pregnanolone were added, is due to the very low chromogenicity of this steroid. Pregnanediol is approximately 40 times more chromogenic in the sulphuric acid reaction.

A gravimetric method of determination would therefore yield fictitiously high results.

Whereas androsterone and isoandrosterone precipitated out as large plaques, the precipitate formed by pregnanolone was a fine dispersion and considerable loss occurred in the syphoning off of the supernatant solution.

(5) Allopregnanolone: allo-pregnan-3(α)-ol-20-one

Standard solution of the pure compound,

m.p. 191° - 195° (corr.)

19.957 mg. in 20 ml. ethanol.

Allo-pregnan-olone added (mg.)	Spekker.	Mg. Recovd. as pregnanediol.	%	Mg. Recovd. as allo-pregnano-lone.	%
0.5	0.002	0.005	1.0	0.25	50
0.5	0.005	0.007	1.4	0.40	80
1.0	0.010	0.012	1.2	0.77	77
1.0	0.012	0.015	1.5	0.95	95
2.0	0.017	0.020	1.0	1.40	70
2.0	0.020	0.022	1.1	1.60	80
4.0	0.045	0.055	1.4	3.72	93
4.0	0.032	0.040	1.0	2.65	66

CONCLUSION: As in the case of pregnan-3(α)-ol-20-one the very low recovery of apparent pregnanediol depended almost entirely upon the low chromogenicity of this steroid. Recovery of the allopregnanolone was actually higher than the recovery of pregnanolone but the former substance is even less chromogenic than the latter - possessing approx. one-eightieth of the chromogenicity of pregnanediol.

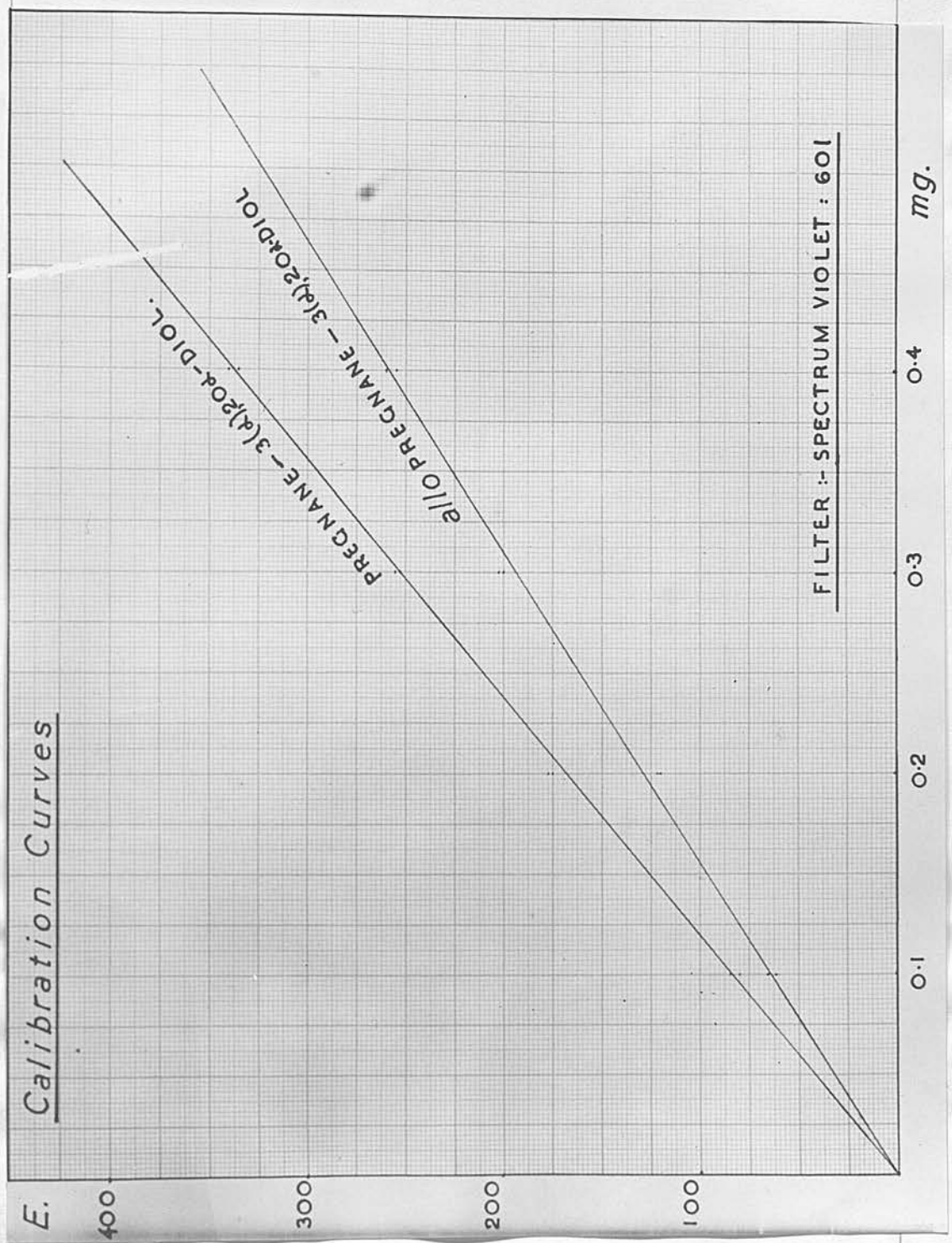
(6) Allopregnanediol; allopregnane -3(α),20α-diol.

Although it has not yet been proved that this compound occurs as a glucuronide in pregnancy urine, it has been isolated from that source and as it might be expected to behave as its stereo-isomer its behaviour in the purification process was of particular interest.

Standard solution: 10.078 mg. in 50 ml. ethanol.
m.p. 242° - 243° (corr.)

CALIBRATION CURVES.

Pregnanediol and allopregnanediol.



Allopregnanediol added (mg.)	Spekker.	Mg Recovd as pregnanediol.	%	Mg. Recovd. as allopregnanediol.	%
0.1	0.018	0.020	20	0.027	27
0.1	0.012	0.014	14	0.020	20
0.2	0.105	0.125	63	0.162	81
0.2	0.106	0.125	63	0.162	81
0.3	0.182	0.215	72	0.280	93
0.3	0.179	0.212	71	0.277	92
0.4	0.252	0.298	75	0.388	97
0.4	0.247	0.292	73	0.380	95

Standards	mg.	Spekker		Mg.	Spekker.
Allopregnanediol	0.1	0.065	pregnanediol	0.1	0.085
	0.1	0.061		0.1	0.080
	0.2	0.120		0.2	0.177
	0.2	0.122		0.2	0.173
	0.3	0.200		0.3	0.252
	0.3	0.202		0.3	0.255
	0.4	0.255		0.4	0.341
	0.4	0.260		0.4	0.335

CONCLUSION: Allo-pregnane-3(α),20α-diol behaves like pregnane-3(α),20α-diol in pure solution. The apparent recovery of pregnanediol is 20% less, however, due to the fact that allopregnanediol is less chromo-:genic to this degree. As this observation on the chromogenicity had not been made previously, the experiment was repeated with fresh standard solutions and, after checking the purity of the allo-pregnane-3 (α), 20α-diol, almost identical calibration curves were obtained. It may be concluded therefore that the method determines a mixture of the two stereoisom-:ers of pregnane-3(α),20α-diol.

In other experiments on specificity it seems likely that by using pure solutions of the steroids, as described, the conditions would rather favour interference and be a severe test of the specificity of the method. In this case, however, where a quantitative recovery is obtained in pure solution it will be necessary to repeat the experiment in the presence of N.F.M.U. It might be that greater or less loss would occur during purification in the presence of N.F.M.U. than is the case with pregnane-3(α), 20α-diol. This will be done as soon as sufficient allopregnanediol has been prepared

(7) $\Delta^{2 \text{ or } 3}$ ANDROSTENE-17-ONE.

Standard solution: 20.155 mg. in 20 ml. ethanol.

$\Delta^{2 \text{ or } 3}$ Androstene-17-One added (mg.)	Spekker.	Mg. Recovd. as pregnanediol.	%	Mg. Recovd. as $\Delta^{2 \text{ or } 3}$ Androstene-17-One.	%
0.5	0.005	0.007	1.4	0.12	24
0.5	0.010	0.012	2.4	0.20	40
1.0	0.006	0.008	0.8	0.12	12
1.0	0.008	0.010	1.0	0.18	18
2.0	0.025	0.030	1.5	0.50	25
2.0	0.035	0.042	2.1	0.70	35
4.0	0.180	0.212	5.3	3.55	89
4.0	0.162	0.192	4.8	3.20	80

CONCLUSION: The apparent pregnanediol recovery obtained when this compound was subjected to the triple precipitation process was satisfactorarily low. This

result was, however, to some extent due to the fact that this 17-keto steroid has approx. one-twentieth of the chromogenic power of pregnanediol in the sulphuric acid reaction.

(8) $\Delta^{3,5}$ ANDROSTADIENE - 17 - ONE.

Standard solution : 39,282mg. in 20 ml Ethanol.

$\Delta^{3,5}$ Androstadieneone added (mg.)	Spekker.	Mg Recovd. as pregnanediol.	%	Mg. Recovd. as $\Delta^{3,5}$ Androstadiene.	%
1.0	0.021	0.027	2.7	0.037	3.7
1.0	0.035	0.045	4.5	0.057	3.7
2.0	0.072	0.082	4.1	0.150	7.5
2.0	0.045	0.055	2.8	0.095	4.8
4.0	l=5 0.090	0.53	13.1	0.95	23.8
4.0	0.120	0.70	17.5	1.28	32.0
8.0	l=10 0.318	3.75	46.9	6.75	84.4
8.0	0.195	2.30	28.8	4.18	52.3

CONCLUSION: Elimination is fairly adequate when this steroid is present in amounts corresponding to less than 10 mg./24 hr. Apparent pregnanediol recoveries of ca. 20% were obtained in the presence of amounts corresponding to 20-30 mg/24 hr. Data is not available concerning the possibility that amounts of this substance in excess of 10mg/24 hr might occur in pathological urine, but it is unlikely that this rather rare steroid will interfere with determinations in urines other than those in which very large amounts of steroids of adrenal origin are present.

(9) CHOLESTEROL.

Standard solution: 40.030 mg. in 20 ml. ethanol.

Cholesterol added (mg.)	Spekker.	Mg. Recovd. as pregnanediol.	%	Mg. Recovd. as Cholesterol.	%
1.0	0.008	0.010	1.0	0.022	2.2
1.0	0.010	0.012	1.2	0.025	2.5
2.0	0.012	0.014	0.7	0.030	1.5
2.0	0.006	0.008	0.4	0.015	0.8
4.0	0.080	0.095	2.4	0.202	5.1
4.0	0.124	0.148	3.7	0.315	7.9
8.0	1:5 0.135	0.80	10.0	2.22	27.8
8.0	0.110	0.65	81	1.65	20.6

CONCLUSION: Cholesterol precipitated out at 75° in the tubes containing 4.0 and 8.0 mg. in pure solution. Very poor sedimentation occurred and at the end of centrifugation the supernatant solution was cloudy and opalescent. The majority of the precipitate was lost in this way, and the apparent recovery as pregnan-:ediol was very low except when amounts corresponding to 40 mg./24 hr were added. Such amounts would not be found in human urine but the observation indicates the difficulty which would be encountered in an attempt to determine the excretion of pregnanediol in human bile. Cholesterol gave an orange colour with sulphuric acid and had about one-third of the chromogenicity of pregnanediol under the conditions of colorimetry employed.

SUMMARY of SECTION II.

The methods of urinary pregnanediol determin-:ations which have hitherto been described are either insufficiently sensitive or insufficiently specific to permit of strictly quantitative studies upon the excretion of urinary pregnanediol during the menstrual cycle, the first trimester of pregnancy or upon that excretion of pregnanediol which results from the administration of progesterone to the human subject. The method described should make such studies more nearly possible. Most of the attributes of a suitable method as envisaged in the Introduction have been attained. The method is simple and determinations can be carried out daily and in duplicate upon one-fifth of a 24 hr. urine specimen. Although two eight-hour working days are necessary for the completion of a single determination, the technique entailed on the second day's work is such that at least six determina-:tions can be carried through daily by one person.

The method permits of the accurate determina-:tion of more than ca.0.4 mg. of pregnanediol in one-fifth of a 24 hr. specimen.

Several steroids which might be present in normal or pathological urines have been investigated as to their behaviour in the purification process. With the exception of the C_5 stereoisomer of pregnane-:diol itself, none of these compounds is likely to cause

serious interference unless present in very large amounts. The relatively specific nature of the method depends upon a providential state of affairs whereby substances of high chromogenicity are eliminated, and substances which are not completely eliminated are weakly chromogenic. Substances of the latter group - especially pregnan - 3(α)-ol-20-one and allopregnan-3(α)-ol-one which occur in pregnancy urine as glucuron-:ides will give fictitiously high results when present in urine where pregnanediol is being determined as its glucuronide or by a gravimetric method.

SECTION III.

A RAPID METHOD for the DETERMINATION of URINARY PREGNANEDIOL SUITABLE for ROUTINE CLINICAL USE.

Introduction.

The Guterman (1944, 1945) qualitative test for urinary pregnanediol, which is based on the quantit-:ative procedure developed by Astwood and Jones (1941) and Talbot, Berman, MacLachlan and Wolf (1941), has been widely used in recent years for the early diagnosis of pregnancy and for the prediction of the fate of threatened abortion. While there is some measure of agreement that the method may be useful for the latter purpose (Guterman, 1946; Bender, 1947,1948; Merivale, 1948), opinions on its value for the diagnosis of pregnancy are sharply divided. Guterman himself (1944, 1945) and McCormack (1946) have claimed an accuracy as great as that of the Friedman method; on the other hand, Reinhart and Barnes (1946) and Merivale (1948) have reported high percentages of both false positives and false negatives.

It seems probable that the unsatisfactory results obtained by the last mentioned authors can be ascribed, to some extent, to technical deficiencies of the method employed - some of which are avoidable.

A general criticism that can be justifiably made of the Guterman method is that a quantitative signifi-

:cance has been attached to the results of a purely qualitative test. Attempts have been made by a number of workers to make the test more quantitative by examining the colour spectrophotometrically instead of visually. (Davis and Fugo, 1947; Bender, 1947, 1948; Kullander, 1948; Merivale, 1948; Guterman, 1948).

Mack and Parks (1946, 1947) have described a qualitative test based upon the Astwood-Talbot and Guterman procedures, but purification of the precipitate and estimation of the final product are omitted and a result arrived at by the mere inspection of the precipitate. These workers state that "in samples containing 0.75 mg. or more of pregnanediol, a precipi-:tate is evident immediately". If no precipitate is visible the precipitation mixture (acetone is used) is cooled rapidly in an ice-water bath for ten minutes, removed from the bath and observed once more. "Roughly quantitative estimates of the precipitate" are obtained by centrifugation of the precipitate in a graduated tube and the results are expressed by these workers in ml. pregnanediol per specimen.

Since, as has been shown in the previous section, there are inherent in these methods, a considerable number of factors conducive to inaccuracy, it seemed possible that by replacing these by more favourable conditions a reasonably quantitative method might be

evolved which would be no less rapid and simple than such unsatisfactory procedures.

An attempt was therefore made greatly to reduce the time taken for a single determination by modification of the method described in Section II. This could only be achieved at the expense of the accuracy and specificity of the method. It was hoped that a reasonable degree of accuracy could be maintained by retaining essential features of the original procedure. A high degree of specificity in the presence of abnormal amounts of such substances as cholesterol and the neutral 17-ketosteroids would not be demanded of a method employed in the routine determination of urinary pregnanediol excretion in pregnancy. The destruction of pregnanetriol by acid hydrolysis and the very low chromogenicity of pregnanolone and allopregnanolone would make interference by these substances unlikely.

(1) Male Urine Blanks: The most effective way in which reduction in time from two days to a few hours could be achieved was by elimination of the 2nd and 3rd pptns. The most serious consideration involved in such a step was the effect upon the elimination of the chromogenic material of the N.F.M.U. It seemed possible that by slow cooling of the precipitation mixture followed by filtration of the large "crystals" through a filter of such a porosity as to permit adequate elimination of these chromogens with retention of the precipitated pregnanediol, that high "blank" values would be avoided.

A sintered glass filter of av. por. diameter 20μ-30μ proved satisfactory for this purpose. In addition treatment of the final product with charcoal was carried out as in the original method.

Blank determinations were carried out in triplicate on eight 100 ml. samples from 24 hr. urine specimens collected from seven different normal men, and the apparent pregnanediol determined by the shortened procedure. The results are shown in Table 13.

CONCLUSION: The values for apparent pregnanediol excreted per 24 hours varied from 0.13 to 0.61 mg. The highest of these values is so much smaller than the lowest amount of pregnanediol which could be determined by the proposed method that error due to this cause would be negligible.

(2) Rapid method adopted after modification of the original method.

The procedure was worked out bearing in mind the results of the recovery experiments which led up to the adoption of the original method. For example, the possibility of omitting overnight incubation of the precipitation mixture was suggested by the results shown in Table 9. Little loss was anticipated by the omission of the second and third extractions of the hydrolysed urine with consequent reduction in the time taken to evaporate down the neutral water-washed extract.

TABLE 13.

Subject.	Volume of 24 hr specimen (c.c.)	Spekker.	Apparent pregnanediol mg/100 ml.		mg./24 hr.	
D.T.	840	0.018	0.020	av. 0.031	0.17	av. 0.25
		0.032	0.038		0.28	
		0.030	0.035		0.29	
R.W.	1850	0.014	0.017	av. 0.016	0.32	av. 0.29
		0.015	0.018		0.33	
		0.010	0.012		0.22	
W.A.	2040	0.020	0.025	av. 0.020	0.24	av. 0.33
		0.012	0.015		0.31	
		0.018	0.021		0.43	
D.P.	1970	0.028	0.031	av. 0.028	0.61	av. 0.54
		0.020	0.025		0.49	
		0.023	0.027		0.53	
J.S.	1220	0.032	0.038	av. 0.029	0.46	av. 0.35
		0.030	0.035		0.43	
		0.011	0.014		0.17	
D.D.	2615	0.003	0.005	av. 0.008	0.13	av. 0.197
		0.006	0.007		0.18	
		0.009	0.012		0.28	

METHOD.

Into a 500 ml. flask fitted with an interchangeable ground-glass joint and a reflux condenser is placed 100 ml. of a 24 hour urine specimen, the volume of which is measured, and 50 ml. of toluene ('sulphur-free', redistilled). The contents are heated to boiling and after the addition of 10 ml. concentrated HCl (A.R. quality) the boiling is continued for exactly 10 minutes. The mixture is cooled, transferred to a 250 ml. separating funnel and shaken. After standing for about 5 minutes the lower urine layer is run off and discarded, and the upper toluene-emulsion layer filtered through a Buchner funnel with gentle suction using a Whatman No. 1 paper in order to break the emulsion. The filtrate is then transferred back into the separating funnel and the lower urine layer that separates from the emulsion is run off and discarded.

The toluene layer is washed in the funnel twice with 15 ml. lots of N/1 NaOH and twice with 15 ml. lots of water. It is then transferred to a 200 ml. round-bottomed flask fitted with a bent ground-glass 'socket adapter' (Quickfit and Quartz) and evaporated to dryness under reduced pressure in a boiling water-bath.

The residue in the flask is quantitatively transferred with warm ethanol to a test-tube (1" diam) which has a graduation mark at the 5 ml. level. The solution is then evaporated to 5 ml. in a warm water-bath under a gentle stream of air. After placing the

tube in a beaker of water at 75°C, 20 ml. of N/10 NaOH are added slowly from a burette over a period of 3 minutes while stirring gently with a glass rod. After a further one minute the beaker and tube are transferred to an incubator at 37°C and left for 2 hours.

The contents of the tube are then filtered through a sintered glass funnel (3 cm. diam. plate; average por. diam. 20-30 microns), the tube and filter being subsequently washed liberally with water to remove all traces of alkali from the precipitate. The funnel is removed from the filter flask and fitted to a test-tube (1" diam.) fitted with a side-arm. The precipitate is then washed through into the tube with three 5 ml. lots of boiling alcohol using gentle suction.

To the filtrate in the filter-tube is added about 1 - 2 mg. of 'Norit' charcoal, the mixture heated in a water-bath for 2 minutes, and filtered through a Whatman No. 1 paper in a conical filter (2" diam.) into a test-tube (1" diam.). The filtrate is finally evaporated to dryness in a water-bath under a stream of air.

To the dry residue in the tube are added 10 ml. of concentrated H_2SO_4 (A.R. quality). The tube is placed in a constant temperature bath at 25° and left for 20 minutes with occasional shaking. The intensity of the yellow colour produced is then measured in a 'Spekker' photoelectric absorptiometer using a 'spectrum violet' No. 601 light filter. The absorptiometer readings

TABLE 14.

	Recovery of pregnane-3(α),20α-diol added to men's urine after acid hydrolysis.				
	Mg. pregnanediol added.		Mg. pregnanediol recovered (corrected for average male urine blank).		
Urine	per 100 ml. urine.	per 24 hrs urine.	per 100 ml.	per 24 hrs urine.	% recovery.
			av.	av.	
A	-	-	0.01)	0.17)	
	-	-	0.02) 0.02	0.25) 0.23	
	-	-	0.02)	0.28)	
B	-	-	0.02)	0.27)	
	-	-	0.03) 0.02	0.54) 0.40	
	-	-	0.02)	0.40)	
A	0.20	2.8	0.10	1.3	48
	"	"	0.05	0.71	26
	"	"	0.06	0.88	32
B	"	3.6	0.17	3.1	87
	"	"	0.11	1.9	53
	"	"	0.19	3.4	94
B	0.30	5.4	0.31	5.5	102
	"	"	0.25	4.5	83
	"	"	0.28	5.2	94
A	0.40	5.6	0.28	3.9	70
	"	"	0.24	3.3	60
			0.34	4.7	85
B	0.40	7.2	0.35	6.3	88
	"	"	0.34	6.1	85
	"	"	0.39	7.0	98
A	0.60	8.4	0.53	7.5	89
	"	"	0.47	6.6	86
	"	"	0.51	7.1	85
B	0.50	9.0	0.41	7.3	82
	"	"	0.43	7.7	86
	"	"	0.45	8.1	90

are interpreted by reference to frequently reconstructed calibration curves as in the original method.

(3) Two 24 hour urine specimens (A. and B.) were collected on successive days from the same normal male. (I.F.S.) Twelve 100 ml. samples were taken from specimen A and fifteen from B. After hydrolysis of these with HCl, amounts of pure pregnane-3(α),20α-diol varying from 0.2 to 0.6 mg. were added in ethanolic solution, three samples from each specimen being reserved as triplicate blanks. The pregnanediol in each sample was then determined by the method described and the recoveries calculated after correcting for the appropriate blank.

The results are given in Table 14.

CONCLUSION: The method developed permits of the reasonably accurate quantitative determination in less than 3 hours of the pregnanediol in 100 ml. of urine, provided that the level of excretion is above about 5 mg. per 24 hours. The method is advocated for the routine determination of pregnanediol excretion in pregnancy but not for quantitative studies in non-pregnant urine, or in urine containing abnormally high amounts of neutral 17-ketosteroids or in the study of the excretion of administered progesterone as pregnanediol. For all such studies, where higher degrees of accuracy and specificity are required the longer method should be employed. This rapid method

has been used for several months in the Department of Obstetrics and Gynaecology (Edinburgh University) for a study of the possible value of pregnanediol determinations in the assessment of prognosis in threatened abortion. Satisfactory duplicate results are being obtained but it is as yet too early to draw any conclusions from this work. All the determinations reported in this thesis were carried out by the longer but more sensitive and specific method.

SECTION IV.

SECTION IV.

THE CONVERSION of PROGESTERONE to URINARY PREGNANEDIOL by the HUMAN SUBJECT.

Introduction.

Since the suggestion by Venning and Browne (1938) that some sort of quantitative relationship might exist between progesterone production in the body and pregnane-:diol excretion in the urine, several workers have studied the excretion of urinary pregnanediol after the administration of progesterone to human subjects. These studies will be considered in some detail but it may be said at the outset that they indicate a marked variation in the extent of conversion among different individuals and in the same individual at different times. The amount of pregnanediol glucuronide appearing in the urine in such investigations appears to be of the order of 10% of the administered progersterone but published data vary from 0% to 50%.

Two facts suggest the possibility that this apparent variation may not be a true statement of the extent of conversion in the normal individual. In the first place, as has been indicated in Section II, methods hitherto employed have not permitted the satisfactory quantitative determination of less than ca. 10 mg./24 hr and these methods have been applied to material in which this level has rarely been attained. (If the % conversion is ca. 10% then 100 mg. would have to be administered daily to produce

this level of urinary pregnanediol). Secondly, for a proper assessment of this conversion under physiological conditions it is obviously desirable that a series of normal subjects should be investigated, and such studies are not to be found in the literature.

These facts and the completion of the work on the improved method for the determination of urinary pregnanediol suggested a reinvestigation of the problem.

The subjects chosen for the first phase of the investigation were either healthy young men or healthy post-menopausal women. The procedure was as follows:- A control period was followed by the administration of 60 mg. of progesterone (Organon; 10 mg. per ml.) on two successive days either by intra-muscular injection or by capsules orally. The urinary pregnanediol was determined during the control period and for six to eight days after the first administration, by which time the excretion of apparent pregnanediol had returned to the "blank" value. The determinations were carried out daily and in duplicate by the original method (Somerville, Gough and Marrian, 1948) on aliquot portions of 24 hour urine specimens and the % conversion calculated after correction of the apparent pregnanediol excretion for the control period blank.

All twenty-four hour specimens of urine of volume less than 2500 c.c. were diluted with distilled water to that volume and two lots of 500 c.c. used for the

duplicate determinations. Where the urine volume was more than 2500 c.c., 500 c.c. lots were taken and the result corrected for the additional volume.

Ovariectomised women were the obvious choice for investigation of the conversion of administered progesterone to urinary pregnanediol with a view to obtaining a control series for the interpretation of subsequent studies.

Healthy ovariectomised women were not available, however, at this time and there was no possibility of collecting a sufficient number of such cases on which to carry out the studies envisaged. Moreover, bearing in mind the indications for this operation, it seemed unlikely that the terms "healthy" or "normal" could properly be ascribed to the majority of ovariectomised women.

On the other hand it was possible to select a sufficient number of apparently healthy women from among those who had experienced the physiological withdrawal of ovarian activity at the menopause. As volunteer post-menopausal women were not forthcoming, women were chosen in whom there was a mild or moderate degree of prolapse of the uterus.

The investigation was carried out in each patient's home and care was taken to select subjects whose temperament and home conditions made it seem likely that they would collect complete twenty-four hour specimens

of urine. The cases agreed to stay in or around their homes during the investigation. Collection of urine under these conditions was found to be more reliable than collection by the nursing staff in the busy wards of the gynaecological unit. It was necessary to check the completeness of 24 hr specimens collected in hospital by daily determination of the urinary creatinine by the method of Folin (1914). The use of this method for this purpose was reviewed by O.W. Smith in 1942.

(1) ADMINISTRATION OF PROGESTERONE TO HEALTHY POST-MENOPAUSAL WOMEN.

Determination of pregnanediol in the urine after the administration of progesterone to a series of healthy post-menopausal women has not been reported, but investigations in women in whom there was reason to believe that endogenous progesterone production was absent or very low, were first described by Venning and Browne (1938). Thus 12% ov the adminis-:tered dose was recovered from the urine as pregnane-:diol glucuronide from a young ovariectomised woman.

Cope (1941) found a 6% recovery in a case of hypoplasia of the uterus and 9% in a case of anovulat-:ory menstruation.

Seegar Jones and TeLinde (1941) report a recovery of 10% in the pre-ovulatory phase of the menstrual cycle.

Hamblen, Ashley and Baptist (1939) and Stover

and Pratt (1939) were unable to detect pregnanediol in the urine after progesterone administration.

For the present purpose, three post-menopausal women aged 65, 70 and 71 were chosen. All three were in excellent health and had normal menstrual and obstetric histories. The results are given in table 15.

Subject.	Age.	Progesterone administered (mg.)	Pregnanediol recovered (mg. corr.)	% Recovery.
M.E.	65	120	18.73	15.61
A.	70	120	18.67	15.56
J.N.	71	120	19.17	15.98.
			Av. :	15.72.

CONCLUSION: The finding of such closely similar patterns of excretion of pregnanediol and % conversion in three random cases suggested the presence of some very definite metabolic process which could be studied in terms of this metabolite - pregnanediol - by this type of experiment. Pregnanediol disappeared from the urine within three to four days of the second injection of progesterone.

(ii) HEALTHY YOUNG MEN.

The excretion of urinary pregnanediol after the administration of progesterone to males was first reported by Buxton and Westphal (1939). 17% of injected progesterone was recovered from the urine of

a normal male as pregnanediol and much larger amounts (67% on one day) from a case of Addison's disease. Hamblen, Cuyler and Hirst (1940) reported a variation in the degree of conversion of from 0% to 42.5% with markedly differing extent of conversion in one male on different occasions: this case was given 120 mg., 120 mg., 80 mg. and 40 mg. of progesterone and conversion to 25%, 39%, 4% and 7% respectively resulted.

Dorfman, Ross and Shipley (1948) report the results of administration of progesterone by mouth (progesterone as dispensed for intra-muscular administration given orally by capsules) to one male with Addison's disease and to one with Diabetes Mellitus. In the former, conversion to 3% and 7% of the administered dose occurred and, in the latter, to 18.3%, 8% and 19.5%. These results and the others mentioned above were determined by the method of Venning. Dorfman et al. (1948) confirmed the work of Marrian and Gough (1946) and established preg-3(α)-ol-20-one as a metabolic product of progesterone by isolating this 20-ketosteroid from the final product. They concluded that 10% of the final product was pregnan-3(α)-ol-20-one.

The present investigation was carried out on three normal male volunteers aged 22 to 23 years. The procedure was carried out on four occasions on subject D.C.P. (Table 16,) and the results, which show

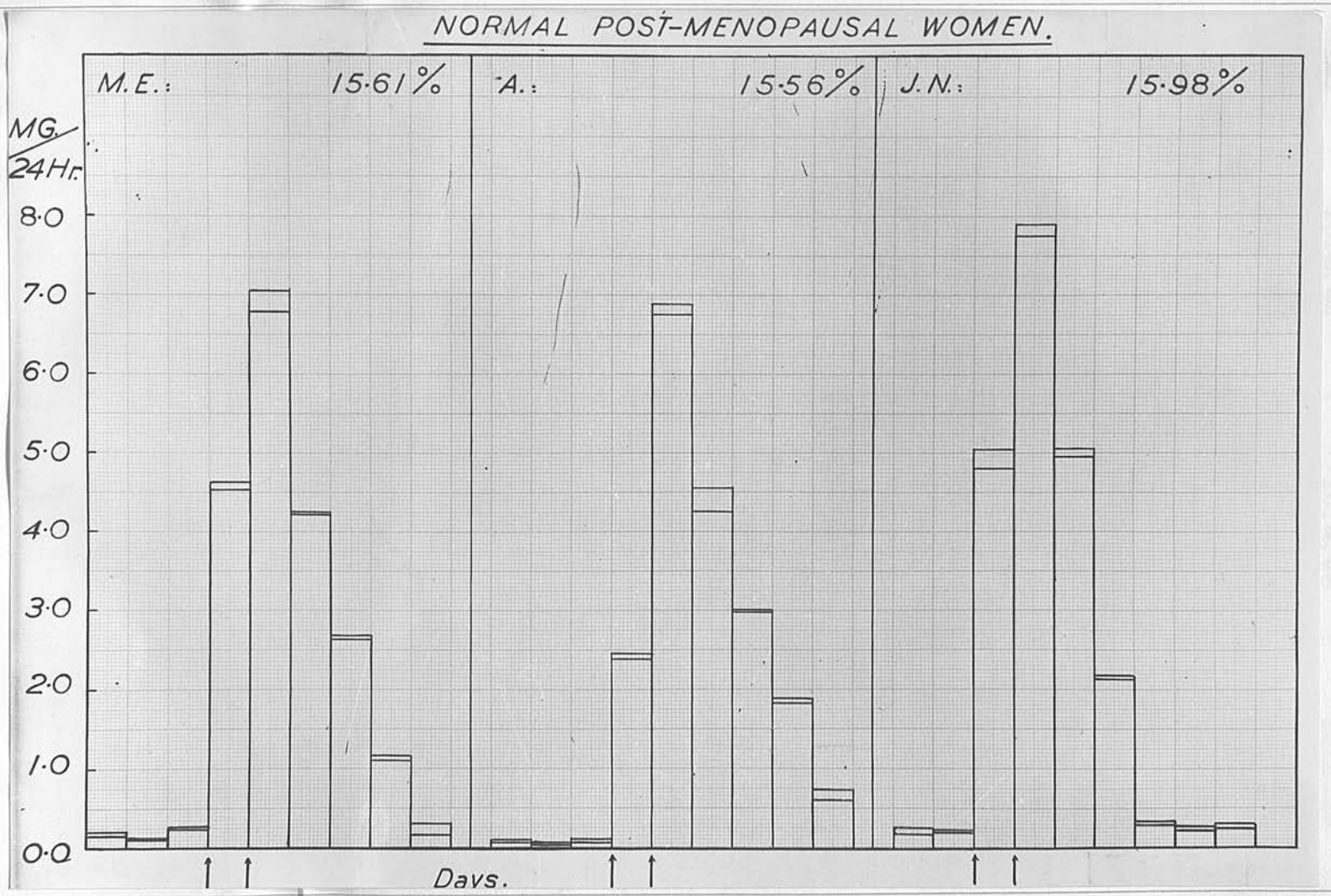

NORMAL POST-MENOPAUSAL WOMEN.
M.E.: 15·61%
A.: 15·56%
J.N.: 15·98%
MG./24Hr.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
Days.

FIGURE 1.

TABLE 15.

NORMAL POST-MENOPAUSAL WOMEN.

Subject:	E.		A.		N.	
Date:	10.2.48		10.4.48		21.4.48	
Admin.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.
-	1290	0.20 0.15	2310	0.07 0.06	-	-
-	2000	0.11 0.10	2420	0.04 0.06	1400	0.16 0.24
Prog. 60 mg. I.M.	1270	0.26 0.23	2125	0.08 0.05	1380	0.21 0.23
"	1470	4.52 4.62	2305	2.38 2.45	1800	4.80 5.03
-	1600	7.05 6.75	2720	6.88 6.75	1460	7.75 7.90
-	1250	4.22 4.25	2380	4.55 4.25	1395	4.95 5.05
-	910	2.63 2.68	2370	2.98 3.00	940	2.18 2.13
-	1260	1.16 1.10	2240	1.81 1.88	1200	0.28 0.33
-	970	0.28 0.14	2605	0.74 0.58	1615	0.26 0.21
App. preg. recov. mg.		19.63		19.13		20.22
" " corr. for "blank mg.		18.73		18.67		19.17
% recovery.		15.61		15.56		15.98

TABLE 16.

NORMAL MEN - PROGESTERONE I.M.

Subject:	D.P.		J.P.		A.R.	
Date:	13.1.48		17.12.47		14.1.48	
Admin.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c. 24 hr.	Pregnane-diol mg./	Vol. c.c.	Pregnane-diol mg./ 24 hr.
-	1340	0.23 0.20	1300	0.14 0.19	1800	0.33 0.28
Prog. 60 mg. I.M.	1030	0.26 0.34	1090	0.34 0.25	2000	0.20 0.24
" "	1400	2.85 2.94	1400	3.50 3.70	2570	3.55 3.70
-	2410	5.80 5.45	1305	8.80 9.05	3660	4.47 4.57
-	1500	2.80 2.73	1265	4.00 3.75	1850	2.23 2.15
-	1350	2.18 2.23	1180	1.33 1.43	1980	1.31 1.28
-	1430	0.31 0.24	1315	1.03 0.93	2175	0.59 0.68
-	1330	0.26 0.25	1060	0.36 0.30	1600	0.18 0.24
-	1800	0.16 0.24	-	-	2580	0.24 0.20
App. preg. recov. mg.		13.51		19.09		12.28
" " corr. for "blank".		12.47		17.65		11.13
% Recovery.		10.39		14.71		9.28

TABLE 16a.

Pregnanediol recovered from urine of normal men after the administration of progesterone (2 x 60 mg.)

Subject.	Age.	Date of expt.	Route of administration.	% recovery of pregnanediol (corr. for control period blank).	
D.P.	22	12.1.48	intramuscular.	10.4)	
		10.4.48	"	10.0)	10.3
		17.4.48	"	10.8)	
		22.4.48	"	10.0)	
		11.5.48	by mouth	12.8	
J.P.	23	17.12.47	intramuscular.	14.7	
		17.4.48	by mouth	18.6)	
		22.4.48	"	17.4)	18.5
		27.4.48	"	19.6)	
A.R.	22	12.1.48	intramuscular.	9.3	
		12.8.48	by mouth	11.5	

TABLE 17.

REPEATED PROGESTERONE INJECTION. (MALE: D.P.)

Admin.	Vol. c.c.	Preg. mg./24 hr.
-	1230	0.16 0.13
(a) Prog.60 mg.I.M.	1680	0.08 0.06
" "	1520	2.05 1.96
-	2315	4.48 4.58
-	1420	2.75 2.81
-	1445	2.09 2.04
(b) Prog.60 mg.I.M.	1380	1.21 1.15
" "	1530	4.30 4.22
-	1980	5.12 4.98
-	2546	2.62 2.75
-	1740	1.38 1.28
(c) Prog.60 mg.I.M.	1440	0.16 0.26
Prog.60 mg.I.M.	1630	2.50 2.38
-	1620	5.28 5.10
-	1530	2.66 2.76
-	1600	1.63 1.73
-	1980	0.60 0.35
-	1320	0.21 0.11

	App. Preg. Recov. mg.	Corr. for "blank"	% Recovery.
(a)	12.57	12.02	10.01
(b)	13.54	12.99	10.82
(c)	12.49	11.94	9.97

FIGURE 2.

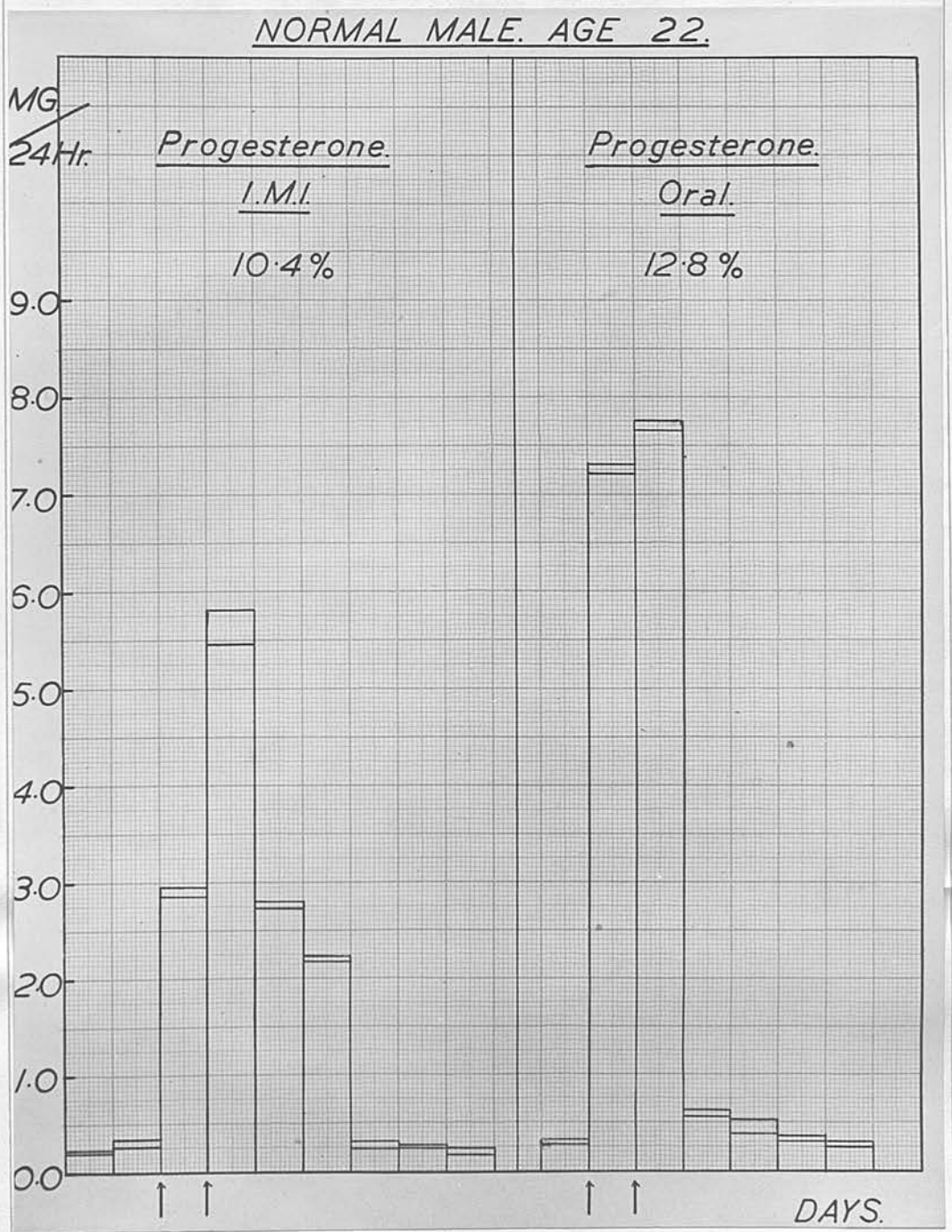

NORMAL MALE. AGE 22.
MG
24Hr.
Progesterone.
I.M.I.
10·4%
Progesterone.
Oral.
12·8%
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
DAYS.

TABLE 18.

REPEATED PROGESTERONE ORAL ADMINISTRATION
(MALE: J.P.)

Admin.	Vol. c.c.	Preg. mg./24 hr.
(a) Prog.60 mg.Oral	1358	0.28 0.16
	1540	0.30 0.24
Prog.60 mg.Oral	1550	6.05 6.45
	1790	9.58 9.63
	1310	6.90 6.80
	985	0.71 0.58
(b) Prog.60 mg.Oral	1300	0.33 0.26
Prog.60 mg.Oral	1455	7.50 7.75
	2040	9.50 9.73
	1970	4.05 3.75
	1820	0.66 0.48
(c) Prog.60 mg.Oral	1275	0.34 0.38
Prog.60 mg.Oral	1580	9.75 9.38
	1720	9.88 9.95
	1590	4.50 4.40
	1530	0.56 0.64
	2700	0.26 0.28

	App.Preg. Recov.mg.	Corr.for "blank"	% Recovery.
(a)	23.56	22.31	18.59
(b)	22.09	20.84	17.37
(c)	24.54	23.34	19.62

TABLE 18(a).

PROGESTERONE : ORAL ADMINISTRATION.

Subject	A.R.			D.P.
Date:	12.8.48			10.5.48
Admin.	Vol. c.c.	Pregnanediol mg./24 hr.	Vol. c.c.	Pregnanediol mg./24 hr.
-	3300	0.19 0.13	-	-
Prog.60 mg.Oral	2020	0.11 0.11	1840	0.33 0.26
Prog.60 mg.Oral	1940	2.14 2.10	1500	7.30 7.20
-	2685	11.50 10.60	1160	8.15 8.25
-	2395	0.25 0.28	1190	0.56 0.63
-	1860	0.13 0.12	1400	0.54 0.38
-	-	-	1060	0.36 0.30
-	-	-	1615	0.25 0.29
App. preg. recov. mg.		14.26		16.83
" " corr. for "blank" mg.		13.84		15.33
% Recovery		11.53		12.75

a remarkable degree of reproducibility are given in Table 16(a).

Oral administration of progesterone in capsules resulted in more rapid absorption and excretion of progesterone as pregnanediol and a slightly higher rate of conversion. Oral administration was repeated on three occasions in Subject J.P. (Table 17) and again a high degree of reproducibility was obtained. There is a quite convincing parallelism between the conversion by either route in the three individuals. Although two subjects gave a conversion ca. 5% lower than that found in post-menopausal females, the extent of conversion by the third was similar to that of the females. The slightly higher rate of conversion which followed gastric administration was a constant feature and is consistent with the view - which will be discussed later - that the liver plays an important part in the metabolism of progesterone.

(iii) The Effect of Oestrogen Administration on the Conversion of Administered Progesterone to Urinary Pregnanediol.

Venning and Browne (1940) studied the excretion of pregnanediol glucuronide after progesterone administra-:tion in two cases of secondary amenorrhoea with hypoplastic endometria. Small doses of progesterone were given i.e., 8 mg. daily for three days; and 10 mg. daily for eleven days. The percentage of admin-:istered progesterone excreted as urinary pregnanediol

in these cases was 0% and 1.7%. After pretreatment with Oestradiol Banzoate of the order of 10,000 I.U. for six to twelve days the recovery of pregnanediol rose to 18% in the first case and to 17% in the second case. These workers concluded that the excretion of pregnanediol was facilitated by the building up of the endometrium with oestrogens.

Cope (1939) using a similar dose of progesterone - 10 mg. daily for five days, determined the urinary pregnanediol in a similar case - secondary amenorrhoea with hypoplasia uteri - using a slightly modified version of the Venning method. The result was the direct opposite of that reported by Venning and Browne. Before Oestrogen pretreatment, 6% of the administered dose was recovered as pregnanediol in the urine but after administration of 20,000 I.U. of Oestradiol Benzoate daily for three weeks, only 3% was recovered.

Smith and Smith compared the effect of progesterone alone and of progesterone plus oestradiol benzoate upon the excretion of pregnanediol in pregnancy and suggested that oestrogen favoured the conversion of progesterone to pregnanediol. These workers stress the importance of this observation and it forms one of the fundamental "facts" in a wide-reaching hypothesis; yet the experimental evidence is not entirely convincing. Endogenous progesterone production complicates interpretation of the few data which are submitted for consideration. (1940)

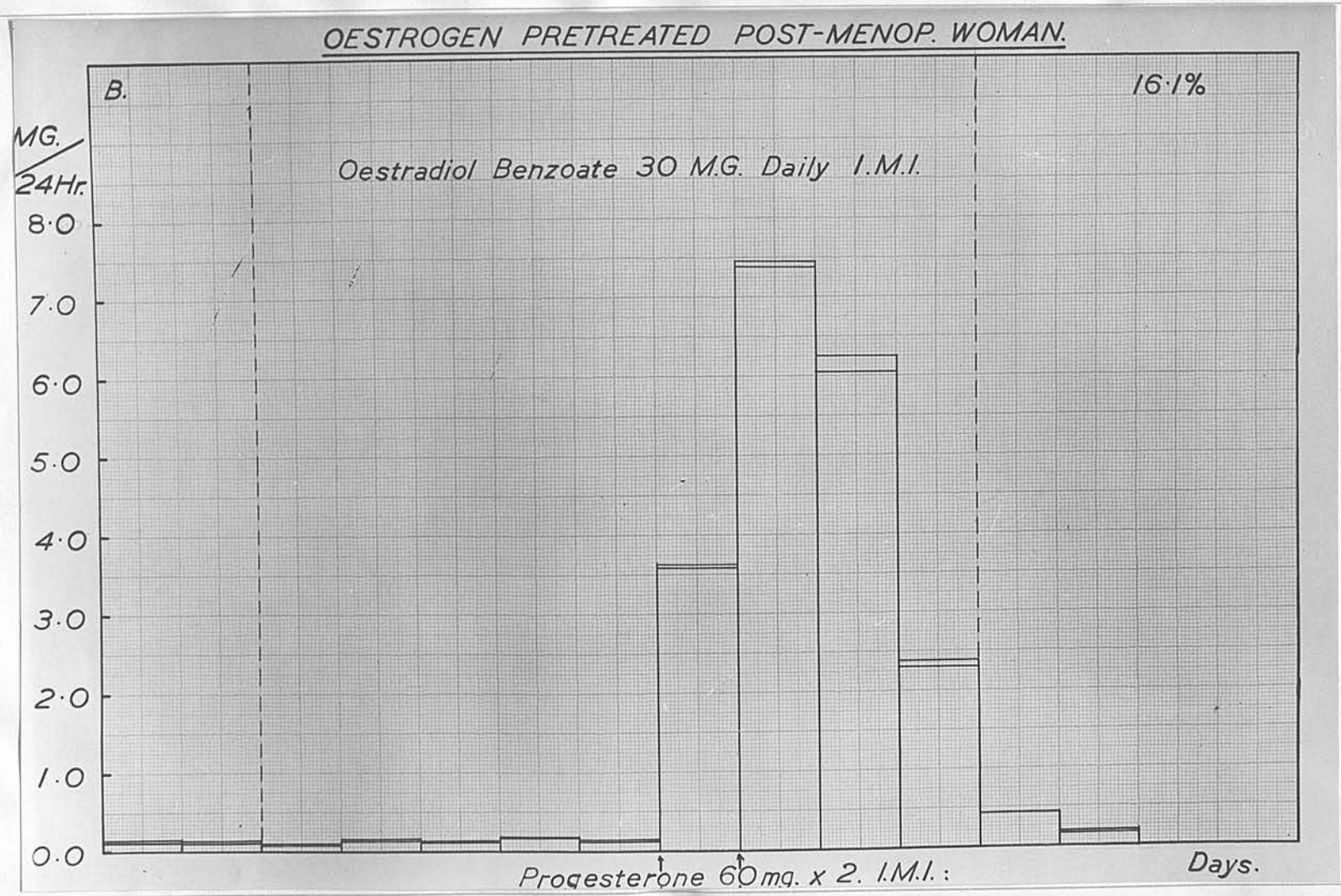

OESTROGEN PRETREATED POST-MENOP. WOMAN.
B.
16·1%
MG./24Hr.
Oestradiol Benzoate 30 M.G. Daily I.M.I.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
Progesterone 60 mg. x 2. I.M.I. :
Days.

FIGURE 3.

In the present work, three healthy postmenopausal women of the type used in series (i) were selected.

Progesterone (60 mg. intra-musc.) was administered on two consecutive days after six daily injections of 30 mg. Oestradiol Benzoate to two of the subjects. In the third, two injections of 120 mg. progesterone were administered after twenty daily injections of oestradiol benzoate. The daily injections of Oestradiol Benzoate were continued for the two days of progesterone administration and for two subsequent days.

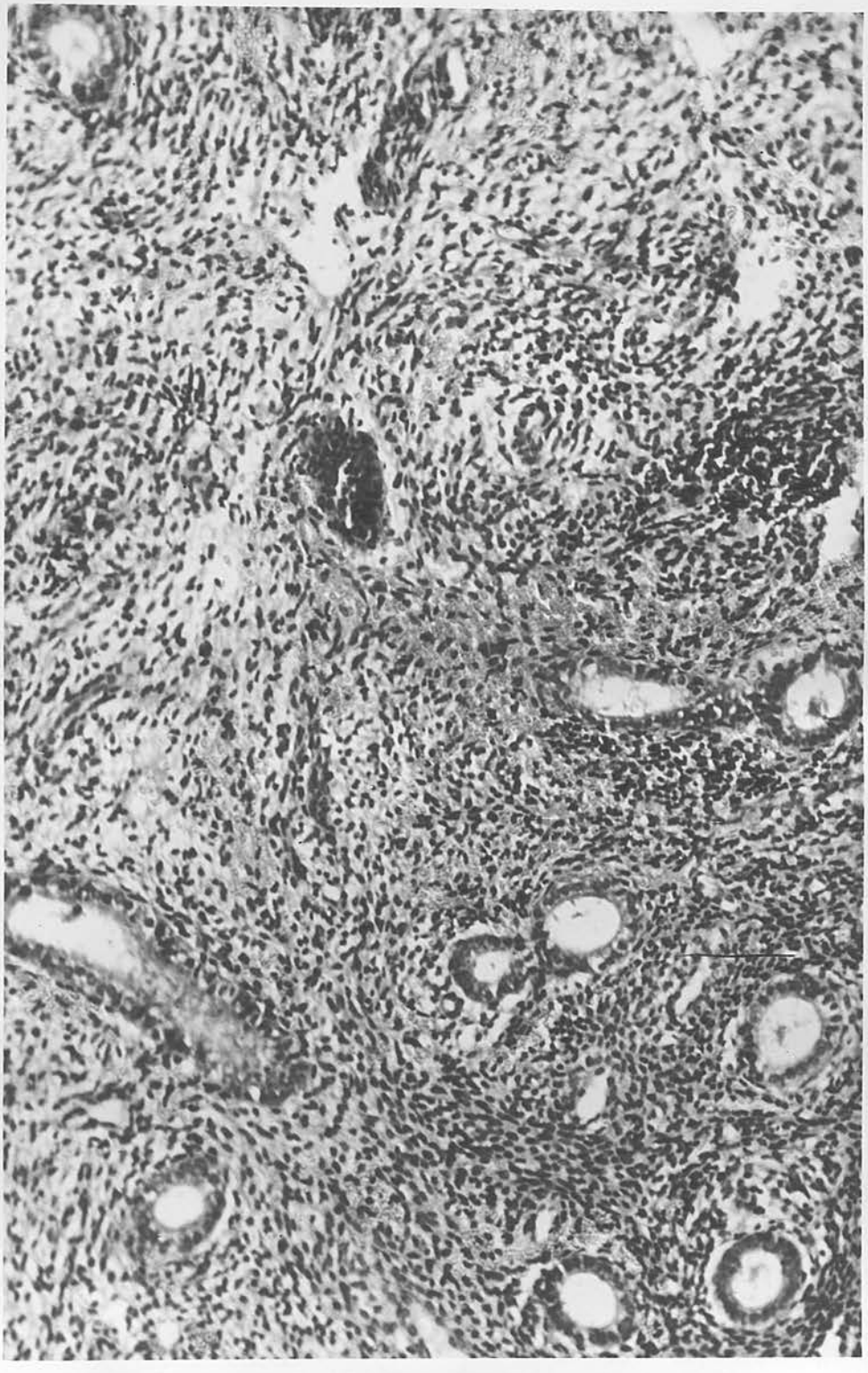
The results are given in Table 19 and compared with those obtained from the untreated postmenopausal women in Table 19(a).

In subjects H. and B., total hysterectomy and in subject K. endometrial biopsy were performed five to seven days after the last injection of Oestradiol Benzoate. For the interpretation of the microscopic sections of these endometria we are indebted to Dr W.I.C. Morris, F.R.C.S., F.R.C.O.G.

Effect on Endometrium:

(a) Subject H. : A microphotograph of the uterine scrapings is shown opposite. The appearance is that of a typical simple atrophic endometrium. There was a simple erosion of the cervix with a mild inflammatory reaction.

SUBJECT "H."



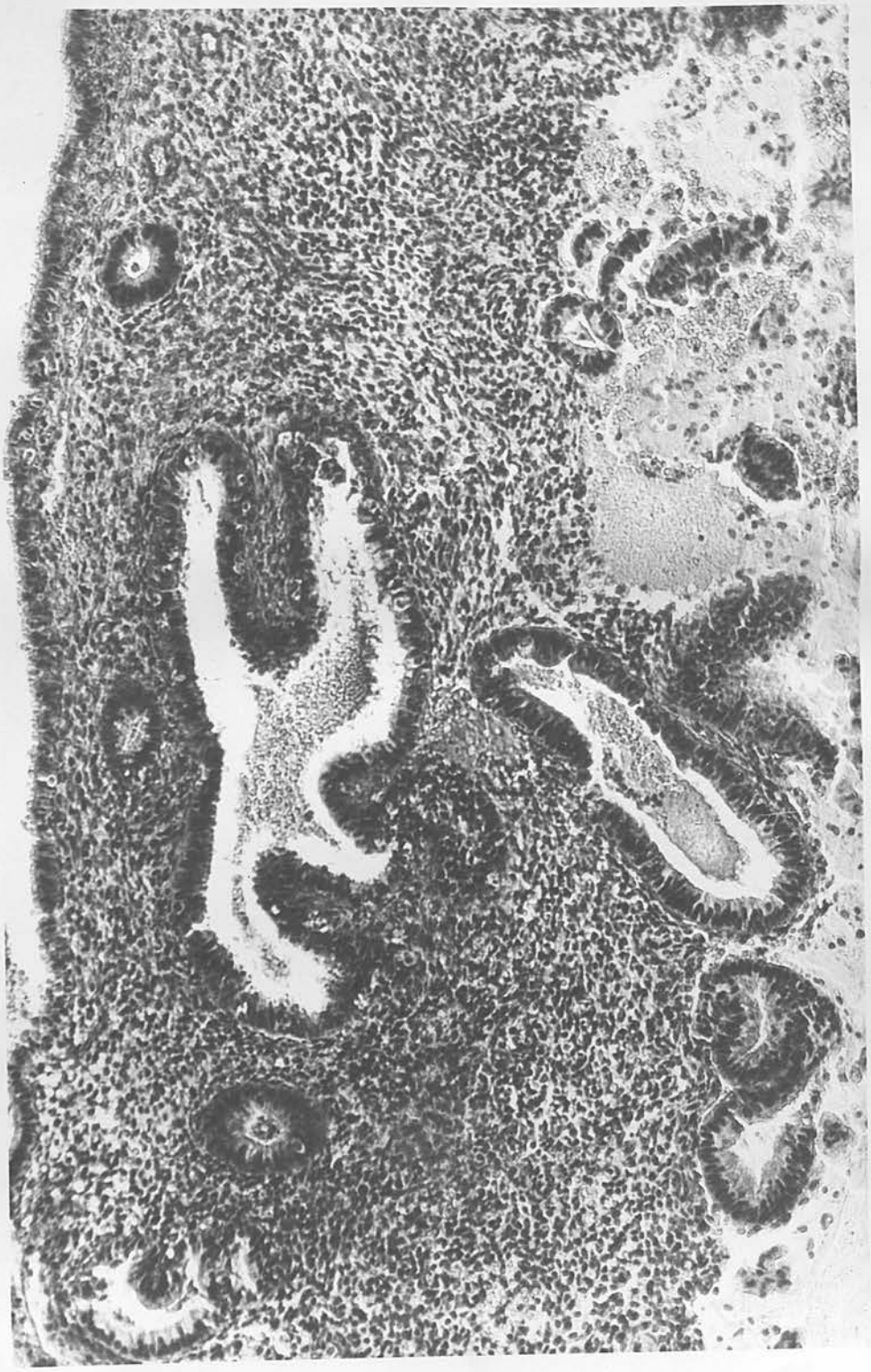
x 240.

TABLE 19.

OESTROGEN PRETREATED POST-MENOPAUSAL WOMEN.

Subject:	B.		H.		K.	
Date:	19.5.48		25.5.48		.2.12.47	
Admin.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.
OE. Benz. 30 mg. I.M.	1320	0.16 0.14	850	0.09 0.05	-	-
"	1890	0.11 0.15	1295	0.13 0.15	-	-
"	840	0.09 0.10	980	0.07 0.10	-	-
"	940	0.15 0.13	840	0.09 0.11	-	-
"	1180	0.11 0.13	700	0.06 0.07	-	-
"	1080	0.16 0.16	880	0.05 0.09	1160	0.36 0.30
" plus prog. I.M.	1680	0.11 0.13	770	0.06 0.05	1060	0.45 0.54
" " "	1240	3.58 3.62	1000	6.38 6.40	1580	9.80 10.13
OE. Benz. 30 mg. I.M.	1460	7.45 7.38	1550	7.65 7.88	1520	15.50 15.38
" "	850	6.25 6.05	1030	3.60 3.53	1115	9.13 9.30
" "	720	2.37 2.30	940	1.84 1.75	1460	2.98 3.07
" "	895	- 0.42	1100	0.37 0.20	1000	0.66 0.64
" "	1005	0.15 0.18	745	0.07 0.10	900	0.21 0.20
App. preg. recov. mg.		20.08		19.81		38.31
" " corr. for "blank" mg.		19.30		19.36		36.21
% recovery		16.08		16.13		15.09

SUBJECT "B".

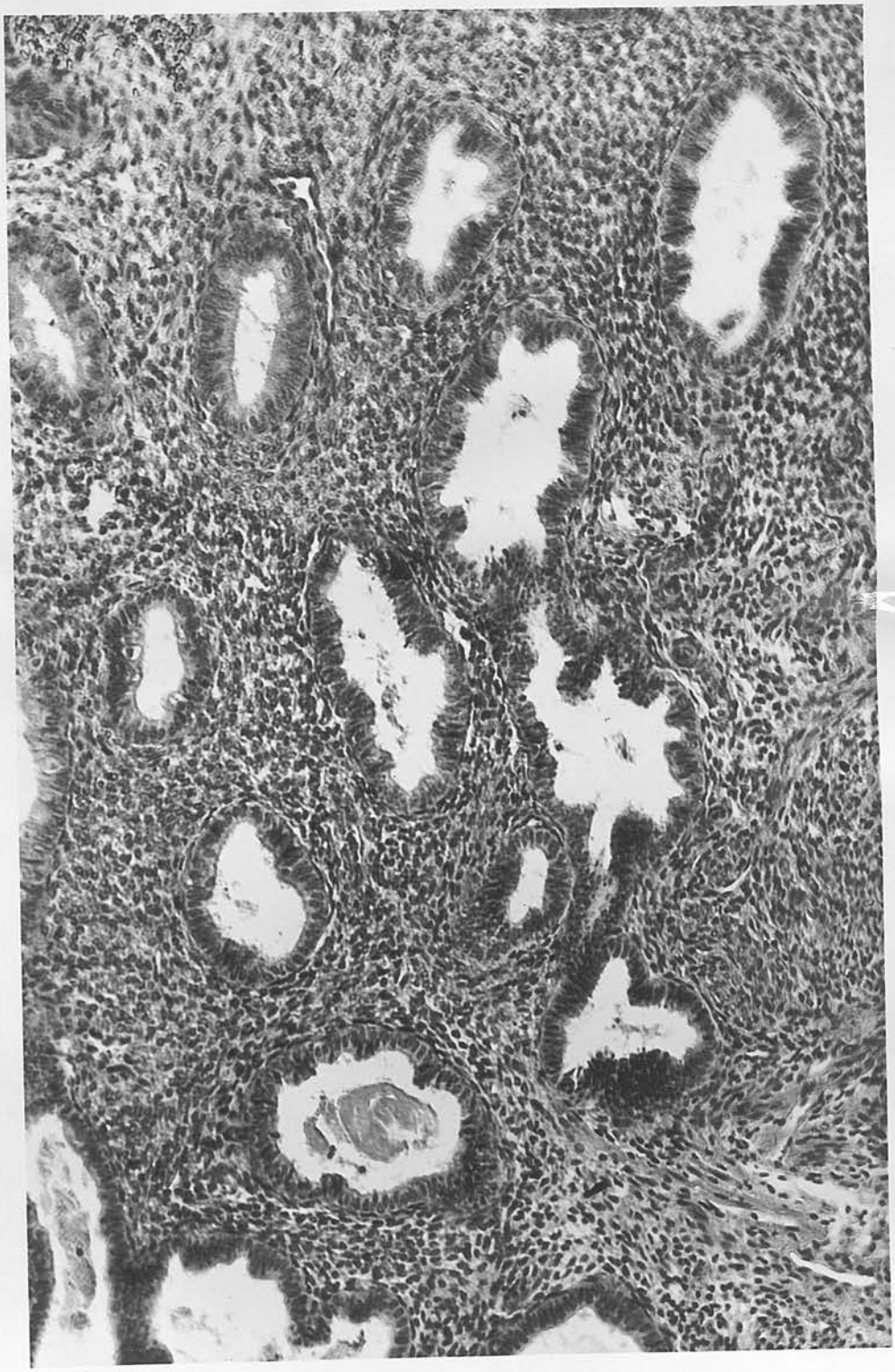


x240.

(b) Subject B. : The microphotograph shows a condition of the endometrium markedly different from that in the previous case. There is a thick active endometrium in which the majority of glands show proliferative activity. "In a few spaces secretory changes are manifest i.e., single layer of cells, sagellate outline displacement of nuclei towards the basement membrane, globules of secretion in the superficial parts of the cell, and cytoplasm crumbling down into the gland lumen. Even in such secretory glands, mitotic figures are occasionally seen. The stroma is in general that of the proliferative phase, numerous closely packed cells with densely staining nuclei and scanty cytoplasm. The cervix shows hyperkeratosis but not more than would be expected in a case of prolapse. No sign of activity in cervical glands, no vigorous proliferation in squamous epithelium. The vaginal skin shows definite hyperkeratosis more than might have been anticipated in such a case not receiving oestrogentherapy. No evidence of unusual mitoses in the deeper layers of vaginal epithelium."

(c) Subject K. : The endometrium in this subject also shows features of the proliferative phase and the pathologist reported as follows: "A proliferative phase endometrium, largely necrotic with some evidence of inflammation signified by the presence of plasma cells infiltration. Glands are cylindrical with marked pseudo-stratification. Very occasional mitoses.

SUBJECT "K."



x 240.

Many cells show vacuoles, some subnuclear but the majority supranuclear. The stroma is cellular with spherical and polygonal cells but no mitoses."

CONCLUSION: As shown above, the recovery of pregnanediol from the urine expressed as a % of the dose of progesterone administered was almost identical to that found in the untreated post-menopausal women. This occurred despite the administration of 240 mg. of oestradiol benzoate over a period of eight days to subjects H. and B. and 600 mg. of oestradiol benzoate over a period of twenty days to subject K. and despite the differing effects which this and the progesterone therapy appeared to produce in the endometria of the postmenopausal uteri. The results are strongly suggestive of the view that the oestrogen pretreatment of the postmenopausal subject in general and uterine endometrium in particular does not influence the conversion of administered progesterone to urinary pregnanediol. This will be further investigated. It might be argued that a threshold dose of oestrogen had been administered to subjects H. and B. which would not be high enough to ensure an effect on the endometrium in all cases. It seems more likely however that this short series illustrates the variation in response which occurs between individuals who are subjected to the same hormonal therapy and how unwise it would be to assume that because therapy has been dispensed the

the desired biological effect will be attained. The possibility that oestrogen administration might influ-:ence progesterone metabolism under different experimen-:tal conditions was considered and it is believed that in preliminary work described later in this section conditions obtain which more closely resemble some aspects of the interrelationship of those two hormones during active reproductive life.

(iv) Conversion of Progesterone to Pregnanediol by HYSTERECTOMISED POSTMENOPAUSAL WOMEN.

In 1938, Venning and Browne reported that pregnanediol glucuronide could not be recovered from the urine after the administration of progesterone to two hysterectomised women - even after the injection in one case of 24 mg. progesterone daily for four days. In a third case (1940) in whom supravaginal hysterec-:tomy had been performed 9.6% of the injected progester-:one (30 mg. daily for three days) was recovered.

Hamblen (1939) reported that pregnanediol, normally present during the luteal phase of the menstrual cycle, did not appear in the urine following curettage of the uterus.

Buxton, 1940, administered progesterone by intra-muscular injection to four hysterectomised women, in the form of 30 mg. daily for three days. In two cases no pregnanediol glucuronide was detected in the urine but in the third and fourth cases, 3.7 mg. and 4.9 mg.

were determined constituting 4% and 5% of the adminis-:tered dose respectively.

Seegar Jones and TeLinde (1941) reported recovery of 15.6% from a panhysterectomised woman and 12.9% and 10.9% from two women after supravaginal hysterectomy in whom 40 mg. of progesterone were injected on three successive days. Of great interest was the determina-:tion of 6.1 mg. of pregnanediol glucuronide on the 16th and 17th days of a menstrual cycle in a woman who had a total hysterectomy on the first day of that cycle.

In the present work, progesterone administration and the determination of urinary pregnanediol were carried out as in the previous series. Subject H. (series iii) was investigated five weeks after vaginal (total) hysterectomy had been performed. Subject L. (aged 42) who was not menopausal had been hysterectomised three weeks previously because of large uterine fibro-myomata. Subject R. was also suffering from uterine fibro-myomata and was investigated two weeks and again twenty-four weeks after vaginal hysterectomy and bilateral ovariectomy. Subject M. was a post-menopausal woman in whom vaginal hysterectomy was carried out prior to pelvic floor repair in the treat-:ment of prolapse of the uterus. Subject M. was investigated two weeks and again 20 weeks after hysterectomy.

The results and certain data relating to these women are given in Table 20.

CONCLUSION: The possibility that irregular results might be found in this series was anticipated since it was obviously impossible to investigate a normal postmenopausal woman who had undergone hysterectomy. In addition some postoperative factors might influence the result and while these would be of considerable interest in themselves, they would obscure the point at issue which was to find out whether the hysterecto-:mised woman differed from the intact postmenopausal woman in her ability to convert administered progester-:one to urinary pregnanediol.

Two cases investigated within two weeks of hysterectomy converted the administered progesterone to amounts of pregnanediol higher than had been previously observed in our subjects. Whatever the fundamental cause of this change it appears to have been related to the short period which had elapsed since the operation and when the investigations were repeated in the same woman 20 to 24 weeks later, figures very similar to those of the intact women were obtained. The trauma of the operation and the tissue repair which followed might alone have some bearing upon the problem and some speculation upon the relationship of the enzyme glucuronidase - the

TABLE 20a.

HYSTERECTOMISED POST-MENOPAUSAL WOMEN.

Subject:	M.		M.	
Date:	3.3.48		16.8.48	
Admin.	Vol. c.c.	pregnanediol mg./24 hr.	Vol. c.c.	pregnanediol mg./24 hr.
	850	0.20 0.22	1555	0.16 0.20
Prog. 60 mg. I.M.	1040	0.13 0.14	1340	0.05 0.08
Prog. 60 mg. I.M.	1210	5.42 5.35	1670	3.45 3.53
	1300	9.95 10.05	1280	7.20 7.25
	980	4.82 4.88	1180	4.08 4.28
	1160	2.61 2.68	1570	1.47 1.46
	1025	1.40 1.43	1110	0.23 0.16
	1400	0.51 0.46	1010	0.11 0.10
	1580	0.19 0.08	-	-
	950	0.14 0.19	-	-
App. preg. recovery.		24.79		16.59
" " corr. for "blank".		23.71		15.99
% Recovery.		19.78		13.33

TABLE 20b.

HYSTERECTOMISED POST-MENOPAUSAL WOMAN.

Subject:	R.		R.	
DATE:	10.2.48		24.7.48	
Admin:	Vol. c.c.	pregnanediol m.g./24 hr.	Vol. c.c.	Pregnanediol mg./24 hr.
	1100	0.34 0.25	1390	0.14 0.13
Prog. 60 m.g. I.M.	950	0.14 0.13	1400	0.18 0.20
Prog. 60 mg. I.M.	800	2.62 2.72	1280	4.03 4.10
	910	6.63 6.50	900	10.08 10.28
	880	5.20 5.10	980	4.00 3.90
	1490	6.05 6.05	960	0.43 0.31
	1160	2.69 2.64	1400	0.18 0.15
	1380	1.24 1.18	-	-
	1300	0.41 0.55	-	-
	1120	0.19 0.13	-	-
App. preg. recov. mg.		24.73		18.57
Corr. for "blank" mg.		23.19		17.89
% Recovery		19.33		14.91

TABLE 20c.

Subject:	H. (Hysterectomised post meno-pausal).		L. (hysterect-ovariect. pre-menopausal).	
Date:		14.7.48		17.7.48.
Admin:	Vol. c.c.	pregnanediol mg./24 hr.	Vol. c.c.	pregnanediol mg./24 hr.
	995	0.21 0.18	1920	0.13 0.09
Prog. 60 mg. I.M.	870	0.15 0.14	2375	0.23 0.19
Prog. 60 mg. I.M.	1280	4.55 4.80	2700	2.44 2.36
	970	8.00 8.05	840	3.85 3.80
	1010	3.65 3.60	1650	3.48 3.42
	1160	1.16 1.17	1820	1.30 1.20
	1140	0.34 0.28	1795	0.55 0.66
	1320	0.13 0.18	1455	0.16 0.18
App. preg. recov.		17.82		11.81
Corr. for "blank"		16.97		10.91
% Recovery.		14.14		9.09

concentration of which is raised in damaged and regenerating tissues - to progesterone metabolism are discussed in Section VIII. During the immediate postoperative period the women had been subjected to therapy by a variety of drugs over which we had no control. Apart from the anaesthetic, which was a barbiturate (pentothal), sulphonamides, penicillin, and salicylates were administered to these two cases alone. During the investigation subject R. developed a Pulmonary Embolism and Heparin was administered on the day following the injection of progesterone and for one week subsequently. As the high recovery in this case was due to an increased output of pregnanediol during the time of Heparin administration the possibil-:ity was considered that Heparin was the cause of this result. Since Heparin contains Glucuronic acid the possibility that it might play some part in the little understood mechanism of conjugation of pregnanediol was considered. These speculations were discouraged, for the time being, by the very similar result obtained in subject M. who did not receive Heparin. The matter cannot be dismissed, however, since as mentioned above, this case was receiving a host of medications/both patients exhibited hyperpyrexia during the investigations due to pulmonary infection in subject R. and urinary infection in subject M. and it is not beyond the bounds of possibility that

the associated toxaemia might be the factor responsible.

If those subjects who were investigated in the immediate postoperative period are omitted from the present series then the following figures remain, and are comparable with those of the intact postmenopausal controls:

Subject.	% Recovery.
H	14.1
M(b)	13.3
R(b)	14.9

If coincident administration of other drugs is the factor responsible for the high recoveries in R. and M. then the younger women, L. with a recovery of 9.1% should be included in the series.

In either case the conclusion is quite definite and it is that the postmenopausal uterus does not play an essential part in the conversion of administered progesterone to pregnanediol. It cannot be assumed however that the physiologically active uterus plays no part in the metabolism of progesterone and an attempt has been made to examine this wider problem.

(vi) Conversion of Progesterone to Pregnanediol in cases of HYPERTENSION.

It was not intended to study a group of cases of this type but three cases of hypertension found their way into our investigations in the following manner: Subject S., aged 61, was a postmenopausal woman upon whom hysterectomy had been recently performed and

hypertension was not noted until the investigation had begun. Blood pressure was of the order of 200/115 mm. Blood Urea was 40 mg. As the patient was in Edinburgh for only a few days after the interesting result of the investigation had been obtained, it was not practicable to repeat the investigation at a later date as in subjects R. and M.

The other two cases were studied in connection with the possible effect of Heparin upon the conversion of progesterone to pregnanediol and it was not consider-:ed justifiable to administer large doses of Heparin and the usual 120 mg. of progesterone to healthy subjects. The two cases available to us were as follows:-

Subject A : A young man (age 49) with hypertension and severe bilateral Raynaud's disease affecting the hands.

Subject H : An older man (age 47) with hypertension and intermittent claudication following traumatic arterial thrombosis.

The results are given in Table 21.

Subject.	Age Sex	Blood Pressure mm. Hg.	Pregnanediol recovered mg. corr.	% Recovery (corr.)
S.	61 Fem.	200/115	5.52	4.6
A.	29 male.	168/96	5.06	4.2
H.	47 male.	156/110	9.07	7.6.

Table 21.

HYPERTENSIVE CASES.

Subject:	S.		A.		H.	
Date:	13.1.48		28.2.48		23.2.48	
Admin:	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	Pregnane-diol mg./ 24 hr.
	1580	0.23 0.21	1370	0.15 0.08	1790	0.05 0.04
Prog. 60 mg. I.M.	1305	0.16 0.22	1400	0.10 0.06	1280	0.04 0.06
" "	1180	1.39 1.34	1620	0.66 0.73	1600	1.48 1.40
	920	2.16 2.13	1580	2.62 2.68	1810	6.30 6.50
	1220	1.43 1.46	1400	0.87 0.78	980	1.16 1.11
	1380	1.18 1.26	1690	0.74 0.78	1580	0.19 0.15
	1060	0.35 0.29	1620	0.65 0.56	1200	0.19 0.10
	1020	0.15 0.11	1750	0.21 0.11	1400	0.07 0.07
App. preg. Recov.		6.48		5.86		9.37
Corr. for "blank"		5.52		5.06		9.07
% Recovery		4.60		4.21		7.56

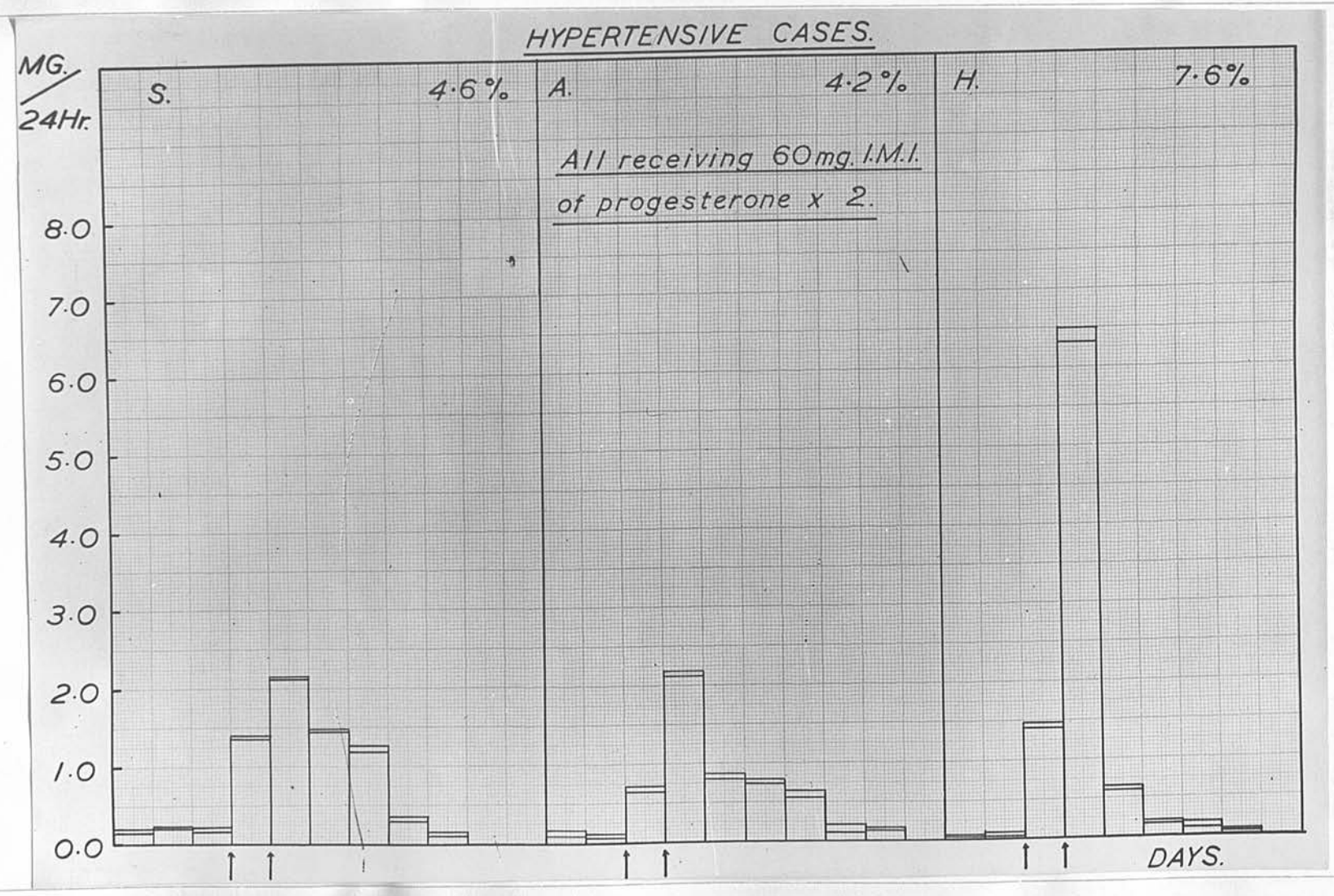

HYPERTENSIVE CASES.
MG./24Hr.
S.
4·6%
A.
4·2%
H.
7·6%
All receiving 60mg. I.M.I. of progesterone x 2.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
DAYS.

FIGURE 4.

CONCLUSION: It seems unlikely that the low yield of pregnanediol in the two males studied was a result of the administration of Heparin in view of the high (19.3%) yield in subject R. (series V) who received similar therapy. It cannot be concluded on results obtained in such unsatisfactory material that hyper-:tension or the underlying or associated phenomena which it signifies is responsible for a diminished capacity to convert administered progesterone to pregnanediol but the suggestion is there and further investigations are pending. Very careful assessment of the renal function in cases A and H was carried out in the Department of Surgery and no impairment of renal function was detected.

One conclusion which may be drawn from this group is the importance of selecting apparently healthy subjects wherever possible where an attempt is being made to elucidate a problem of physiology, although the fact that such variations occur suggests interest-:ing possibilities for future studies in pathology.

It was interesting to note that clinical improvement in the vascular insufficiency of the hands in case A appeared to follow the injections of proges-:terone.

(v) RECOVERY of PREGNANEDIOL after administration of PREGNANEDIOL and of SODIUM PREGNANEDIOL GLUCURONI-DATE.

Venning and Browne (1938) failed to recover pregnanediol glucuronide from the urine after intra-

muscular injection of pregnanediol to two hysterectomised women. Injection of sodium pregnanediol glucuronidate (as prepared by Venning and containing ca. 20% sodium pregnanolone glucuronidate) resulted in the excretion of 58% and 43% of pregnanediol in the same two cases. These were the cases which did not excrete pregnanediol after injection of Progesterone. The "NaPG" was injected in aqueous solution - 20 mg. daily on three successive days.

Hamblen, Cuyler and Hirst (1940) administered Sodium Pregnanediol glucuronidate (prepared by Venning's method) to six males by mouth. The recovery of the compound in the urine was extremely variable - 0% to 100% but the excretion in three of these subjects fell within the range of 30% to 50%.

In the present work the same three males as in series two were investigated. The procedure was identical to that observed on the previous occasion, but on the two consecutive days on which progesterone had been administered, pure pregnane-3(α),20α-diol in olive oil was administered orally in capsules. The solution of pregnanediol in olive oil was prepared in the follow-:ing manner: 140.3 mg. pure pregnane-3(α),20α-diol was dissolved in 5 ml. ethanol and approximately 30 ml. olive oil added. The solution was heated until the pregnanediol was completely dissolved and the ethanol had boiled off. The olive oil was then cooled to room temperature and made up to the 50 ml. mark. 20 ml. of this solution contained 56.12 mg. of pregnanediol and

TABLE 22.

PREGNANEDIOL BY MOUTH.

Subject:	D.P.		J.P.		A.R.	
Date:	20.7.48.		29.7.48.		9.7.48.	
Admin:	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Vol. c.c.	pregnane-diol mg./ 24 hr.
	1760	0.12 0.13	1665	0.25 0.24	1980	0.20 0.21
Preg.Oral	1560	0.11 0.19	960	0.29 0.26	3400	0.15 0.26
" "	1170	7.10 6.95	1160	9.30 9.33	1810	7.63 7.53
	1265	5.80 5.85	1400	8.75 8.73	1960	5.18 5.10
	1300	1.32 1.31	1600	2.29 2.15	2155	0.25 0.23
	1820	0.08 0.12	1910	0.38 0.28	2365	0.21 0.15
App. preg. Recov. mg.		14.18		20.61		12.96
Corr. for "blank" mg.		13.76		19.53		12.79
% Recovery		12.26		16.28		13.32

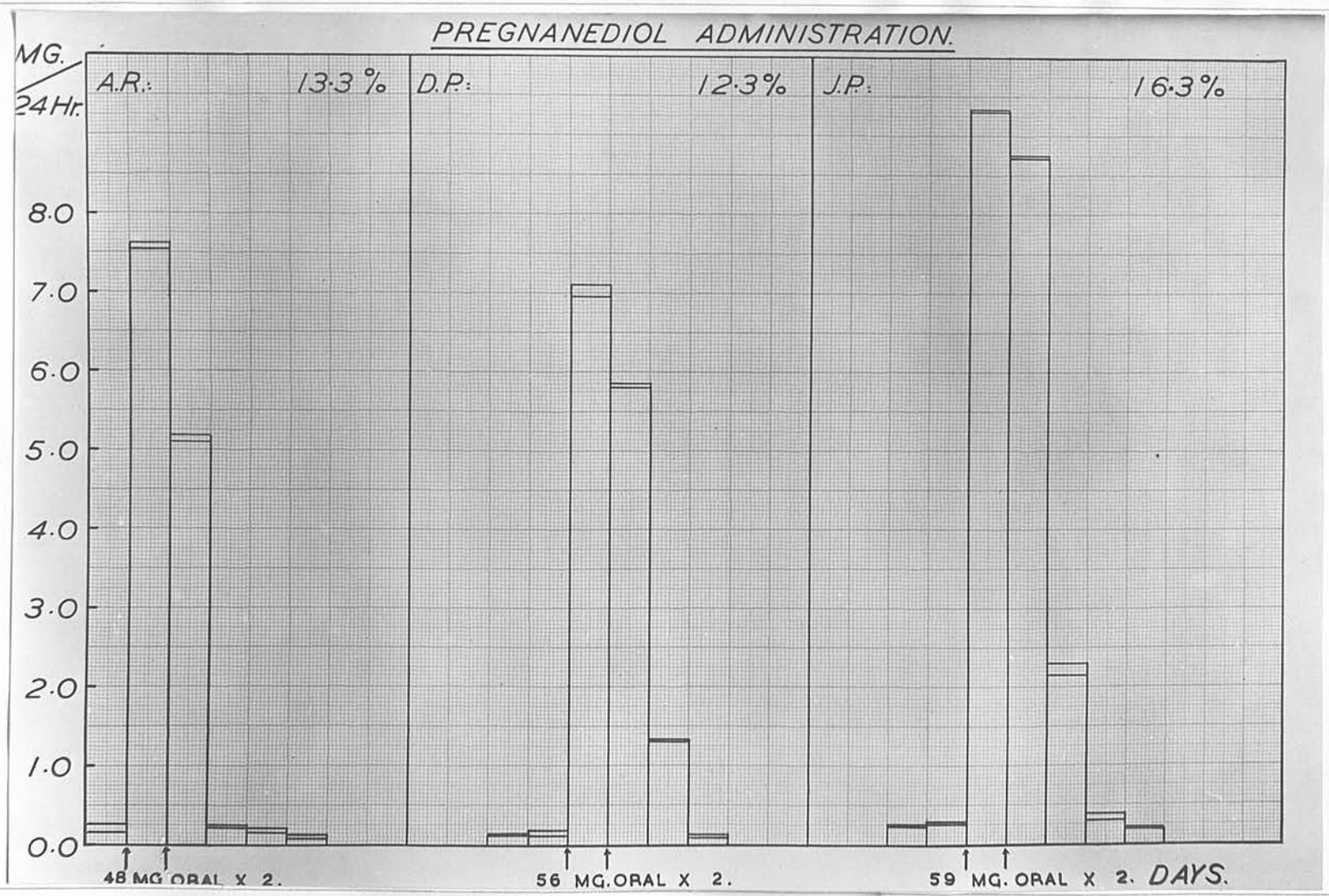

PREGNANEDIOL ADMINISTRATION.
MG. / 24Hr.
A.R.: 13·3 %
D.P.: 12·3%
J.P.: 16·3%
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
48 MG ORAL X 2.
56 MG. ORAL X 2.
59 MG. ORAL X 2.
DAYS.

FIGURE 5.

TABLE 22(a).

Percentage of administered Progesterone or Pregnanediol recovered as Pregnanediol in the Urine.

Subject.	Progesterone: I.M.	Progesterone: Oral.	Pregnanediol: Oral.
D.P.	10.3 (av.)	12.8	12.3
J.P.	14.7	18.5(av.)	16.3
A.R.	9.3	11.5	13.4

was administered in gelatine capsules. The results are given in Table 22 and compared with those obtained after administration of progesterone in Table 22(a).

CONCLUSION: The results of this investigation are compared with those obtained after the administration of progesterone by two routes to the same subjects. There is a striking parallelism in the amounts of unconverted progesterone and preconverted progesterone (pregnanediol) which are excreted in the urine as pregnanediol. It seems highly unlikely that this similarity would have been produced if the small amount of pregnanediol recovered in the present experiment was a result of incomplete absorption by the alimentary tract. The implication that the factors determining the amount of pregnanediol excreted in the urine operate after the conversion of exogenous, and possibly endogenous progesterone, to pregnanediol is worthy of consideration and is discussed in Section VIII.

(b) Administration of Sodium Pregnanediol Glucuronidate.

Pure sodium pregnanediol glucuronidate (the trihydrate) isolated by the method of Sutherland and Marrian (1946, 1947) was prepared by Miss Sutherland. The original intention was to dissolve ca.420 mg. of $NaPG.3H_2O$ in ca. 30 ml. of 0.9% saline (pyrogen free). This would be equivalent to ca.250 mg. of pregnanediol and 5.0 ml. of the solution would contain 40 mg. pregnanediol. With heating and even after increasing

Table 23.

SODIUM PREGNANEDIOL GLUCURONIDATE I.M. (D.P.)

Admin.	Volume.	Pregnanediol mg./24 hr.
	1590	0.30 -
71.47 mg. I.M.	1080	0.25 0.25
71.47 mg. I.M.	1310	1.85 1.89
	2650	3.30 3.18
	1800	4.13 4.25
	,1800	4.62 4.58
	1480	4.83 4.88
	1070	0.75 0.80
	1600	0.23 0.23

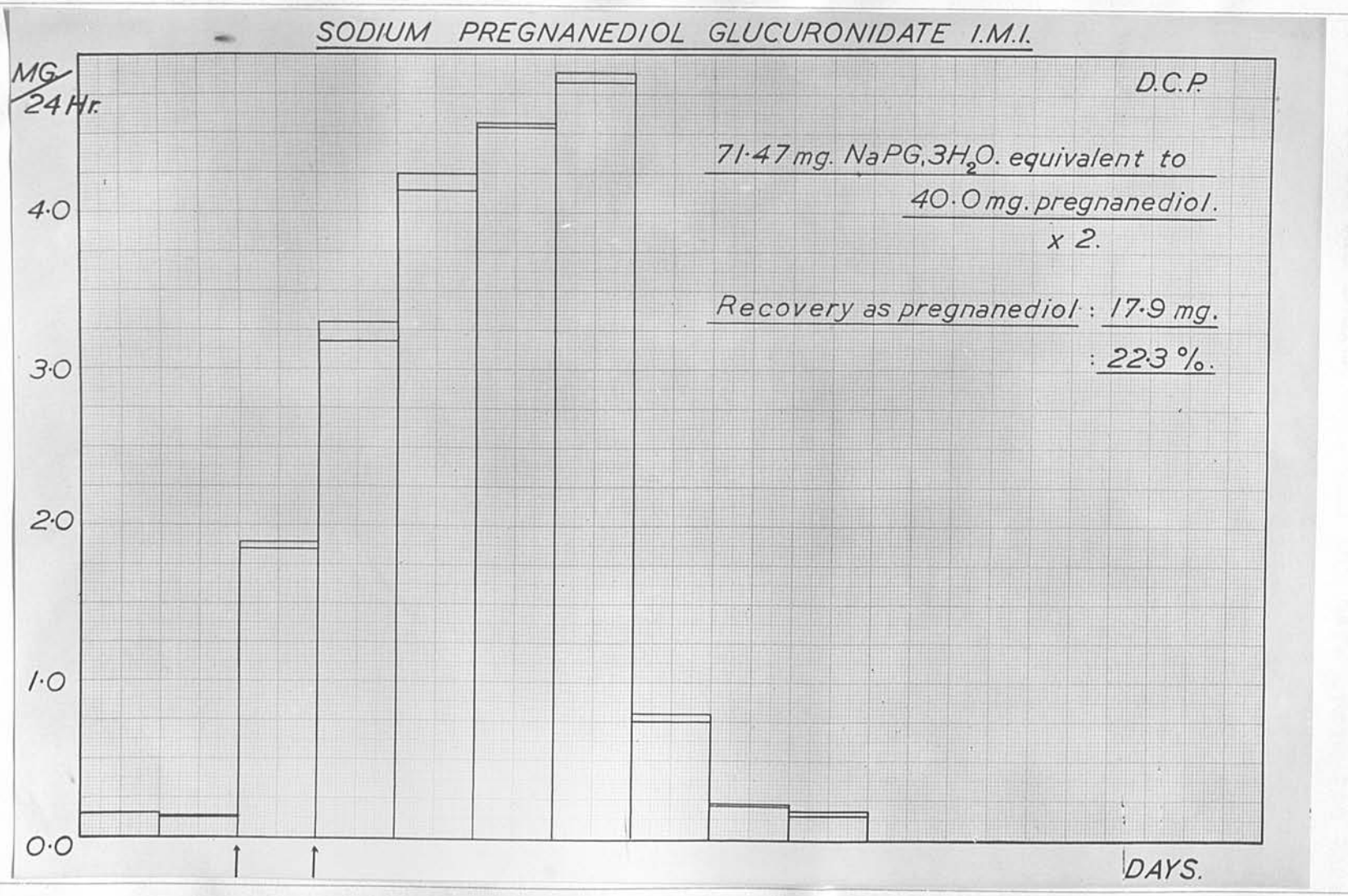

SODIUM PREGNANEDIOL GLUCURONIDATE I.M.I.
MG/24 Hr.
4·0
3·0
2·0
1·0
0·0
D.C.P.
71·47 mg. NaPG, 3H2O. equivalent to
40·0 mg. pregnanediol.
x 2.
Recovery as pregnanediol : 17·9 mg.
: 22·3 %.
DAYS.

FIGURE 6.

the proposed injection to 7.0 ml. the NaPG.$3H_2O$ would not go into solution, whereas it might reasonably have been expected to do so in aqueous solution. (The presence of the sodium chloride may have prevented this by a common ion effect). As it seemed unlikely that further dilution within the range of a reasonable injection dose would ensure solution of the NaPG$3H_2O$ - and this subsequently proved to be the case - it was decided to administer it as a suspension. Accordingly, 7 ml. of this suspension each containing 71.5 mg. of NaPG.$3H_2O$ and equivalent to 40.0 mg. of pregnanediol was injected intramuscularly on two successive days in one of the normal males (D.P.) previously studied.

As has been found in the case of injection of other crystalline suspensions the site of injection became painful and there was a febrile reaction - although the suspension was sterile and no inflammation ensured. The results are given in Table 23.

CONCLUSION: The recovery of pregnanediol from the urine was approximately twice as great as had been found after the administration of progesterone or pregnanediol to this normal male. The figure was not as high, however, as either of those reported by Venning and Browne (1938, 1940). It seems reasonable to suppose that this discrepancy is related to the form in which the glucuronide was injected. Slower absorption and excretion resulted from the administration of the suspension in our case than was found after the admin-

:istration of "NaPg" by Venning and Browne. Destruction during sterilisation is unlikely but if hydrolysis of the slowly absorbed material occurred in the tissues and pregnanediol was liberated, then lower recoveries as pregnanediol in the urine would be explained.

THE EXCRETION of PREGNANEDIOL RESULTING from CONTINUED DAILY ADMINISTRATION of PROGESTERONE.

Data upon the excretion of urinary pregnanediol in the urine of subjects receiving daily administration of progesterone for more than a few days are not presented in the literature. Venning and Browne (1940) administered doses of the order of 10 mg. progesterone daily for four to eight days. The recoveries of pregnanediol glucuronide were low and irregular and no significant trend was observed in the pattern of excretion.

Cope (1940) in a case of secondary amenorrhoea previously discussed (section iii) injected 10 mg. on the first of five days and 5 mg. on the remaining four days. Only doubtful traces of pregnanediol glucuron-:ide were detected during the first five days but 2.5 mg. was determined on the sixth day. In a case of anovular menstruation 10 mg. of progesterone was injected on each of five consecutive days. Pregnanediol glucuronide was not detected in the urine on the first three days but small amounts, adding up to 4.5 mg.

were detected on the fourth and fifth days. Cope offered the suggestion that there may be "a kind of saturation phenomena" under these conditions comparable to that described in the excretion of ascorbid acid taken by mouth.

In the first two cases studied in the present work, progesterone was administered daily to two healthy postmenopausal women aged 65 and 72 respectively. Both women received 60 mg. daily, by intramuscular injection in subject F., and orally by capsules in subject N. After a control period, progesterone was administered daily for 18 days in subject F. and for 22 days in subject N. Oestradiol Benzoate was adminis-:tered (30 mg. daily) on the 11th and 12th days of progesterone administration of the first experiment and on the 15th and 16th days of progesterone administra-:tion in the second. The results are given in Table 24 and compared in Fig. VII.

CONCLUSION: In both cases a plateau of pregnanediol excretion was found corresponding to a conversion of progesterone to pregnanediol of the extent previously observed in other healthy postmenopausal women. This plateau level of excretion was not maintained, however, and after a time-lag of six days in subject F. and ten to twelve days in subject N. the excretion of pregnane-:diol began to rise. During the plateau period subject F. (intra-muscular) was excreting 9 to 10% of the administered dose and this rose to 17% by the 10th

TABLE 24.

CONTINUED I.M. ADMIN. of PROGESTERONE to POST-MENOP. WOMAN (F.)

(contd.)

Admin.	Vol. c.c.	Pregnanediol mg./24 hr.	Admin.	Vol. c.c.	pregnanediol mg./24 hr.
-	1560	0.15 0.14	Prog. 60 mg.	1510	10.93 11.25
	1464	0.20 0.15	"	1700	11.50 11.70
	1665	0.10 0.15	"	1665	9.05 8.88
	1645	0.20 0.13	"	1660	8.13 7.85
Prog. 60 mg. I.M.	1750	0.18 0.20	"	2100	7. 80 8.00
"	1420	1.69 1.65	"	2005	7.65 7.70
"	1960	5.25 5.15	"	1250	5.45 5.75
"	1210	6.05 5.75	"	1530	5.30 5.15
"	1280	5.75 6.20	"	1300	6.25 6.45
"	1530	6.40 6.05	-	1530	6.95 6.90
"	1300	6.50 6.20			
"	1530	6.20 6.55			
"	1580	6.93 7.25			
"	1460	8.38 8.50			
Prog. 60 mg. + OE.B. 30 mg.	1840	10.08 10.38			
" "	1620	10.80 11.05			

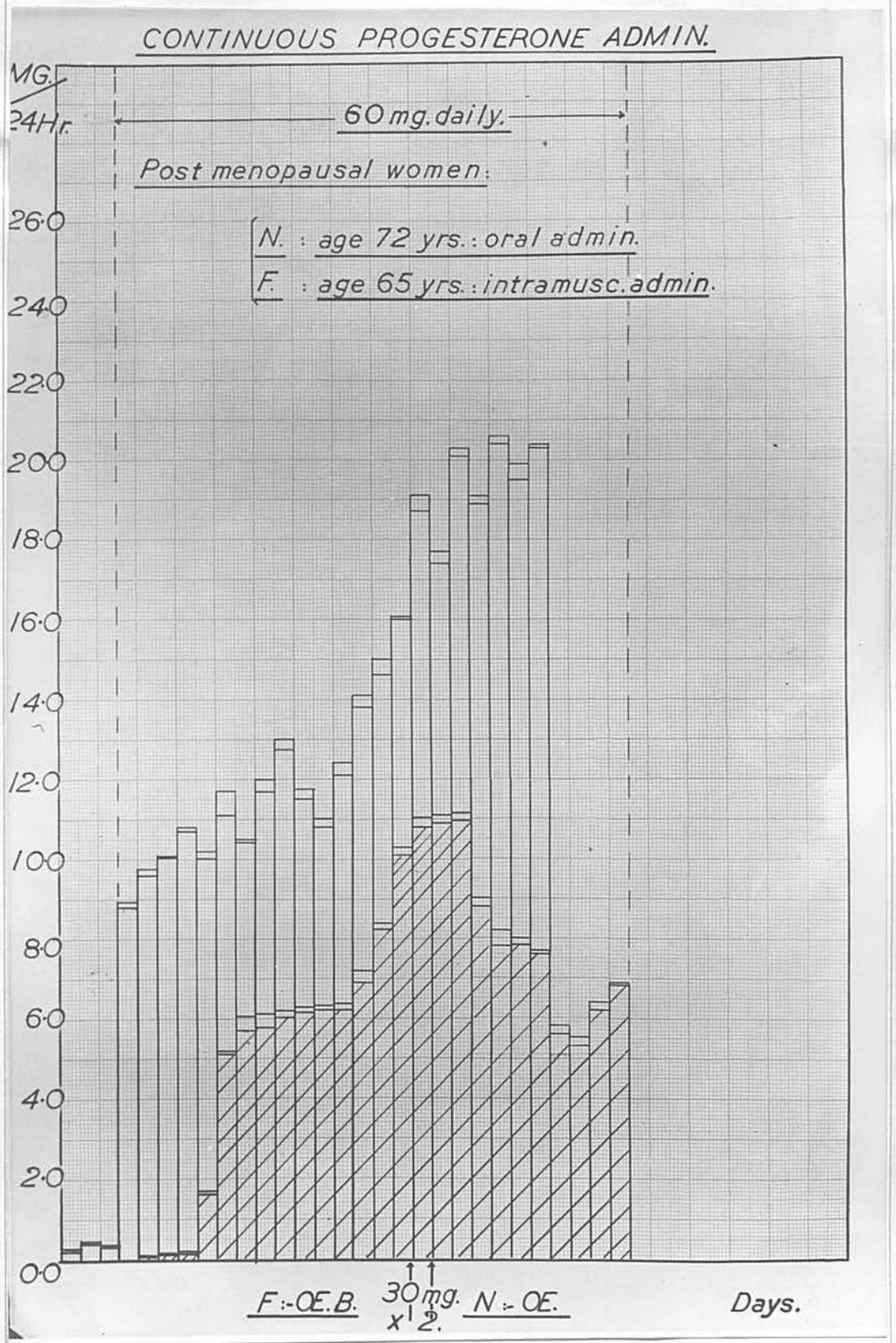
TABLE 24a.

CONTINUED ORAL ADMIN. of PROGESTERONE to POST-MENOPAUSAL WOMAN (N.)

(contd.)

Admiń.	Vol. c.c.	pregnane-diol mg./ 24 hr.	Admin.	Vol. c.c.	pregnanediol mg./24 hr.
-	2600	0.19 0.15	Prog. 60 mg.	1710	12.10 12.40
-	1860	0.23 0.20	"	1315	13.75 14.10
Prog. 60 mg.	1350	0.21 0.21	" plus Oestra-:diol 30 mg.I.M.	1300	15.00 14.65
"	1240	8.80 8.95	" " "	1680	16.10 16.00
"	1650	9.60 9.75	Prog. 60 mg.	1490	18.75 19.10
"	1170	10.50 10.50	"	1240	17.35 17.65
"	1800	10.80 10.75	"	1360	20.40 20.15
"	1630	10.00 10.25	"	1410	19.10 18.85
"	1110	11.05 10.75	"	1300	20.50 20.65
"	1350	10.50 10.40	"	1630	19.90 19.50
"	1610	12.00 11.65	-	1250	20.51 20.60
"	1480	12.75 13.00	-	1280	5.80 5.50
"	1820	11.40 11.75	-	990	1.94 1.91
"	820	11.00 10.80	0	1430	0.15 0.18

FIGURE 7.


CONTINUOUS PROGESTERONE ADMIN.
MG.
24Hr.
60 mg. daily.
Post menopausal women:
N. : age 72 yrs. : oral admin.
F. : age 65 yrs. : intramusc. admin.
26·0
24·0
22·0
20·0
18·0
16·0
14·0
12·0
10·0
8·0
6·0
4·0
2·0
0·0
F :- OE. B.
30 mg.
x 1 2.
N :- OE.
Days.

During the plateau period subject N. (oral) was excret-:ing about 17 to 18% of the administered dose daily and this rose to about 25% by the 15th day. Subject N. was one of the postmenopausal controls and yielded a recovery of 15.98% in series (i). This case therefore parallels closely that of the male (J.P.) who produced 14.7% on intramuscular injection and 17.4 to 19.6% on oral administration.

After the injection of 30 mg. oestradiol benzoate in the first experiment the pregnanediol continued to rise for two or three days although by smaller incre-:ments than in the last days of the progesterone control period. On the second day after the second oestrogen injection the pregnanediol excretion fell sharply and continued to fall until it had reached a level of the same magnitude as the previous "platuea". If this experiment could be confirmed an advance in our understanding of this problem would have been made. Several considerations delayed an immediate attempt to repeat it. In the first place we recognised how fortunate we had been in our subject (F.) who allowed us to inject 6 ml. of oil into the buttock every morning for twenty six days with 12 ml. on the 15th and 16th days. An attempt was therefore made to obtain more concentrated solutions of the two hormones, and in the meantime an attempt was made to repeat the result (in subject N.) by oral administration of progesterone. A second consideration was doubt as

to the desirability of injecting such large doses of steroids into healthy subjects. Oral administration seemed a more innocuous procedure, but this also had its disadvantages in that the experiment broke down completely should the patient vomit or fail to take a capsule. Furthermore, absorption from the alimentary tract might vary with the taking of alcohol or in response to unknown dietetic conditions.

With these considerations in mind the investigation along the lines described above was carried out in subject N. That all the capsules were swallowed was ensured by watching this process in the home, and once more we were very fortunate in receiving so much co-operation. No conclusion can be reached at this stage as to the apparent lack of effect produced by Oestradiol, in this experiment. This was chosen in place of benzoate to facilitate the estimation of oestrogens in the urine and because of its more rapid action. It is recognised however that the biological effect of the pure substance is less potent than that of the benzoate. Whatever the explanation, the pregnanediol excretion rose steadily and continued to rise for several days after the effect of the oestradiol must have passed off, and from the 19th to the 23rd days of the experiment a second plateau of about 34% was present.

If the oestrogen had its biological effect in this experiment and yet did not affect the conversion

of progesterone to pregnanediol then the explanation may lie in the choice of route of administration. Progesterone administered orally is stated to have very little biological effect (Dorfman et al, 1948) and if the effect of oestrogen on the "priming" phenomenon is bound up with this biological effect upon certain tissues then it would not be surprising to find the two different responses exhibited by these two subjects.

Before beginning a systematic investigation of this possible interaction of progesterone and oestrogen, further studies on the "priming" phenomenon, obtained during the continued administration of progesterone, were carried out. These studies are still in progress but the results of an attempt to reproduce this effect in men have just been obtained.

The first male to be investigated (J.M.) aged 30 (Table 25) was given 40 mg. of progesterone daily by mouth for 18 days and determinations carried out daily and in duplicate. This smaller dose was tested in an attempt to reduce the cost of such experiments. Urine collection by this subject was exemplary and the result is striking. The pregnanediol remained at a constant level of ca. 10% throughout this long period. Not only does this indicate that "priming" does not occur in this male - whereas the plateau level can be attained by male or female - but it is strong evidence against the possible view that the "priming" phenomenon results from saturation. A source of fallacy lay

however in the fact that this case received only 40 mg. of progesterone daily, and accordingly, a second male (J.P.) was given 60 mg. of progesterone by mouth daily for 16 days. Throughout many experiments this male had consistently converted a higher proportion of administered progesterone to pregnanediol than had similar subjects. In the present experiment a level of about 19% - as in previous experiments - was produced and this was maintained throughout the sixteen days.

CONCLUSION: When progesterone was administered daily for more than about six days to healthy postmeno-:pausal women, a rapid and progressive rise in the proportion excreted as urinary pregnanediol resulted. There is a suggestion in the results obtained from subject N. that this rise continues until a second plateau of about 30% or more is reached.

This effect was not observed in similar experi-:ments in young men. It seems unlikely that age is the vital factor but this has not been excluded. No direct evidence upon the role of the uterus is afforded by these experiments and a hysterectomised woman will be investigated.

While definite conclusions cannot be drawn from this preliminary work on progesterone "priming" the results will be discussed and some speculation submitted in Section VIII.

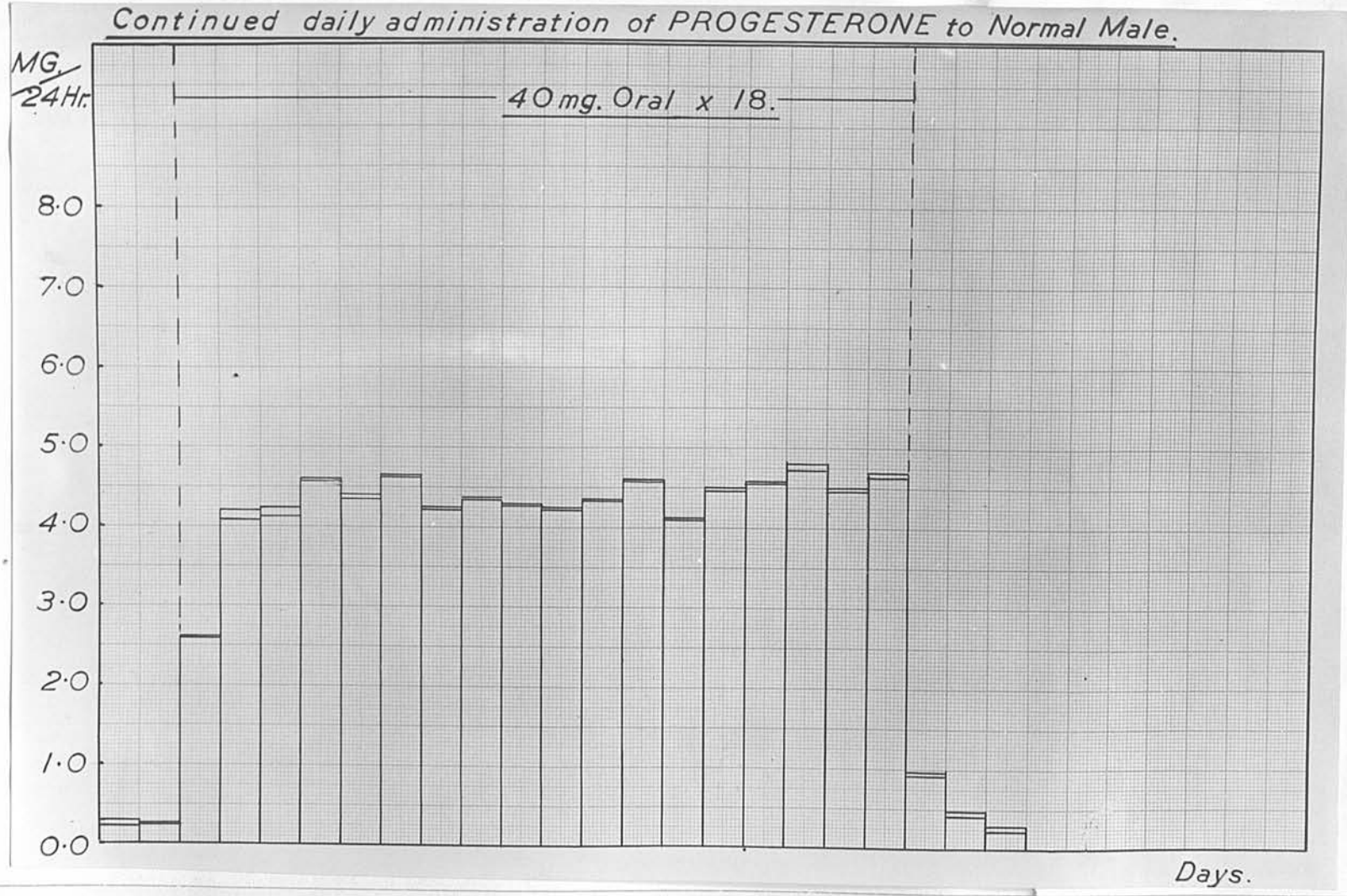

Continued daily administration of PROGESTERONE to Normal Male.
MG./24Hr.
40 mg. Oral x 18.
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
Days.

FIGURE 8.

TABLE 25.

CONTINUED ORAL ADMIN. of PROGESTERONE To MALE (J.M.)

(contd.)

Admin.	Vol. c.c.	Pregnanediol mg./24 hr.	Admin.	Vol. c.c.	Pregnanediol mg./24 hr.
-	1890	0.29 0.21	40 mg. Oral.	1600	4.33 4.35
-	1240	0.22 0.23	"	1900	4.60 4.61
40 mg. Oral	2685	2.60 2.61	"	2350	4.10 4.13
"	3860	4.08 4.20	"	1600	4.45 4.50
"	1520	4.20 4.13	"	2050	4.56 4.57
"	1510	4.60 4.59	"	1200	4.80 4.72
"	1800	4.35 4.40	"	1800	4.46 4.50
"	2110	4.65 4.63	"	1200	4.62 4.67
"	1500	4.25 4.23	-	2540	0.92 0.96
"	1900	4.37 4.35	-	1800	0.49 0.43
"	1250	4.29 4.30	-	2010	0.28 0.24
"	2400	4.25 4.27			

SECTION V.

SECTION V.

THE EFFECT of PROGESTERONE upon the EXCRETION of PREGNANEDIOL DURING NORMAL PREGNANCY.

The earliest observations on this subject were made by Venning and Browne in 1940. These workers injected 10 mg. of progesterone on the 44th, 45th and 46th days of a normal pregnancy and later injected 20 mg. on the 59th and 60th days of the same pregnancy. On both occasions a greater increase in pregnanediol excretion resulted than was found in patients similarly injected in whom endogenous progesterone production was absent or very low. The first increase in excretion represented ca. 38% of the injected progesterone and the second, ca. 50%. This observation was in accord with similar high recoveries obtained by these workers after the injection of progesterone during the luteal phase of the menstrual cycle. Venning and Browne concluded that the recovery of pregnanediol from administered progesterone was greater when "all the normal mechanisms involved in its excretion are operative".

More recently, Davis and Fugo (1947) carried out a similar investigation in three normal pregnancies at about the 12th week. In two cases, 50 mg. was injected on each of four days and in the third case 120 mg. was injected on one day. Pregnanediol determinations were infrequent and were determined by a semi-quantitative method (Davis & Fugo 1947), but

these workers concluded that recoveries of additional pregnanediol were obtained which corresponded to 30 to 35% of the injected progesterone.

The view has been expressed by Guterman (1946) and subsequently by Bender (1948) that the administration of progesterone to pregnancies in which there is no sign of progesterone deficiency, as indicated by a "normal" level of pregnanediol excretion, tends to precipitate abortion rather than to afford additional prophylaxis against it. Influenced by this view, obstetricians were rather reluctant to allow us to investigate the effect of progesterone injection in normal pregnancy. Conversely it seemed undesirable to investigate the effect on abnormal cases. The dilemma was solved by the discovery of a women who was at the 27th week of her 8th normal pregnancy and was found to be suffering from cardiac decompensation and mitral stenosis. It was considered inadvisable to induce therapeutic abortion by hysterotomy at this stage of pregnancy, but had our investigation resulted in termin-:ation of the pregnancy this would have been a satisfac-:tory outcome.

In experiments reported in Section VII very constant day to day excretion has been found in cases of pregnancy with normal levels, and even abnormal levels, of pregnanediol excretion. This is in accord with

the findings of Bachman, Leekley and Hirschmann (1941). With this in mind and with an approximate idea as to the duration of excretion resulting from two consecutive daily injections of 60 mg. progesterone, an attempt was made to carry out the experiment in five phases each lasting five days with progesterone administration during the second and fourth phases. In this way it was hoped that a calculation of the "additional" pregnanediol would be possible. To facilitate this, Creatinine excretion was determined daily by the method of Folin and the daily excretion of pregnanediol corrected to the average creatinine value. As the patient was confined in a busy antenatal ward, 24 hour specimens were not always complete but as shown in Fig. IX, after correction in this way, a remarkably constant and slightly rising level of pregnanediol excretion was obtained.

Results:-

Day of Experiment.	Total pregnanediol recovered.
(1) 1 - 5	,94.20 mg.
(A) 5 - 10	138.20 mg.
10 - 15	98.40 mg.
(B) 15 - 20	158.24 mg.
20 - 25	112.40 mg.

(A) Theoretical endogenous yield during 1st administration ..... 96.30 mg.

Therefore pregnanediol derived from injection ..... 41.90 mg.

= 34.9%

(B) Theoretical endogenous yield during 2nd administration ..... 105.38 mg.

Therefore pregnanediol derived from injection ..... 53.46 mg.

= 44.5%

CONCLUSION: Previous studies on the effect of progesterone upon the excretion of pregnanediol in pregnancy have been confined to early pregnancy. The results in this case constitute a definite confirmation of such studies and extends their application into the second and third trimesters of pregnancy. The tendency in the "priming" experiments to attain a similar extent of conversion of administered progesterone to pregnane-:diol is of the greatest interest and possible implica-:tions of these observations are discussed in Section VIII.

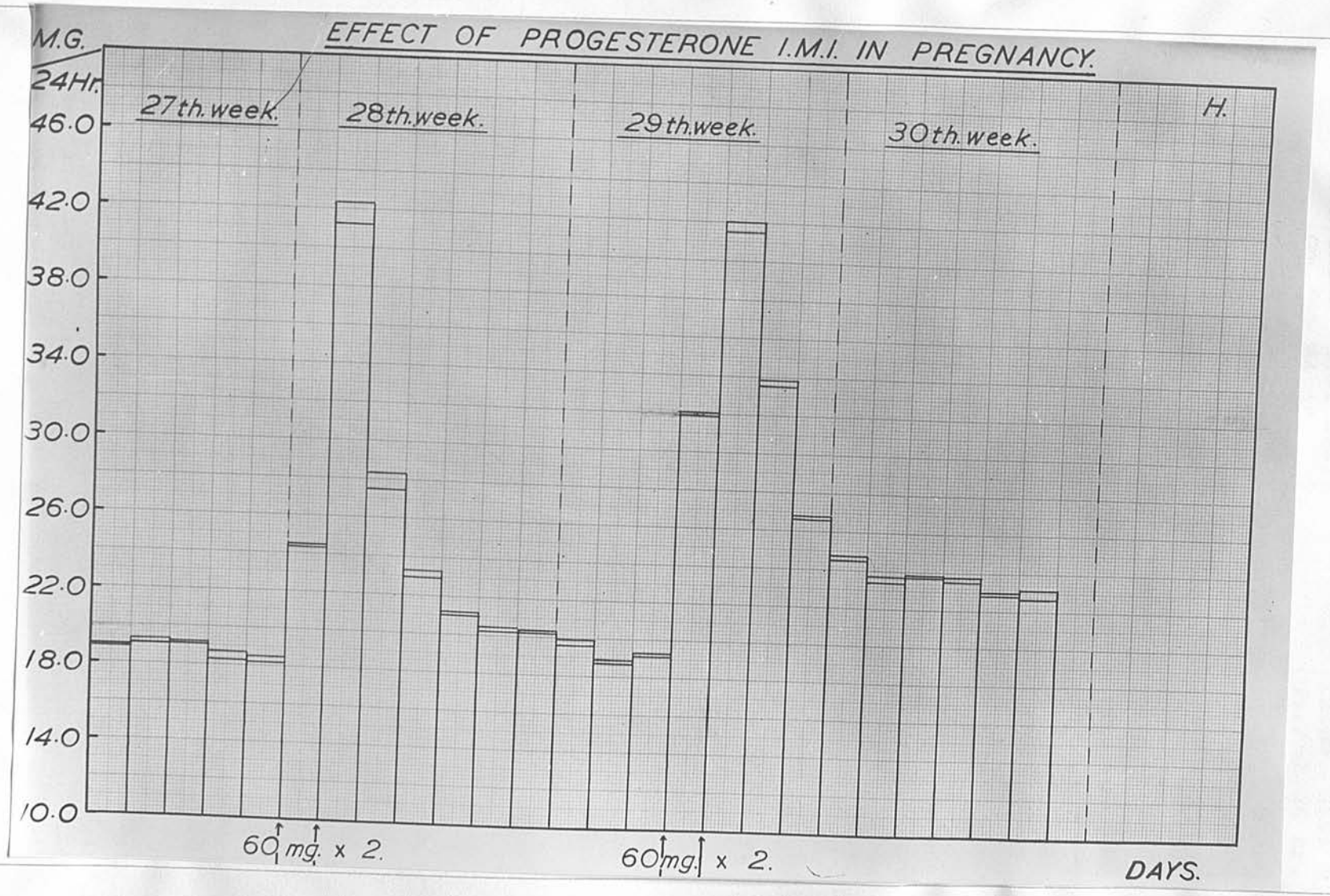

EFFECT OF PROGESTERONE I.M.I. IN PREGNANCY.
M.G.
24Hr.
46.0
42.0
38.0
34.0
30.0
26.0
22.0
18.0
14.0
10.0
27th. week.
28th. week.
29th. week.
30th. week.
H.
60 mg. x 2.
60 mg. x 2.
DAYS.

FIGURE 9.

TABLE 26.

PROGESTERONE ADMIN. in PREGNANCY.

Admin.	Vol. c.c.	Creatinine Gm./24 hr.	Pregnanediol Mg./24 hr. (corr.)	Admin.	Vol. c.c.	Creatinine Gm./24 hr.	Pregnanediol Mg./24 hr. (corr.)
-	610	0.61	18.95 18.80	-	1280	0.68	18.97 18.83
-	730	0.52	19.06 19.36	Prog. 60 mg. I.M.	980	0.47	19.16 19.09
-	900	0.50	19.22 19.28	" "	860	0.68	32.09 31.90
-	790	0.62	18.36 18.62	-	1060	0.63	41.50 42.00
Prog. 60 mg. I.M.	970	0.57	18.26 18.48	-	1500	0.67	33.48 33.85
" "	1160	0.69	24.34 24.25	0 -	2480	0.63	26.75 26.85
-	990	0.55	41.04 42.41	-	2150	0.64	24.50 24.75
-	1130	0.67	27.55 28.32	-	2050	0.63	23.75 23.40
-	1180	0.58	22.95 23.31	-	2750	0.66	23.75 23.81
-	1400	0.68	21.16 21.06	-	2450	0.65	23.57 23.76
-	880	0.68	20.23 20.41	-	2100	0.63	22.85 23.00
-	1480	-	20.35 20.20	-	2360	0.65	23.04 22.80
-	825	0.45	19.95 19.60				

SECTION VI.

SECTION VI.

THE EXCRETION of PREGNANEDIOL FOLLOWING EXCISION of the CORPUS LUTEUM of PREGNANCY.

Many cases of excision of the corpus luteum of pregnancy have been reported. In 1926, Ask-Upmark reported a series of fifty-one cases of excision of the corpus luteum during the first eight weeks of pregnancy in whom abortion followed in only seventeen cases. Pratt (1927), Douglass (1931) and Corbet (1932) published similar accounts of the continuation of pregnancy after this excision in pregnancies not more advanced than the sixth week. On the other hand DeLee (1916) reported two cases followed by abortion and Wilson (1937) reported abortion following excision of the C.L. in five cases ranging from the fifth to the twelfth week of pregnancy. As pointed out by Jones and Weil (1938) the fact that abortion may or may not follow abdominal operation makes it difficult to assess the importance of removal of the corpus luteum in such cases.

While these findings are contrary to conclusions reached by Corner (1928) Robson (1937) and others who have studied the maintenance of pregnancy in animals, species variation must not be overlooked.

Considerable indirect evidence exists which indicates an extra-ovarian source of progesterone in pregnancy. (Newton 1935). Such evidence was provided

by the experiments of Haterius (1936) in which it was shown that pregnancy could be maintained in the ovar-:iectomised rat provided that all but one of the foetuses were removed while all the placentae were left in utero. Whereas the presence of the ovary and corpus luteum are essential for the maintenance of pregnancy in the rabbit, this is less true of higher species and the implication is that this extra-ovarian source, which is probably the placenta, has acquired wider functions in the course of evolution. (Robson, 1947).

Attempts to detect progesterone in placental tissue or to detect the production of progesterone by placental cells grown in vitro (Jones, Gey and Gey, 1943) (Stewart, Sano and Montgomery, 1948) have been unsuccessful.

Strong evidence in favour of extra-ovarian proges-:terone production in pregnancy would be provided by the determination of pregnanediol in the urine of ovariect-:omised pregnant women. Data are published concerning the determination of pregnanediol glucuronide by the method of Venning in two cases following excision of the corpus luteum of pregnancy.

Jones and Weil (1938) report the removal of the corpus luteum at the eighth week of pregnancy. Abortion did not occur and a normal living child was delivered at term. Pregnanediol determinations were detected for about twelve days subsequently but it then

reappeared in the urine in increasing amounts. It seems probable that during the time when no pregnanediol glucuronide was detected, small amounts were in fact present in the urine but that these were not great enough to be detected by the Venning method.

Venning and Browne (1937) reported a similar case in which the pregnanediol fell to a low level and then rose again to normal limits for the remainder of the pregnancy. (Details are not published).

Cope (1940) reported a rather different case in that pregnanediol disappeared from the urine within five days of resection of the corpus luteum at the ninth week of pregnancy. Abortion followed several days later.

The case investigated in the present work was a pregnancy at the ninth week. An ovarian cyst was present on the left side and torsion of its pedicle occurred with congestion of the left ovary and the C.L. of pregnancy. Intense pain resulted and the clinical picture resembled that of imminent rupture of a tubal pregnancy. The whole of the left ovary and corpus luteum were removed. Pregnanediol determinations were carried out daily until abortion occurred nine days after the operation. The results are shown in Table 26 and Fig X. Complete 24 hour urine specimens were not obtained in the first three days after operation, and the pregnanediol recovery on these days was corrected for the average daily

excretion of creatinine which was 1.15 mg./24 hr.

CONCLUSION: For six days after the excision of the corpus luteum at the tenth week of pregnancy, a small but constant amount of pregnanediol was excreted in the urine. The pattern of excretion and the length of time during which this level was maintained exclude the possibility that this might represent clearance from the body of pregnanediol formed from progesterone produced by the corpus luteum. A fall in pregnanediol excretion occurred coincident with commencing detachment of the uterine contents and pregnanediol disappeared from the urine after their expulsion. The probability is that the pregnanediol determined was formed from progesterone produced by the still immature placenta.

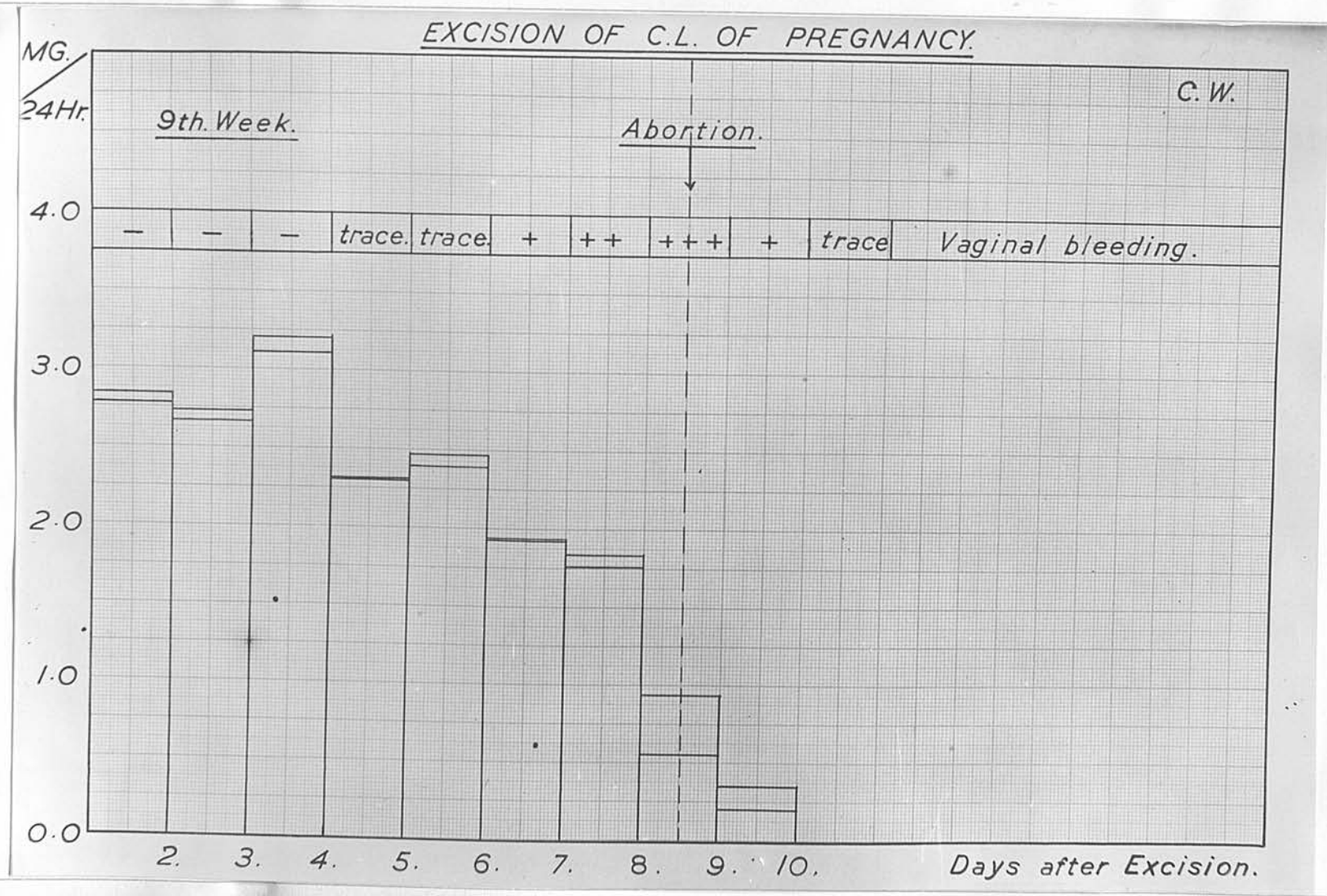
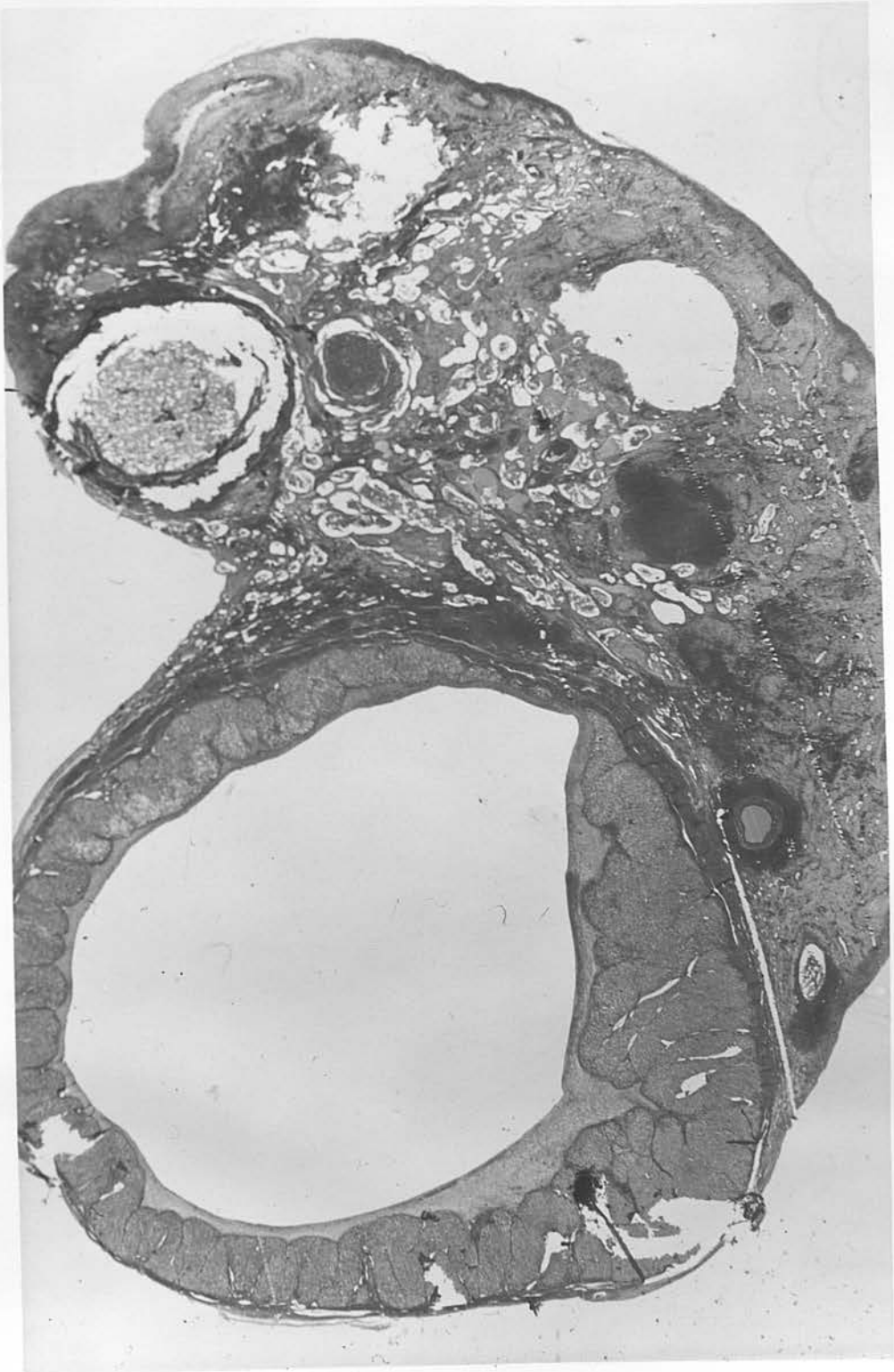

EXCISION OF C.L. OF PREGNANCY.
MG./24Hr.
C.W.
9th. Week.
Abortion.
4.0
3.0
2.0
1.0
0.0
— — — trace. trace. + ++ +++ + trace Vaginal bleeding.
2. 3. 4. 5. 6. 7. 8. 9. 10.
Days after Excision.

FIGURE 10.

SUBJECT "W".



x7.

Table 27.

RESECTION of C.L. of PREGNANCY (C.W.)

Day Post-Op.	Vol. c.c.	Creatinine mg./24 hr.	Pregnanediol mg./24 hr.	Corr.
2	890	0.82	2.05 1.96	2.87 2.77
3.	640	0.97	2.25 2.30	2.66 2.73
4.	760	0.72	2.00 1.95	3.20 3.11
5	1840	1.23	2.49 2.46	2.31 2.30
6	2750	1.10	2.36 2.29	2.47 2.39
7	2100	1.13	1.89 1.90	1.92 1.93
8	1190	1.16	1.85 1.78	1.83 1.76
9	435	-	0.55 0.93	-
10	1395	-	0.35 0.20	-

SECTION VII.

SECTION VII.

DIETHYLSTILBOESTROL ADMINISTRATION during PREGNANCY.

Effect upon the urinary excretion of pregnanediol in normal and diabetic Pregnancy.

In a series of papers published over the period 1931-1948, O.W. Smith and G. van S. Smith have developed a theory concerning the interdependence of oestrogen and progesterone metabolism. These workers believe that oestradiol and oestrone are partly converted in the body into oestrogenically inactive oxidation products and that this conversion is favoured when there is progesterone deficiency. They further believe that these oxidation products stimulate the secretion of progesterone and other "sex-steroids". (Smith and Smith, 1941). They suggest that this effect is mediated in the non-pregnant woman by an increased secretion of pituitary gonadotrophin and in pregnancy by "increased utilization of chorionic gonadotrophin". (1948).

In the belief that premature senility of the placenta with premature withdrawal of its hormonal secretion is a vital factor in the pathogenesis of such accidents of pregnancy as pre-eclampsia, these workers have found a therapeutic application for their earlier work in an attempt to modify this premature "syncytial-steroid" aberration in human pregnancy. Thus Smith, Smith and Hurwitz (1944) suggested that it might be possible to forestall an incipient deficiency of progesterone and oestrogen in pregnancy by administering

sufficient oestrogen to supply an increased amount of stimulating oestrogen break-down products.

The finding by Smith and Smith (1944) that diethylstilboestrol is one hundred times more active than oestrone in stimulating the pituitary of the rat, suggested to them that the synthetic oestrogen might also be effective in stimulating a defective secretion of placental "sex-steroids" in pregnancy.

To investigate this possibility, Smith, Smith and Hurwitz (1946) studied the urinary excretion of pregnanediol and endogenous oestrogen in a pregnant diabetic woman with a history of pre-eclampsia in her two previous pregnancies. Diethylstilboestrol was administered daily from the 17th to the 35th week, with intermissions at the 19th and 25th weeks. The dose administered daily rose from 30 mg. at the 17th week to 125 mg. at the 32nd week. During this time the excretion of urinary pregnanediol was determined on twenty occasions by the method of Venning (1937,1938).

The results appeared to be conclusive; during the periods when diethylstilboestrol was administered the pregnanediol excretion rose steadily and dropped each time it was withdrawn. It is noteworthy that the pregnanediol level during periods of diethylstilboestrol administration was considerably higher than previously reported normal values (Venning 1938, Smith and Smith 1941, and Hain 1942).

Smith, Smith and Hurwitz (1946) suggest that the rise in pregnanediol excretion which follows the

administration of diethystilboestrol is a result of stimulation of progesterone secretion.

More recently, Davis and Fugo (1947) using a method of pregnanediol determination based on the procedures of Astwood and Jones (1941), Talbot et al (1941) and Guterman (1944, 1945) have studied the effect of large doses of diethylstilboestrol - from 50 mg. to 200 mg. daily - on pregnanediol excretion during early pregnancy. These workers claim that this treatment had no effect on the pregnanediol excretion.

The observations ofDavis and Fugo (1947) were of especial interest since urinary pregnanediol was being determined after acid hydrolysis and not as its glucuronide. The data published by these workers is not, however, entirely convincing. The levels of pregnanediol excretion as determined by their method (1947) were more irregular than those observed by us in normal or abnormal pregnancy, and as control periods prior to diethylstilboestrol administration are not included in their publication, interpretation of the effect upon pregnanediol excretion is rather difficult. Smith and Smith (1948) criticising this work draw attention to the fact that the dosage employed was greatly in excess of their own recommendations and suggest that such large doses early in pregnancy may inhibit progesterone secretion by depressing the utilisation of chorionic gonadotrophin.

In the present work the effect of diethylstilboes-:trol upon the urinary excretion of pregnanediol has

been studied in three diabetic pregnant women and one normal pregnancy. Pregnanediol was determined by the method described in Section II. All determinations were carried out daily and in duplicate upon aliquot portions of 24 hour urine samples.

The completeness of all but a few of these specimens was indicated by a constant daily excretion of urinary creatinine as determined by the method of Folin (1914). The value of creatinine determinations upon the urine of women receiving sex hormone therapy has been discussed by O.W. Smith (1942).

The dose of diethylstilboestrol the week of pregnancy during which it was administered, and the effect upon the excretion of pregnanediol are given in Figs. XI - XV and Tables 29 - 31.

The normal pregnancy studied - subject D - was a primipara aged 30. It was interesting to note the high degree of tolerance to diethylstilboestrol which is found in pregnancy. No symptoms resulted from the administration of 50 mg. daily at the 14th week of pregnancy. Administration was repeatedat the 22nd and 24th weeks of pregnancy. The pregnancy continued without mishap until the 40th week when a healthy male child was delivered.

Subject H. was also a primipara aged 30. Diabetes Mellitus had been present since the age of five and had been controlled by insulin during the inter-:vening years.

A diagnosis of diabetes mellitus was made during

the previous pregnancy of subject P. That pregnancy - which occurred two years prior to the one under investigation was terminated by the delivery of a large stillborn infant. Intrauterine death was suspected four days antepartum. Althouth diabetes was only recognised and insulin therapy commenced during the first pregnancy, there was a definite history of thirst, polyuria, boils, and pruritus, in preceding years.

In subject B, diabetes mellitus was recognised and its control by insulin begun eleven years previous-:ly. This patient's first pregnancy occurred two years previously, and was terminated by an intrauterine death five days antepartum. Her second pregnancy - one year later - was terminated by a miscarriage at the nineteenth week.

CONCLUSION: The effect upon the excretion of urinary pregnanediol was unequivocal and was reproduced in all four cases studied. Administration of diethylstilboestrol resulted in a sharp fall in pregnanediol excretion. After withdrawal, the pregnanediol rose steadily or after a short delay (as in subject H.) This effect was produced by doses of diethylstilboestrol which were either greater than, less than, or similar to those advocated by Smith and Smith. (A scheme of dosage was received by private communication with these workers).

These results cannot be said to disprove those of Smith, Smith and Hurwitz (1946) since the conditions of the experiments could not be identical but they appear to indicate that a fall in urinary pregnanediol is a common sequel to the administration of diethylstil-:boestrol in pregnancy and although some spontaneous elevation of the pregnanediol may occur during continued administration, this does not appear to constitute a rise even to normal levels.

Possible explanations of the discrepancy between these findings and those of Smith, Smith and Hurwitz are discussed in Section VIII.

FIGURE II.

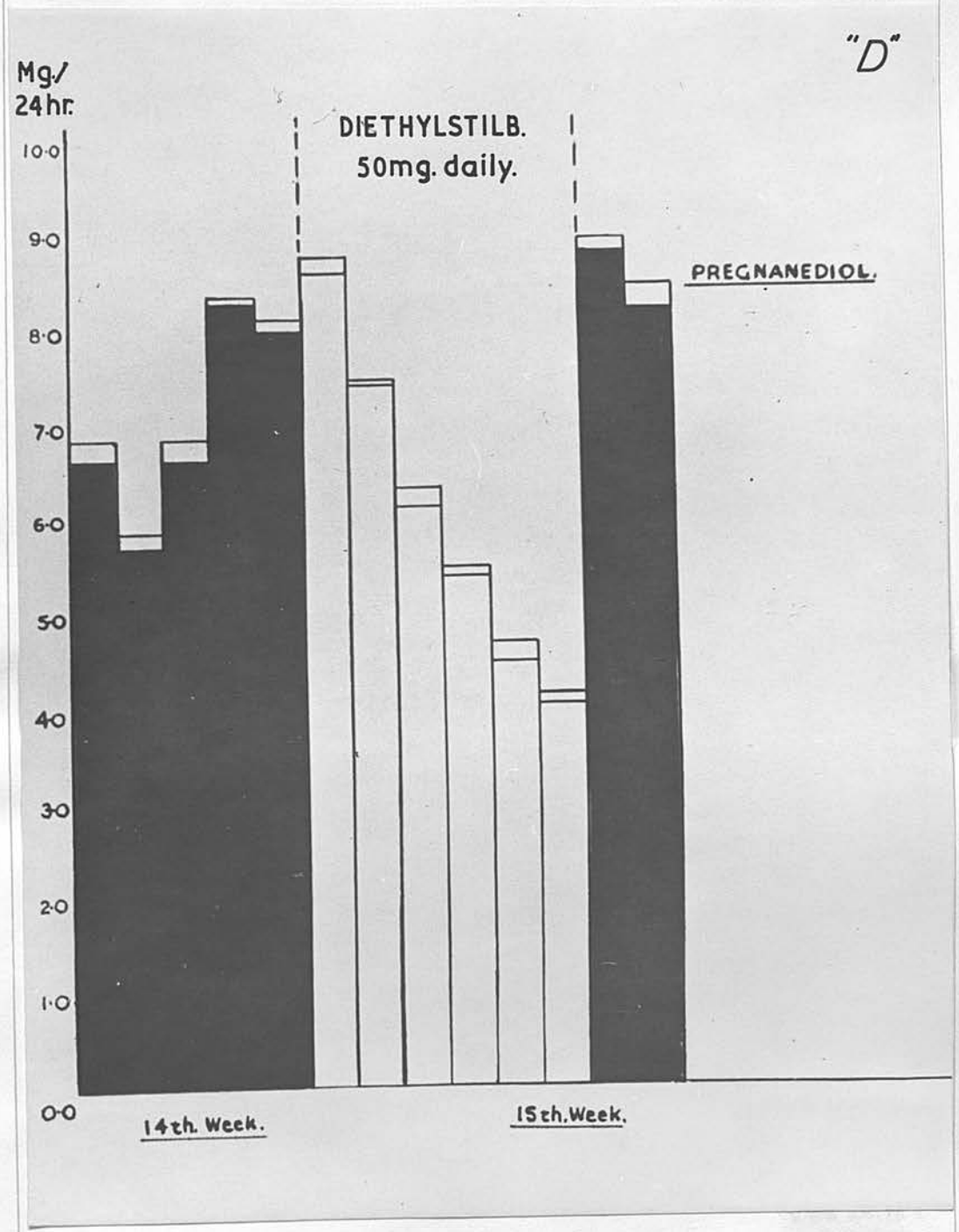
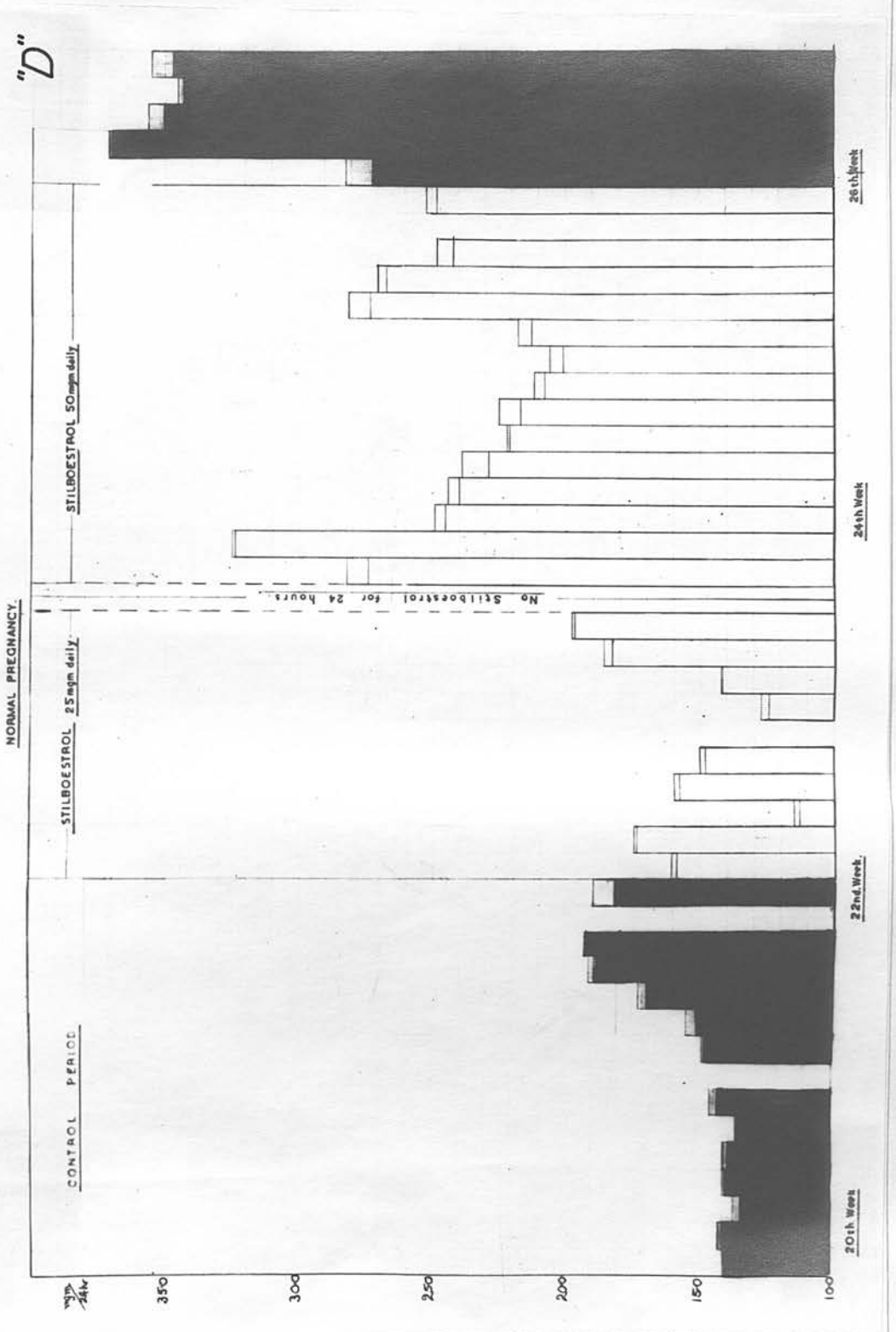

"D"
Mg./
24hr.
DIETHYLSTILB.
50mg. daily.
PREGNANEDIOL.
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
14th. Week.
15th. Week.

FIGURE 12.


"D"
NORMAL PREGNANCY.
CONTROL PERIOD
STILBOESTROL 25mgm daily
No Stilboestrol for 24 hours
STILBOESTROL 50mgm daily
20th Week
22nd Week.
24th Week
26th Week
100
150
200
250
300
350

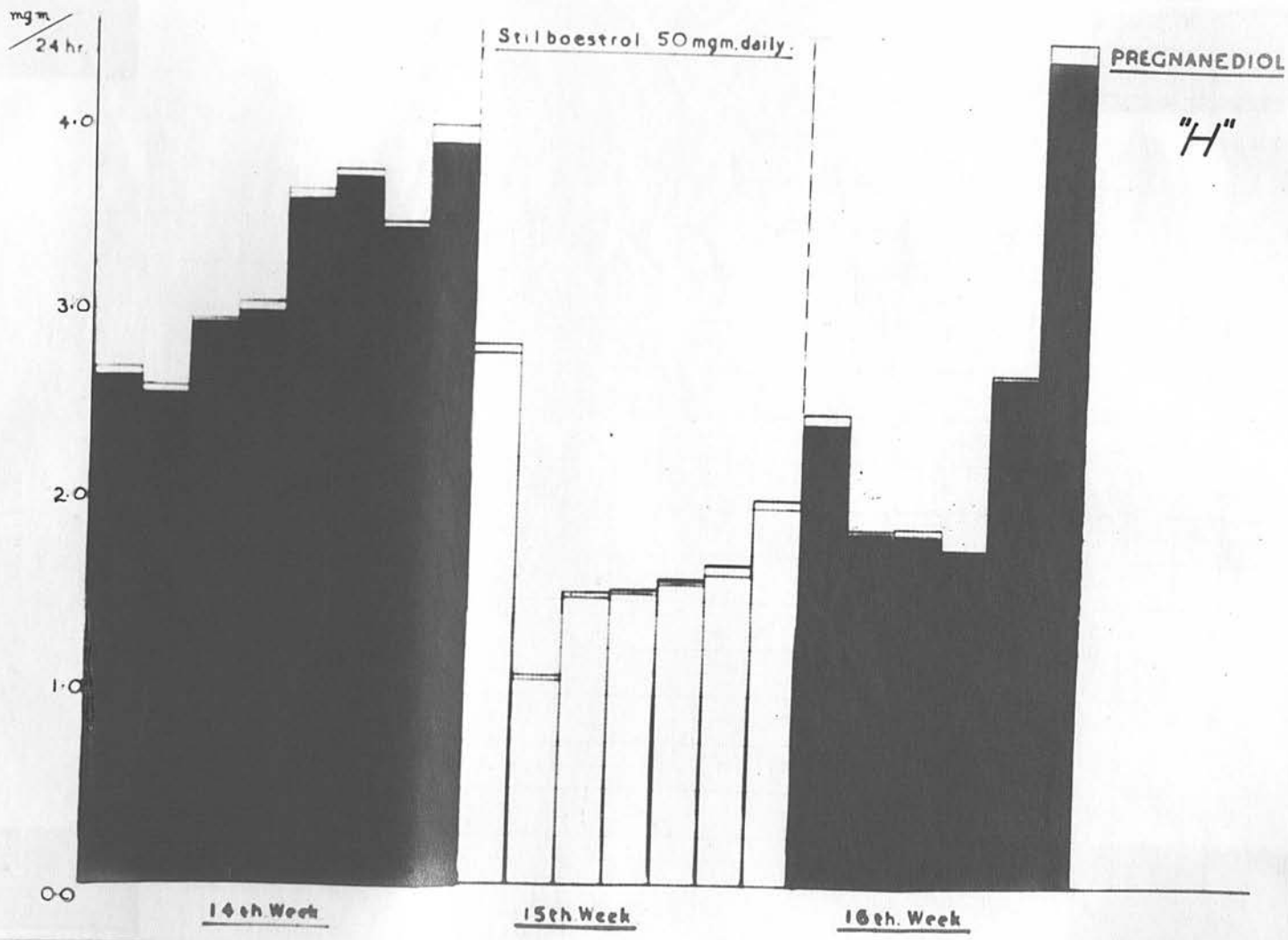

mgm
24 hr.
4·0
3·0
2·0
1·0
0·0
Stilboestrol 50 mgm. daily.
PREGNANEDIOL
"H"
14th Week
15th Week
16th. Week

FIGURE 13.

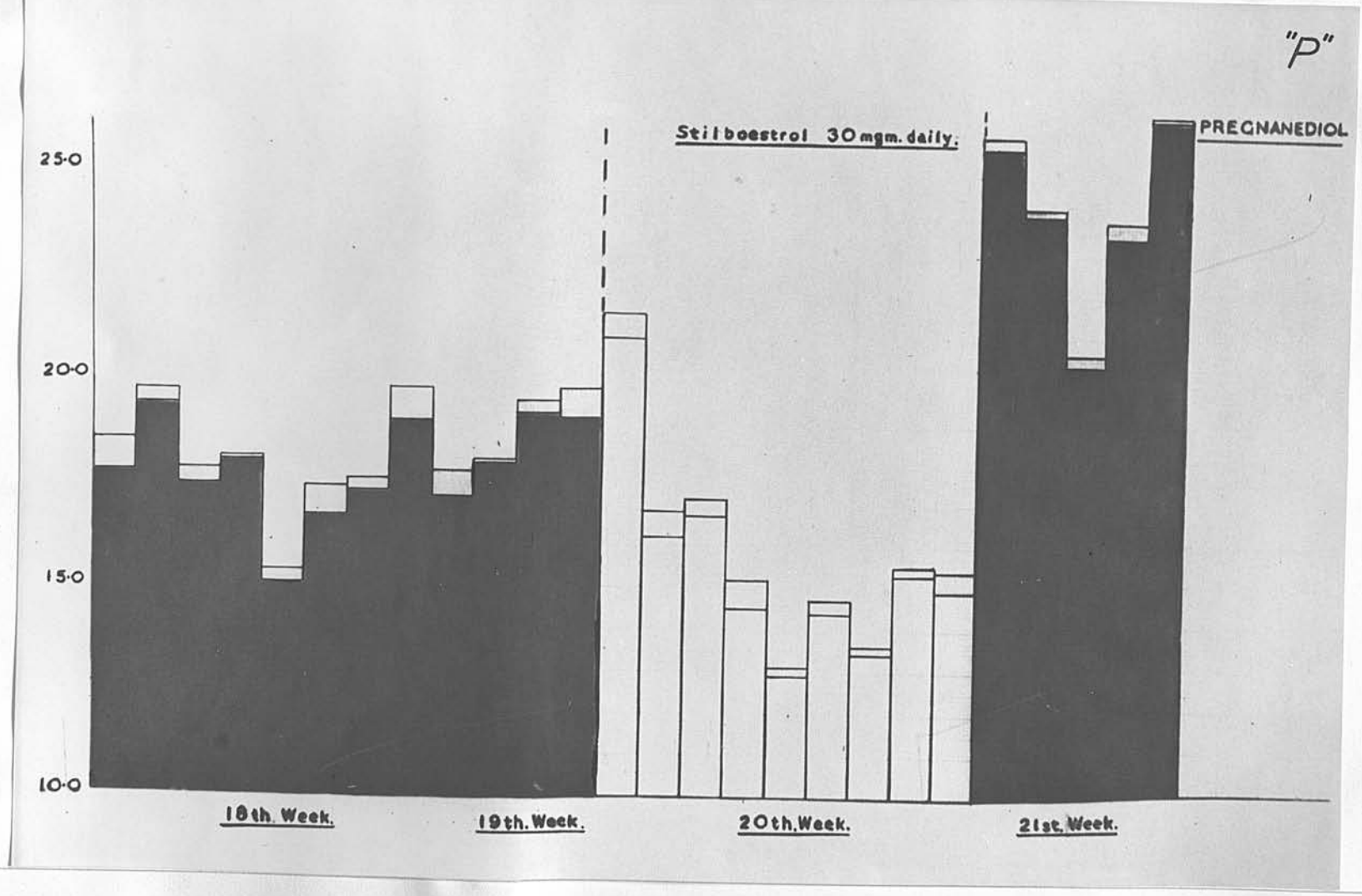

"P"
Stilboestrol 30 mgm. daily.
PREGNANEDIOL
25·0
20·0
15·0
10·0
18th. Week.
19th. Week.
20th. Week.
21st. Week.

FIGURE 14.

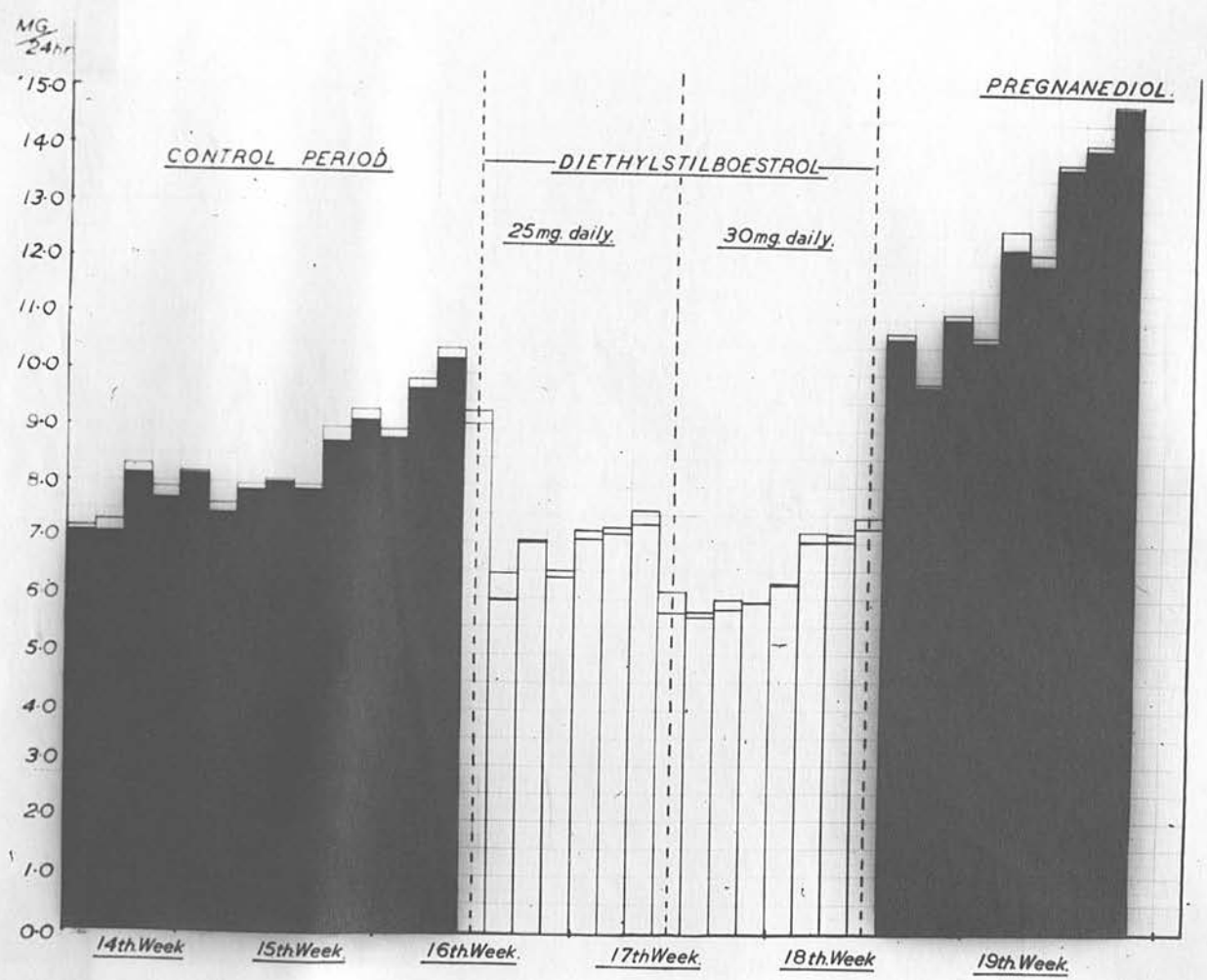

"B"
MG. 24hr
15·0
14·0
13·0
12·0
11·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
CONTROL PERIOD
DIETHYLSTILBOESTROL
25mg. daily.
30mg. daily.
PREGNANEDIOL.
14th Week
15th Week
16th Week.
17th Week.
18th Week
19th Week.

FIGURE 15.

TABLE 28.

DIETHYLSTILB : NORMAL PREGNANCY (20th - 26th Week)(D.)

Admin.	Vol. c.c.	Creat-inine Gm.	Preg. mg./24 hr.
-	2225	-	14.0 13.9
-	1325	-	14.2 14.0
-	1670	-	13.6 13.3
-	1855	-	13.9 14.0
-	1840	-	13.8 14.0
-	1795	-	13.5 13.5
-	1055	-	14.2 14.5
NO COLLECTION.			
-	1625	-	14.7 14.8
-	1510	-	14.8 15.4
			Preg. corr. for av. Creat.
-	1100	0.91	16.9 17.2
-	1200	0.98	18.9 19.1
-	1265	1.05	18.0 19.2
NO COLLECTION.			
Stilb. 25 mg.	1600	1.13	19.0 18.2
"	1880	1.30	16.1 15.9
"	1500	0.96	17.4 17.5
"	1995	1.26	11.5 11.3
"	1818	1.11	16.0 15.8
Stilb. 25 mg.	2460	1.30	15.0 14.8
NO COLLECTION.			
"	1880	1.61	12.4 12.7
"	1930	1.42	14.2 14.2
"	1300	0.97	18.3 18.6
"	1010	1.09	19.7 19.8
NO COLLECTION.			
Stilb. 50 mg.	1840	1.33	28.2 27.4
"	1810	0.97	32.5 32.4
"	1500	1.39	24.9 24.5
"	2060	1.33	24.0 24.0
"	1650	1.31	23.9 22.9
"	1725	1.34	22.1 22.2
"	1400	1.27	22.5 21.7
"	1360	1.39	20.8 21.2
"	1900	1.44	20.6 20.1
"	1800	1.29	21.8 21.3
"	1400	1.01	28.1 27.3
"	1075	0.83	27.0 26.7
"	1920	1.20	24.8 24.2
NO COLLECTION			

.....over/

TABLE 28 (contd.)

Admin.	Vol. c.c.	Creatinine. Gm.	Pregnanediol mg./24 hr.
-	1520	1.24	25.2 24.9
-	2080	1.25	27.2 28.2
-	1075	0.85	37.0 37.1
-	1600	1.03	35.6 35.0
-	1005	1.18	34.5 34.3
-	1800	1.20	35.5 34.7

TABLE 29.

DIETHYLSTILB: PREGNANT DIABETIC (H.)

Admin.	Vol. c.c.	Pregnanediol mg./24 hr.	Admin.	Vol. c.c.	Pregnanediol mg./24 hr.
-	2000	2.65 2.69	Stilb. 50 mg.	2310	1.56 1.53
-	1870	2.56 2.60	"	2505	1.61 1.59
-	2470	2.93 2.96	"	1995	1.68 1.63
-	2160	3.05 2.99	-	1810	2.00 2.04
-	2370	3.65 3.60	-	2560	2.44 2.51
-	2040	3.76 3.72	-	2340	1.89 -
-	1420	3.48 3.45	-	1320	1.90 1.87
Stilb. 50 mg.	2650	3.90 4.00	-	1200	1.78 1.78
"	2200	2.80 2.85	-	1740	2.71 2.73
"	1980	1.08 1.10	-	1520	4.50 4.40
"	2385	1.53 1.51			

TABLE 30.

DIETHYLSTILB. : DIABETIC PREGNANCY (P).

Admin.	Vol.	Creat-inine Gm.	Pregnane-diol Mg./ 24 hr. Corr.	Admin.	Vol.	Creat-inine Gm.	Pregnane-diol Mg./ 24 hr. Corr.
-	1550	-	18.4 17.3	"	2120	1.11	16.2 16.8
-	1370	0.94	19.6 19.2	"	1760	1.15	16.7 17.1
-	1290	1.21	17.7 17.3	"	1740	0.96	15.2 14.5
-	1040	0.82	18.0 17.9	"	1870	1.07	12.9 13.1
-	1980	1.14	15.3 14.7	"	1820	1.14	14.4 14.7
-	1600	1.16	16.6 17.3	"	1760	1.13	13.6 13.4
-	1280	1.03	17.5 17.2	"	1920	1.14	15.3 15.5
-	1340	1.04	18.9 19.7	-	1900	1.12	15.4 14.9
-	1760	1.23	17.7 17.1	-	1760	1.11	25.8 25.5
-	1900	1.15	17.9 18.0	-	1400	0.82	23.9 24.1
-	1920	1.11	19.4 19.1	-	750	0.95	20.3 20.6
Stilb. 30 mg.	1590	1.17	19.7 18.8	-	1720	1.11	23.8 23.4
"	1820	1.19	20.9 21.5	-	1800	1.10	26.4 26.2

TABLE 31.

DIETHYLSTILB.: PREGNANT DIABETIC (B.)

Admin.	Vol. c.c.	Preg. mg./ 24 hr.	Admin.	Vol. c.c.	Preg. mg./ 24 hr.	Admin.	Vol. c.c.	Preg. mg./ 24 hr.
	1360	5.50 5.55		2080	8.75 9.00	Stilb. 30 mg.	2040	5.70 5.75
	2020	7.50 7.58		1850	9.13 9.33	"	2700	6.53 6.26
	2095	6.75 6.90		2480	8.81 8.98	"	2565	6.00 6.00
	2600	6.40 6.15		2500	9.70 9.88	"	2300	6.28 6.30
	1700	7.18 7.00	Stilb. 25 mg.	2580	10.25 10.43	"	1940	7.23 7.08
	2205	7.08 7.30	"	1800	9.10 9.33	"	2465	7.08 7.20
	2765	7.55 7.38	"	3620	5.99 5.45	-	2780	7.45 7.25
	2225	7.90 7.70	"	1950	7.03 7.00	-	2645	10.69 10.81
	2290	8.13 8.18	"	2785	7.22 7.07	-	2480	9.85 9.90
	2450	7.43 7.63	"	2630	7.28 7.08	-	2656	11.04 11.15
	2600	7.75 7.65	"	2730	6.49 6.68	-	2625	10.65 10.74
	2180	8.00 8.05	Stilb. 30 mg.	1980	7.58 7.28	-	2665	12.64 12.26
	2565	7.95 7.88	"	2790	6.17 5.73	-	2750	12.02 12.19

SECTION VIII.

SECTION VIII.

INTERPRETATION.

Certain assumptions are made when a quantitative or even a qualitative significance is attached to the amount of pregnanediol excreted in the urine of human subjects.

The first assumption concerns the identity of the substance estimated. It can by no means be assumed that this is pregnanediol - whether that term be limited to pure pregnane-3(α),20α-diol or be widened to include a mixture of its stereoisomers. No routine method exists for the determination or pregnane-3(α),20α-diol separated from allopregnane-3(α),20α-diol and, in the discussion which follows, the term "pregnanediol" is applied to the group of pregnanediol compounds deter-:mined by the method described in Section II.

No method can be recommended for the determination of pregnanediol in urine containing very large amounts of neutral 17-ketosteroids or of other neutral steroids of adrenal origin. These were not present in the urines investigated in the present work nor would they be likely to cause serious interference in determina-:tions in the menstrual cycle or in pregnancy. For routine determinations in pregnancy, for diagnostic or prognostic pruposes, a less specific method such as that described in Section III may serve a useful purpose.

Reference has already been made to methods for the determination of pregnanediol as its glucuronide

and to their comparative lack of specificity. It was almost inevitable that the application of such methods to clinical material would result sooner or later in fallacious interpretation. Thus the fact that a high degree of specificity is not a feature of the Venning method affords one of several possible explanations for the discrepancy between the findings of Smith and Smith and those reported in the present work, upon the effect of diethylstilboestrol administration on the excretion of pregnanediol in pregnancy. Diethylstil-:boestrol monoglucuronide has been isolated from human urine by Williams (1948), and it cannot be assumed that sodium diethylstilboestrol glucuronidate did not contaminate the final product of the Venning method in the investigation reported by Smith, Smith and Hurwitz (1946). The fact that this final precipitate contains sodium pregnanolone glucuronidate (Marrian and Gough, 1946) as well as pure sodium pregnanediol glucuronidate (Sutherland and Marrian, 1946) constitutes another source of error in inter-:pretation. The possibility may be considered that under certain circumstances, progesterone metabolism may be influenced in such a way that the relative proportion of these two glucuronides in the final precipitate is significantly altered. Thus diethyl-:stilboestrol might conceivably lower the excretion of pregnanediol glucuronide and raise that of pregnano-:lone glucuronide to such an extent that a high result

be obtained by the Venning method and a low result by the more specific method.

Similarly, the apparent fall in the excretion of pregnanediol which occurred on injection of oestradiol benzoate after "priming" with progesterone (case F. Section IV) might be accounted for by diversion of the metabolic pathway of progesterone to metabolites other than pregnanediol. It would be interesting if such a change occurs under physiological conditions. Deter-:minations are being carried out on the excretion of pregnanediol in normal pregnancy. As none of the pregnancies studied has as yet come to full term these results are not reported here, but already there is a suggestion that they are significantly lower than those obtained by the Venning method and reported by Venning (1938), Smith and Smith (1941), Bachman, Leekley and Hirschmann (1941) and Hain (1942). If this prelimin-:ary observation is confirmed, contamination of the final product of the Venning method by ketonic sodium glucuronidates would afford a partial explanation. The possibility should perhaps be considered, however, that alterations in the ratio of non-ketonic and ketonic glucuronides may be physiological phenomenon in pregnancy. Thus one might speculate that during pregnancy, and possibly under the influence of endogenous oestrogen, there is a progressive shift in the metabol-:ism of progesterone from non-ketonic to ketonic metabolites. Investigation of such a hypothesis awaits the elabora-:tion of a method for the determination of sodium

pregnanolone glucuronidate in human urine.

A second assumption is concerned with the sources of progesterone or its metabolites in the experimental subject. The possibility that extra-ovarian or even unsuspected sources of these compounds may be present in the body cannot be overlooked.

Pregnanediol has been isolated from the urine of men by Westphal (1944) and of women with hyperplasia of the adrenal cortex by Venning, Weil and Browne (1937). Furthermore, Cuyler, Ashley and Hamblen (1940) and Horwitt, Dorfman, Shipley and Fish (1944) observed the appearance of pregnanediol in the urine after the administration of desoxycorticosterone to human subjects. Desoxycorticosterone may be produced and, of course, closely similar substances are produced normally by the adrenal cortex.

In the control periods of the investigations reported in the present work the apparent excretion of pregnanediol rarely exceeded 0.5 mg. per 24 hour specimen. Furthermore, it is probable that a proportion of this "blank" reading was attributable to substances other than pregnanediol. Traces of neutral 17-ketosteroids and other chromogenic steroids and traces of urinary pigments which had survived the purification procedure would act as chromogens in the sulphuric acid reaction. It was unlikely, therefore, that significant amounts of progesterone were produced by the ovaries or adrenals of the post-menopausal women studied, in view of the control period "blank"

values which were observed before and after each investigation.

It may be mentioned in parenthesis that the work of Corner (1945) and Greulich (1946) indicates that the theca interna cells as well as the granulosa cells not only contribute to corpus luteum formation, but may exhibit luteinisation and progesterone formation prior to ovulation.

Thus while it may be said that sources of progesterone formation other than the corpus luteum caused negligible interference in these quantitative studies, it is conceivable that in certain circum-:stances such sources may be stimulated.

The production of progesterone by the maturing placenta was implied by the maintenance of excretion of pregnanediol which occurred after resection of the corpus luteum of pregnancy, and the rapid disappearance of pregnanediol from the urine after detachment and expulsion of the uterine contents (Section VI.)

A third assumption in the interpretation of urinary pregnanediol excretion has arisen from the tentative suggestion by Venning and Browne (1938, 1940) that a parallelism exists between progesterone production and pregnanediol excretion. Their pioneer work demonstra-:ted a striking qualitative relationship and established pregnanediol as an essential metabolite of progesterone but it could not be assumed that a simple quantitative relationship existed between progesterone production and pregnanediol excretion. This has been made,

however, by other workers. Thus Smith, Smith and Hurwitz (1946) concluded that the rise in pregnanediol excretion which they observed following diethylstil-:boestrol administration was due to an increase in endogenous progesterone production, and the therapeutic indication for the administration of this drug in pregnancy was based upon that assumption.

The experiments in the present work which were concerned with the conversion of administered progester-:one to urinary pregnanediol (Section IV), suggested, at first, that a proportion of the administered progesterone, approximately constant for a given individual, was excreted as pregnanediol in the urine. In men, in post-menopausal women whether normal, hysterectomised, or pretreated with oestrogen, this fraction remained within fairly narrow limits. If it could be assumed that exogenous progesterone is metabol-:ised in the body in a similar manner to endogenous progesterone - and direct evidence is not available on this important point - then it seemed possible that by a simple calculation, the approximate endogenous pro-:duction could be interpreted from the excretion of pregnanediol of endogenous origin. This may, in fact, be justifiable where the body is under the influence of progesterone for short periods only, but the results of experiments on the continued daily administration of progesterone which are reported later in Section IV, and the result of progesterone administration on the

excretion of pregnanediol in normal pregnancy (Section V) throw a different light upon this relationship.

It appears that under the continuous influence of progesterone there results a progressive change in the proportion of administered progesterone excreted as pregnanediol. Furthermore, when progesterone is administered to an individual where endogenous progesterone production is occurring, a much higher proportion of the administered progesterone is excreted as pregnanediol (Section V). The results of the latter type of experiment when carried out in the menstrual cycle (Venning and Browne, 1940) as well as these obtained in pregnancy, are not directly opposed to the existence of a simple quantitative relationship since progesterone production is beyond the control of the observer and since exogenous progesterone may undergo a more complete reduction to pregnanediol in the presence of excess endogenous progesterone. On the other hand, it is of especial interest to find a progressive increase in pregnanediol excretion resulting from the conversion of progesterone from a constant source in subjects in whom endogenous production is absent or minimal. The ultimate extent of conversion which would result from very prolonged administration of progesterone cannot be deduced from these preliminary experiments since the excretion of pregnanediol was rising rapidly at the time of administration of oestrogen, but it may be that a progressive change of this type

represents the physiological relationship between progesterone production and pregnanediol excretion when endogenous progesterone production is prolonged as in pregnancy and to a less extent in the menstrual cycle. The effect of oestrogen upon this phenomenon was therefore of especial interest. The administration of oestradiol benzoate appeared to abolish the "priming" effect which resulted from intra-muscular administration of progesterone, but the administration of œstradiol had less effect on the "priming" which resulted from the administration of progesterone by mouth. The latter investigation must be neglected for the present purpose as it contains too many variables for interpre-:tation, but the former implies a limitation of this spontaneous progesterone "priming" phrnomenon by oestrogenic influences. If this is confirmed then it might find a place in a speculative translation of these findings to endogenous metabolism. It may be that the extent of conversion of endogenous progesterone in pregnancy is limited in accord with the production of endogenous oestrogen or of its metabolic products.

The possible influence of the uterus upon the metabolism of progesterone has been the subject of considerable controversy. The results of previous workers have been reviewed in Section IV. The results of Jones and Telinde (1941) and of the present work show that the post-menopausal uterus does not play an important part in the conversion of administered

progesterone to urinary pregnanediol but do not exclude the possibility that a greater extent of conversion may occur in the presence of the physiologically active uterus. On the other hand pretreatment with oestrogen, resulting in two cases in the development of prolifer-:ative endometria, also did not affect the percentage conversion by post-menopausal women. Approaching the problem from a different angle, Davis and Fugo (1948) have investigated the menstrual cycle of twelve young women in whom total hysterectomy has been performed. Ovulation was indicated by a typical rise in the basal temperature and although pregnanediol determinations were infrequent these workers state that the "luteal phase" was of normal duration with pregnanediol excretion ranging from 5.0 to 17.0 mg. per 24 hour specimen.

With these considerations in mind, it was perhaps surprising to find that the progesterone "priming" phenomenon was not observed in men following continued progesterone administration by mouth. This requires further investigation, however, since many factors other than the absence of the uterus might be respons-:ible, and the "priming" phenomenon following proges-:terone administration by mouth to the post-menopausal woman (N) must be confirmed.

Conflicting reports are found in the literature concerning the possible function of the liver in the metabolism of progesterone. Kochakian et al (1944)

implanted pellets of progesterone in various sites in the rabbit and concluded that the liver was the chief site of inactivation. Selye (1941) and Selye and Stone (1944) observed that the anaesthetic action of progesterone administered orally to the rat is greatly enhanced by partial hepatectomy. This anaesthetic effect was apparent in one of our cases - the post-menopausal woman receiving 60 mg. of progesterone orally daily for three weeks (subject N, Section IV), who became progressively more drowsy from the tenth day of the investigation. That the term "progesterone anaesthesia" is justifiable was indicated by the studies on the brain potentials of rats by Hartman et al (1947).

In 1945 Masson & Hoffman compared the effect upon the rabbit endometrium of progesterone administered subcutaneously or by gavage. These workers found that 400 to 800 times the effective subcutaneous dose was required to produce progestational changes following oral administration and furthermore that the same effect could be obtained with one-eightieth of this intragastric dose following partial hepatectomy. Masson & Hoffman concluded that progesterone adminis-:tered by this route is absorbed and inactivated by the liver before reaching the endometrium. A compari-:son of the excretion of progesterone by oral or intramuscular route had not been reported and the finding, in the present work, of a similar fraction

of pregnanediol after administration by either route supported some of these observations on animals, although it was not possible to study biological effects in our (male) subjects. In view of the reproducib-:ility of the effect, it seems unlikely that poor absorption from the gut explains the ineffectiveness of orally administered progesterone but it is just conceivable that reduction of the progesterone occurred in the gut and that it was absorbed as pregnanediol. A satisfactory method for the determination of minute amounts of progesterone would be of the greatest value in this connection.

The similar outcome following the oral administra-:tion of pregnanediol suggests that the factors limiting the proportion of administered progesterone excreted as pregnanediol operate after the conversion of a large proportion of the administered progesterone to pregnane-:diol, which then undergoes further metabolic change. These observations, therefore, tend to attract attention to possible metabolites of progesterone of which pregnanediol may be an intermediary. One such theoretical possibility is the pregnanetriol isolated from urine of women with the adreno-genital syndrome by Butler & Marrian (1937, 1938). Such findings also stimulate investigations upon the excretion of pregnanediol by routes other than urinary. Allopregnanediol has been isolated from ox-bile by Pearlman (1946) and pregnanediol has been isolated from human bile after oral administra-:tion of Δ^5-pregnen-3(β)-ol-20-one, but studies upon

possible excretion by this route of pregnanediol derived from progesterone have been delayed by technical difficulties.

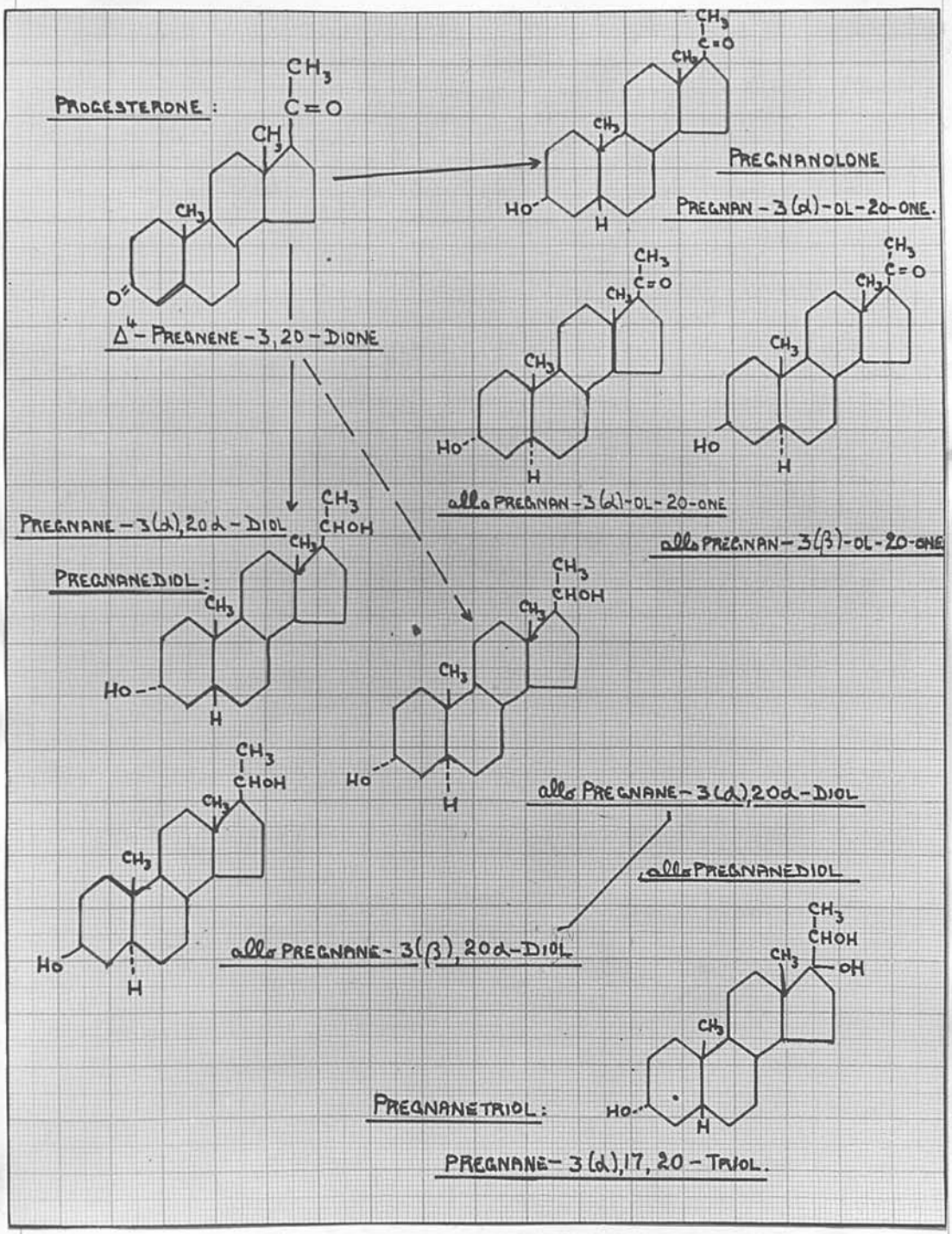
Furthermore, there is the implication that the important factors determining the extent of conversion of progesterone may be involved less with its metabolic reduction than with the conjugation of pregnanediol with glucuronic acid. It is not difficult to visualise a role, hydrolytic, synthetic, or dual, for the enzyme glucuronidase in this process. The evidence in the present work, obtained from the investigations of abnormal cases - recently hysterect-:omised and hypertensive, and the demonstration of the progesterone "priming" phenomenon, clearly indicate that markedly different levels of excretion of urinary pregnanediol may follow the administration of the same dose of progesterone. Thus factors influencing enzyme activity in the liver and in other tissues, may be as important as these influencing hormone production in determining the amount of urinary pregnanediol derived from endogenous and exogenous progesterone.

With this in mind, and while extending these investigations upon clinical material, priority will be given in future work to an attempt to s tudy the biochemical processes involved in the conjugation of pregnanediol with special reference to enzyme activity.

If progress is made in such studies, it may be possible to commence interpretation of the vast amount

of data which is accumulating concerning the excretion of urinary pregnanediol by human subjects in health and disease.

FIGURE 16.


PROGESTERONE :
Δ⁴ - PREGNENE - 3, 20 - DIONE
PREGNANOLONE
PREGNAN - 3 (α) - OL - 20 - ONE.
allo PREGNAN - 3 (α) - OL - 20 - ONE
allo PREGNAN - 3 (β) - OL - 20 - ONE
PREGNANE - 3 (α), 20 α - DIOL
PREGNANEDIOL :
allo PREGNANE - 3 (α), 20 α - DIOL
allo PREGNANEDIOL
allo PREGNANE - 3 (β), 20 α - DIOL
PREGNANETRIOL :
PREGNANE - 3 (α), 17, 20 - TRIOL.

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THE QUANTITATIVE DETERMINATION OF SMALL AMOUNTS OF PREGNANEDIOL IN HUMAN URINE

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Various methods have been described for the quantitative determination of pregnanediol in human urine. Of these the original procedure of Venning [1937, 1938], in which sodium pregnanediol glucuronidate is extracted from the urine and weighed after purification, is still perhaps the most widely employed. This method, although somewhat time-consuming and laborious, gives satisfactory results when applied to urines containing more than *c.* 10–15 mg. of pregnanediol per 24 hr., but suffers from certain serious disadvantages where urines containing smaller amounts of the steroid are concerned. Thus, as pointed out by Astwood & Jones [1941], 'when only small amounts of material are present in the urine, the identity of the final product is sometimes questionable'. Furthermore, it may be necessary to use a full 24 hr. or even a 48 hr. specimen of urine for a single determination in order to obtain sufficient of the glucuronidate to weigh accurately.

Other methods of determining pregnanediol as its glucuronide have been described by Allen & Viergiver [1941], Jayle, Crépy & Wolf [1943], and Bisset, Brooksbank & Haslewood [1947]. These methods would seem to be somewhat less laborious and considerably more sensitive than the Venning method, but they are open to criticism on the grounds of lack of specificity. A further disadvantage of all methods in which pregnanediol is determined as its glucuronide arises from the fact that, unless very special precautions are taken, hydrolysis of the latter by bacterial action may occur during the collection of the urine and subsequently.

A method of a different kind was developed by Astwood & Jones in 1941. In this method the urine is boiled with acid to hydrolyse the glucuronide and the free pregnanediol thus liberated extracted with toluene, purified and weighed. The originality of this method lies in the procedure used for the purification of the pregnanediol in the crude toluene extract. It was shown that from the toluene-soluble fraction, after removal of acidic substances by treatment with sodium hydroxide, nearly pure pregnanediol in almost quantitative yield can be obtained by precipitation from ethanolic solution with four volumes of water or dilute sodium hydroxide solution. The sensitivity of this method was considerably increased subsequently by Talbot, Berman, MacLachlan & Wolfe [1941], who estimated the purified pregnanediol colorimetrically by means of the yellow colour which it yields with concentrated sulphuric acid. Both groups of workers reported reasonably satisfactory recoveries of about 70 % in short series of experiments in which sodium pregnanediol glucuronidate was added to men's urine in varying amounts,* but the evidence that either procedure

* It seems possible that the recoveries obtained by these authors may actually have been somewhat higher than they themselves reported, since Marrian & Gough [1946] have shown that sodium pregnanediol glucuronidate prepared and purified in the usual way [Venning & Browne, 1936] contains only about 80 % of that compound.

would be dependable when applied to urines containing less than *c.* 10 mg. of pregnanediol per 24 hr. was not entirely satisfactory.

A somewhat simplified version of the Astwood & Jones method as modified by Talbot *et al.* has recently been developed by Guterman [1944, 1945] for the detection of pregnanediol in urine as a rapid means of diagnosing pregnancy.

What will for convenience be called the 'Astwood-Talbot' method has several advantages over other methods of determining urinary pregnanediol. The colour reaction which pregnanediol gives with concentrated sulphuric acid is extremely sensitive, thus permitting the accurate determination of small amounts of the substance; and although it is not a specific reaction for pregnanediol it was shown by Astwood & Jones and by Talbot *et al.* that many of the other urinary substances which give it are largely eliminated in the ethanol-water precipitation process of purification. Since the pregnanediol is determined in the free state, elaborate precautions to avoid bacterial hydrolysis of the glucuronide in the urine are unnecessary.

The objective of the work described in this paper was to develop an accurate, specific, convenient and rapid method for the determination of the pregnanediol (1–10 mg./24 hr.) in the urine of women during the luteal phase of the menstrual cycle. Furthermore, in order to permit duplicate determinations of pregnanediol and of other urinary constituents to be carried out it was necessary that the method developed should require not more than one-fifth or one-quarter of a 24 hr. specimen of urine.

This objective has been nearly, but not quite, attained. A procedure based on the Astwood-Talbot method has been elaborated which permits of the reasonably accurate determination of more than *c.* 0·4 mg. of pregnanediol in one-fifth of a 24 hr. urine specimen. Six determinations can be completed in two 8 hr. working days, and the method is such that it could be satisfactorily employed by a trained and competent laboratory technician.

APPARATUS AND MATERIALS

Quickfit & Quartz glassware, with interchangeable standard glass joints, was used throughout in order to avoid contamination of the urinary extracts with coloured or chromogenic material that might be dissolved out of rubber or cork stoppers.

The toluene used for the extractions was 'sulphur-free' and was distilled before use. Ethanol was purified by refluxing over sodium hydroxide and distilling twice.

Pregnane-3(α), 20α-diol was prepared from human pregnancy urine and purified *via* its diacetate. The sample used in the recovery experiments melted at 236–237° (corr.).

Sodium pregnane-3(α), 20α-diol glucuronidate was prepared from human pregnancy urine by the method of Venning & Browne [1936] and freed from ketonic glucuronides by the method of Sutherland & Marrian [1946, 1947]. The preparation used in the recovery experiments melted at 282–283° (corr.) with decomposition and evolution of gas. Samples of the glucuronidate were weighed out after exposure to moist air. As shown by Sutherland & Marrian [1947] material so treated is the *trihydrate* having the composition $C_{27}H_{43}O_8Na.3H_2O$.

For certain of the recovery experiments the toluene-soluble neutral fraction of acid-hydrolysed men's urine was required. This was prepared in the following way: 24 hr.

urine specimens from a number of normal men were pooled and heated to boiling, together with some toluene, acidified with one-tenth of its volume of concentrated hydrochloric acid, and the boiling continued for 10 min. After cooling the mixture was extracted three times with one-fifth volumes of toluene, emulsions being broken by filtration through a Buchner funnel with gentle suction. The combined toluene extracts were washed twice with one-sixth volumes of N sodium hydroxide, three times with one-sixth volumes of water, evaporated to a small volume on a hot plate, and finally taken to dryness under reduced pressure in the water-bath. The brown gummy material so obtained will be referred to as 'male urine neutral fraction'.

MODIFICATIONS MADE IN THE PROCEDURES OF ASTWOOD AND TALBOT

In consequence of a very large number of preliminary experiments, the details of all of which are not reported here, a number of modifications have been made in the original procedures as described by Astwood & Jones [1941] and by Talbot *et al.* [1941]. These modifications were designed to minimize the number of transferences from vessel to vessel during the procedure and to facilitate the rigid standardization of technique which experience has shown is essential if accurate and reproducible results are to be obtained with urines containing less than *c.* 10 mg. of pregnanediol per 24 hr. urine volume.

Removal of acidic substances

Astwood & Jones and Talbot *et al.* removed acidic and phenolic material from the toluene extract of the hydrolysed urine by boiling with methanolic sodium hydroxide and then filtering off the resulting precipitate of sodium salts. In the experience of the present authors it is equally effective and much simpler to remove acidic and phenolic material by washing the toluene extract in a separating funnel with aqueous sodium hydroxide.

Purification of pregnanediol from the neutral fraction by precipitation from ethanol

The accuracy and specificity of the Astwood-Talbot method are very largely dependent upon the efficiency of the precipitation process in separating pure pregnanediol quantitatively from the neutral toluene-soluble fraction of the hydrolysed urine. Accordingly, considerable attention has been paid in the present work to investigating the optimal conditions for carrying out the precipitation and for collecting the precipitated pregnanediol.

Collection of the precipitated pregnanediol. In the Astwood & Talbot procedures and also in the method of Guterman [1944] the precipitated pregnanediol was collected by filtration. To facilitate the quantitative collection of the small amounts of precipitate and in order to avoid as far as possible transference of the material from vessel to vessel, the precipitation process in the present work has been carried out in centrifuge tubes and the precipitate collected by centrifugation. At first some difficulty was experienced in getting the relatively light pregnanediol crystals to pack sufficiently tightly in the tubes to permit the siphoning-off of the supernatant solution. This difficulty was, however, overcome by adding to the precipitation mixture before centrifugation a small amount of the filter-aid 'Hyflo-Super Cel', which effectively entrained the precipitated pregnanediol.

Number of precipitations and volume of precipitation mixture. Astwood & Jones [1941] purified the pregnanediol from the neutral fraction by one precipitation with four volumes of N/10 sodium hydroxide followed by one with four volumes of water. Talbot *et al.* [1941] and Guterman [1944] used a single precipitation with four volumes of N/10 sodium hydroxide. In the experience of the present authors, however, one precipitation with sodium hydroxide and two with water are required in order to obtain the pregnanediol in a satisfactory state of purity. This multiple precipitation technique lengthens the process somewhat, but the loss of time is more than compensated for by the resulting increase in specificity which results.

Numerous preliminary experiments showed that the most satisfactory results were obtained when the neutral fraction from one-fifth of a 24 hr. specimen of urine was dissolved in 4 ml. of ethanol and precipitated with 16 ml. of N/10 sodium hydroxide or water.

Rate of cooling after precipitation in hot solution. The efficiency of the precipitation process has been studied in many series of recovery experiments in which varying amounts of pure pregnane-3(α), 20α-diol were added to quantities of 'male urine neutral fraction' each corresponding to one-fifth of a 24 hr. urine specimen. In the early experiments of this kind it was found that satisfactory recoveries (80% or better) were obtained when more than about 4 mg. of pregnanediol were present (corresponding to 20 mg./24 hr.), but that with smaller amounts of pregnanediol the recoveries were very much lower and very irregular.

The great irregularity of the recoveries suggested that some important variable factor in the purification process was not being properly controlled. Since the rate of cooling of the mixture after precipitation in hot solution might be expected to determine the size of the pregnanediol crystals it seemed possible that this might be the uncontrolled factor.

This possibility was investigated in two series of recovery experiments in which varying amounts of pure pregnane-3(α), 20α-diol were added to portions of 'male urine neutral fraction', each of which was equivalent to one-fifth of a single 24 hr. specimen. The pregnanediol in these mixtures was purified by the triple precipitation procedure, which was as described below (p. 252) with the exception that the cooling conditions following each precipitation were varied. In one series of experiments the precipitation mixtures were cooled rapidly by immediate immersion in an ice-bath and were then left in the refrigerator overnight before centrifugation. In a second series, which were duplicates of the first, the mixtures were cooled slowly by transferring them in beakers of water at 75° to an incubator at 37° where they were allowed to remain for 2 hr. They were then cooled in the refrigerator for 30 min. and centrifuged.

The results*, which are shown in Table 1, are quite conclusive. It will be seen that rapid cooling of the precipitation mixtures gave lower and much more irregular recoveries of pregnanediol than were obtained by the slow-cooling technique. It will also be seen that the difference in recovery due to the rate of cooling was most marked with amounts of pregnanediol corresponding to less than *c.* 20–25 mg./24 hr.

Later experiments, which are not reported here, have shown that the recoveries by the slow-cooling technique are unaffected by the omission of the short period of refrigeration before centrifugation. It has also been found that a longer period at 37°

* A preliminary paper dealing with these findings was read to the Society for Endocrinology on 29 May 1947.

than 2 hr. does not lower the recoveries. In the method finally adopted (p. 252) the actual periods during which the precipitation mixtures are incubated have been adjusted to fit a working day of convenient length.

Table 1. *The effect of varying the rate of cooling after precipitation on the recovery of pregnanediol added to 'male urine neutral fraction'*

'Male urine neutral fraction'	Pregnanediol added (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovered corrected for 'male urine blank' (%)	
		Rapid cooling	Slow cooling	Rapid cooling	Slow cooling
A	0·00	0·056	0·056		
	0·00	0·012 } 0·031	0·055 } 0·050	—	—
	0·00	0·026	0·040		
B	0·00	0·017	0·040		
	0·00	0·015 } 0·014	0·042 } 0·040	—	—
	0·00	0·010	0·037		
A	0·27	0·032	0·123		
	0·27	0·012 } 0·023	0·092 } 0·096	0	17
	0·27	0·025	0·072		
B	0·40	0·016	0·33		
	0·40	0·021 } 0·017	0·38 } 0·34	1	75
	0·40	0·015	0·31		
A	0·53	0·43	0·45		
	0·53	0·42 } 0·41	0·46 } 0·49	66	84
	0·53	0·37	0·57		
B	0·80	0·63	0·86		
	0·80	0·82 } 0·75	0·82 } 0·84	89	99
	0·80	0·80	0·83		
A	4·0	4·0	4·2		
	4·0	3·9 } 3·5	4·1 } 4·1	86	101
	4·0	2·5	4·0		

In view of these findings it is of some interest to consider the precipitation and cooling techniques of previous workers who have used the Astwood-Talbot method. Astwood & Jones [1941] do not appear to have controlled the temperature at which precipitation was carried out very exactly, but they did allow the precipitation mixtures to cool to room temperature before cooling in the refrigerator. Such a procedure in an American laboratory, where 'room temperature' may be 25° or more might be said to provide 'slow cooling'; in the average British laboratory in winter time, however, the procedure might very well give quite rapid cooling. Neither Talbot *et al.* [1941] nor Guterman [1944] controlled the temperature of precipitation, and since in both cases the precipitation mixtures were transferred directly to the refrigerator or into an ice-bath, their procedures must have undoubtedly involved rapid cooling. It can be concluded that the procedures used by all these workers would be liable to give low and erratic results with urines containing less than *c.* 20 mg. of pregnanediol per 24 hr.

Sulphuric acid colour reaction

The finally purified pregnanediol obtained by the precipitation process from the urine of pregnant and of non-pregnant women has occasionally been found to be contaminated with traces of a blue pigment. The nature of this pigment has not been

investigated, but it is suspected that it may be of dietary origin. The presence of this pigment seriously interferes with the sulphuric acid colour reaction and it is therefore necessary to remove it before carrying out the reaction. This can be effectively done without loss of pregnanediol by warming in ethanolic solution with charcoal. In order to maintain a rigid uniformity in procedure this treatment with charcoal has been adopted as a routine whether the pigment is present or not.

Talbot *et al.* [1941] carried out the colour reaction by allowing the purified pregnanediol to stand with 10 ml. of concentrated sulphuric acid at room temperature for 20 min. In the present work, in order to standardize conditions as far as possible, the colour development has been carried out in a water-bath at 25° instead of at 'room temperature'.

METHOD FINALLY ADOPTED FOR THE DETERMINATION OF PREGNANEDIOL IN URINE

A 24 hr. specimen of urine collected with 5 ml. of toluene as preservative is made up to 2500 ml. and duplicate 500 ml. samples removed. Each sample is treated as follows: It is placed in a 1000 ml. flask and after the addition of 100 ml. of toluene brought to boiling point under a reflux condenser. To the boiling mixture is added down the condenser 50 ml. of concentrated hydrochloric acid (A.R.), and the boiling continued for exactly 10 min. The flask is then rapidly cooled in cold water and the contents transferred to a separating funnel of 750 ml. capacity. After shaking and allowing the urine layer to separate, the latter is run off into the original flask and the layer of toluene and emulsion filtered with gentle suction through a Whatman No. 1 paper on a Buchner filter funnel. The urine layer is then returned to the separating funnel and extracted twice more with 100 ml. portions of toluene, each toluene and emulsion layer being filtered in succession through the same filter funnel. The combined filtrates are then transferred to a clean separating funnel, and after running off the small urine layer that separates, the toluene extract is washed twice with 100 ml. portions of N sodium hydroxide and twice with 100 ml. portions of water. The washed toluene extract is run into a 500 ml. round-bottomed flask and is evaporated nearly to dryness on an electric hot plate and then taken completely to dryness under reduced pressure on a boiling-water bath.

The dry residue is transferred quantitatively with warm ethanol to a 20 ml. conical centrifuge tube and the ethanolic solution evaporated to dryness in a water-bath under a stream of air. To the residue in the tube are added exactly 4·0 ml. of ethanol and the tube is placed in a beaker of water maintained at 75°. After stirring with a glass rod for 1 min. to obtain complete solution, 16·0 ml. of N/10 sodium hydroxide are added drop-wise from a burette during 3 min. with stirring, the last 1 ml. being used to wash down the stirring rod into the tube. After a further 1 min. at 75°, the beaker of water containing the tube is transferred to an incubator at 37° and left overnight. Approximately 8–10 mg. of 'Hyflo-Super Cel' (Johns-Manville Co. Ltd.) are added and the mixture stirred with a glass rod. The rod is washed down into the tube with 1 ml. of a 1 : 4 (v/v) ethanol-water mixture and the tube is then centrifuged for 1 hr. (1500 r.p.m.; radius of centrifuge head: 15 cm.). The supernatant solution is finally sucked from the precipitate with the aid of a fine glass tube attached to a slowly running water-pump.

The second and third precipitations are carried out as described above, except that water instead of sodium hydroxide solution is used, and the incubation periods are reduced for convenience to 2 hr. No additional filter-aid is added before the centrifugations following the second and third precipitations.

To the final precipitate are added 5 ml. of ethanol and the pregnanediol dissolved by warming with stirring at about 75°. 'Norite' charcoal (*c.* 1–2 mg.) is then added and the warming continued for 2 min. The mixture is filtered through a small filter (Whatman No. 1 paper) into a test-tube of 1 in. diameter, the centrifuge tube and filter being washed three times with 2 ml. portions of warm ethanol. The filtrate and washings in the tube are evaporated in a water-bath under a stream of air and the residue finally dried by leaving the tube in a vacuum desiccator over calcium chloride for several hours.

The colour reaction is carried out with not more than *c.* 0·5 mg. of the finally purified product. If, therefore, the amount of the latter appears on inspection to be in excess of 0·5 mg. a suitable aliquot portion is removed after solution in a known volume of ethanol. To the dry pregnanediol 10·0 ml. of concentrated sulphuric acid (A.R.) are added from a burette, and the tube is left in a water-bath at 25° for 20 min. with occasional shaking. The intensity of the yellow colour produced is measured in a 'Spekker' photoelectric absorptiometer using a 'spectrum violet' No. 601 light filter.

The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-3(α), 20α-diol varying from 0·1 to 0·5 mg. It is advisable to construct a fresh calibration curve for each batch of unknowns.

RECOVERY EXPERIMENTS

The accuracy of the finally adopted method was tested in a long series of recovery experiments in which pure sodium pregnanediol glucuronidate was added in varying amounts to men's urine. The validity of such tests of accuracy depends upon two assumptions: (*a*) that all the pregnanediol in human urine is present as the glucuronide, and (*b*) that women's urine contains no substances which are not present in men's urine which would interfere with the determination of pregnanediol. Further work will be necessary to see whether these assumptions are justifiable or not.

Twelve 24 hr. specimens of urine were collected from four normal men. Each specimen was made up to 2500 ml. and four 500 ml. samples removed. To each of two of these samples was added an identical amount of sodium pregnanediol glucuronidate dissolved in 80% ethanol, the other two samples being retained for working-up as 'male urine blanks'. All four samples from each specimen were then treated as described in the preceding section (pp. 252, 253). The results in Table 2 show that a pregnanediol content of *c.* 2 mg./24 hr. is a critical one, above which recoveries are excellent, but below which they are poor.

That the loss of pregnanediol when less than *c.* 2 mg./24 hr. is present in the urine occurs mainly during the precipitation process rather than during the hydrolysis or extraction seems to be clear from the results shown in Table 1. This loss must be largely due to an effect of other substances in the neutral fraction upon the solubility of pregnanediol in 20% ethanol, since experiments in pure solution have shown that

80–95 % of pregnanediol can be removed after the triple precipitation process when it is present in amounts corresponding to as little as 0·5 mg./24 hr. It is possible, therefore, that the critical concentration of pregnanediol below which recoveries are unsatisfactory may vary somewhat with different urines. However, the results reported here with a number of different specimens of men's urine suggest that this critical concentration is probably not far from 2 mg./24 hr. in the majority of cases.

Table 2. *Recovery of pregnanediol after the addition of sodium pregnanediol glucuronidate to men's urine*

Men's urine specimen	'Male urine blank' as apparent pregnanediol in $\frac{1}{5}$ of 24 hr. specimen (mg.) (av. of duplicates)	Pregnanediol added as glucuronidate to $\frac{1}{5}$ of 24 hr. urine specimen (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovery (corrected) (%)
			Apparent	Corrected for blank	
C4	0·016	0·2	0·017	0·001	0
		0·2	0·012	—	0
A3	0·008	0·2	0·021	0·013	7
		0·2	0·047	0·039	20
B2	0·024	0·2	0·060	0·036	18
		0·2	0·045	0·021	11
D4	0·035	0·4	0·32	0·29	72
		0·4	0·33	0·29	74
B3	0·015	0·4	0·28	0·27	67
		0·4	0·29	0·28	69
A2	0·018	0·4	0·35	0·33	82
		0·4	0·35	0·33	82
A4	0·044	1·0	0·99	0·95	95
		1·0	0·98	0·93	93
C3	0·019	1·0	0·94	0·92	92
		1·0	0·96	0·94	94
D2	0·077	1·0	1·0	0·92	92
		1·0	0·98	0·90	90
B4	0·030	2·0	1·9	1·9	95
		2·0	2·0	2·0	100
D3	0·017	2·0	2·0	2·0	100
		2·0	1·9	1·9	95
C2	0·026	2·0	1·9	1·9	95
		2·0	1·9	1·9	95

SPECIFICITY OF THE METHOD

The sulphuric acid colour reaction is not specific for pregnanediol; many other steroids give similar colours with varying intensities. The specificity of the method as a whole therefore depends upon the completeness with which other chromogenic steroids originally present in the urine are eliminated in the extraction and purification process.

Some data relevant to this extremely important point have been presented by previous workers. Thus Astwood & Jones [1941] showed that cholesterol and androsterone were completely eliminated by their double precipitation process when present in amounts not exceeding 16 and 8 mg. per litre respectively, while Talbot *et al.* [1941] showed that dehydro*iso*androsterone in amounts up to *c.* 10 mg. per litre

caused no interference. In the course of present work additional relevant data have been accumulated, but since these data are still incomplete they will be referred to at the present time only briefly.

Androsterone, *iso*androsterone, and pregnan-3(α)-ol-20-one are not completely eliminated by the triple precipitation process used, but providentially they are so much less powerfully chromogenic than pregnanediol in the sulphuric acid reaction that their presence in the final product, except in abnormally large amounts, does not introduce any serious error into the pregnanediol determination. Dehydro*iso*-androsterone and pregnane-3(α), 17, 20-triol, on the other hand, are very powerful chromogens, but, providentially again, they seem to be very readily eliminated in the precipitation process. Any possible interference by the pregnanetriol is doubly safeguarded against by the fact that this compound would be largely decomposed during the initial hydrolysis of the urine with acid.

These preliminary findings provide hope that the method may be reasonably specific for pregnanediol when applied to normal urines, but before definitely concluding that it is indeed so it will be necessary to carry out further experiments with other steroids likely to be present. At the present time the method cannot be recommended for pathological urines containing abnormally high concentrations of neutral 17-ketosteroids or of other neutral steroids of adrenal origin. In such cases the pregnanediol present might be considerably overestimated. In passing, it may be remarked that any of the methods in which pregnanediol is determined as its glucuronide are also likely to give fictitiously high results when applied to urines containing abnormally high concentrations of steroids of adrenal origin, since certain of the latter are probably excreted as glucuronides also.

DISCUSSION

The methods of urinary pregnanediol determination which have hitherto been described are either insufficiently sensitive or insufficiently specific to permit of strictly quantitative studies being carried out upon pregnanediol excretion during the menstrual cycle and during the early stages of pregnancy. The method described here should make such studies more nearly possible, and in particular should be of some value in the investigation of causes of female sterility.

Although the method was not designed for use as a means of pregnancy diagnosis, it may be useful for this purpose, and because of its more quantitative nature it may prove to be less subject to both positive and negative errors than the more rapid Guterman [1944, 1945] method. In view of the widespread interest in the latter it would perhaps not be out of place to discuss in the light of the findings in the present work some of the possible sources of the negative and positive errors to which the method seems to be subject [cf. Reinhart & Barnes, 1946].

In his latest paper on the method Guterman [1945] has arbitrarily fixed on an intensity of colour in the sulphuric acid reaction corresponding to 6–8 mg. of pregnanediol per 24 hr. as the lower limit for a positive reaction. It seems probable that at such levels of pregnanediol excretion the unsatisfactory cooling conditions in the precipitation process in Guterman's procedure might lead to low and variable yields of pregnanediol and thus to false negative results. It is likely that a controlled 'slow

cooling' technique in the precipitation process might eliminate some at least of these false negatives.

The findings in the present work suggest that false positives might be caused by the presence of abnormally large amounts of the weakly chromogenic saturated neutral 17-ketosteroids which are incompletely removed even by a triple precipitation procedure, or by the incomplete elimination in the single precipitation of the Guterman method of the strongly chromogenic dehydro*iso*androsterone. In this connexion it is noteworthy that Morrow & Benua [1946] recorded false positives in a case of arrhenoblastoma which was excreting 59 mg. of 17-ketosteroids per 24 hr. It is doubtful whether false positives caused by the presence of abnormally large amounts of 17-ketosteroids could be entirely eliminated by any simple modification in the precipitation process, but it is likely that their number could be reduced if a double instead of a single precipitation procedure were to be adopted.

In conclusion, one may perhaps question whether it is justifiable to accept as diagnostic of pregnancy any arbitrarily fixed low level of pregnanediol excretion associated with amenorrhea. It must be remembered that little is known at the present time about the metabolism of progesterone, and there are in fact few reasons to believe that pregnanediol is even the main metabolic end-product of the latter. The low yields of urinary pregnanediol obtained after the administration to human subjects of progesterone raise the possibility that the latter may be largely metabolized in the body by other routes. It is therefore questionable whether the level of pregnanediol excretion provides a reliable indication of the progesterone production in the body, as has been so widely assumed.

As pointed out by Reinhart & Barnes [1946], it is possible that the greatest source of error in the Guterman test is the 'individual variations in the metabolism of progesterone, both in the pregnant and the non-pregnant woman'.

SUMMARY

A procedure based on the methods of Astwood & Jones [1941] and Talbot *et al.* [1941] has been elaborated which permits of the reasonably accurate determination of more than *c.* 0·4 mg. of pregnanediol in a fifth of a 24 hr. sample of human urine.

This work was carried out at the request of the Committee on Human Fertility of the Medical Research Council. The authors are indebted to the Medical Research Council for a grant from which the expenses of the work were defrayed and for personal grants to two of them (N.G. and I.F.S.). They are also indebted to their colleagues who co-operated in the collection of urine and to Miss E. Sutherland who prepared the sample of pure sodium pregnanediol glucuronidate.

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RAPID DETERMINATION OF URINARY PREGNANEDIOL

METHOD SUITABLE FOR ROUTINE CLINICAL USE

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THE Guterman (1944, 1945) qualitative test for urinary pregnanediol, based on the quantitative procedure developed by Astwood and Jones (1941) and Talbot et al. (1941), has been widely used for the early diagnosis of pregnancy and for prognosis in threatened abortion. Though there is some measure of agreement that the method may be useful in the prognosis of threatened abortion (Guterman 1946, Bender 1947, 1948, Merivale 1948), opinions on its value in the diagnosis of pregnancy are sharply divided. Guterman (1944, 1945) and McCormack (1946) have claimed an accuracy as great as that of the Friedman method; on the other hand, Reinhart and Barnes (1946) and Merivale (1948) have reported high percentages of both false positives and false negatives. In our opinion their unsatisfactory results are possibly due partly to technical deficiencies of the method, some of which are avoidable.

The Guterman test depends on the detection, by means of a colour reaction with sulphuric acid, of an amount of pregnanediol in 100 ml. of morning urine which is stated to correspond approximately to an excretion of 6–8 mg. of the steroid per 24 hours. Guterman considers a pregnancy test to be positive if this amount of pregnanediol or more is detected in the urine during amenorrhœa. Morrow and Benua (1946) have, however, observed "false positive tests for pregnancy" in the luteal phase of the menstrual cycle, and have pointed out that, since there may be a considerable overlap between the lower levels of pregnanediol excreted during early pregnancy and the higher levels excreted during the latter half of the menstrual cycle, there is a danger of obtaining false positive reactions in cycles prolonged by late ovulation. This criticism seems to be valid; but, provided no reliance is placed on positive reactions obtained within a short time of an apparently missed period, the danger of diagnosing a delayed or prolonged luteal phase of the cycle as a pregnancy should not be serious.

Reinhart and Barnes (1946) criticise the method on rather similar grounds, pointing out that "a test which is not accurate in the face of vaginal bleeding is of restricted value as a diagnostic aid in hospital practice." Though this is doubtless true, we feel that their further statement that "the source of error lies not in the interpretation of the colour reaction but in individual variations in the metabolism of progesterone, both in the pregnant and the non-pregnant woman" cannot pass unchallenged. They seem to have overlooked the possibility that a not inconsiderable source of both positive and negative errors may lie in the technique of the stages in the method preceding the final colour assessment, and it is at least possible that they have over-emphasised the importance of individual variations in progesterone metabolism. The methods hitherto used for studying the excretion of pregnanediol during the menstrual cycle and early pregnancy are insufficiently accurate to show whether there are considerable individual variations in the extent to which progesterone is reduced to pregnanediol in non-pregnant and pregnant women.

A general criticism that can be justifiably made of the Guterman method is that a quantitative significance has been attached to the results of a qualitative test. Attempts have been made by several workers to make the test quantitative rather than qualitative by examining the colour spectrophotometrically instead of visually. Such attempts, however, must necessarily fail in their object, since the test is carried out on an unknown fraction of the 24-hour urine output, and since, as recently shown by Sommerville et al. (1948), the procedure used for purifying the extracted pregnanediol is likely to give low and erratic results when applied to urine containing less than about 20 mg. of the steroid per 24 hours.

It seems clear that the value of pregnanediol determinations for diagnosis requires careful reinvestigation with the aid of a method more nearly quantitative and less open to criticism on technical grounds than is the Guterman procedure. The method of Sommerville et al. (1948), which has been shown to be reasonably accurate at levels of pregnanediol * excretion above about 2 mg. per 24 hours, would be suitable for such an investigation but is somewhat too laborious and time-consuming for routine use. Accordingly an attempt has been made to develop a procedure combining the speed and simplicity of the Guterman method with certain of the technical improvements made in the original Astwood and Talbot procedures by Sommerville et al. (1948). The method developed permits of the reasonably accurate quantitative determination in less than 3 hours of the pregnanediol in 100 ml. of urine, provided that the level of excretion is above about 5 mg. per 24 hours. It will probably be less liable than the Guterman method to give erratic results at low levels of pregnanediol excretion, but like the latter it

* Unpublished work by Sommerville and Marrian has shown that *allo*pregnane-3(α), 20α-diol, as well as pregnane-3(α), 20α-diol, is determined by this method. In this paper the term "pregnanediol" is used to designate the mixture of both stereoisomers that is present in urine.

DETERMINATION OF URINARY PREGNANEDIOL

Recovery of pregnane-3(α), 20α-diol added to men's urine after acid hydrolysis

Urine	Pregnanediol added (mg.)		Pregnanediol recovered corrected for average male urine blank (mg.)		
	Per 100 ml. urine	Per 24 hrs. urine	Per 100 ml. urine	Per 24 hrs. urine	% recovery
A	—	—	0·01 } av. 0·02	0·17 } av. 0·25	0·23 ..
	—	—	0·02 }	0·28 }	
B	—	—	0·02 } av. 0·03	0·27 } av. 0·54	0·40 ..
	—	—	0·02 }	0·40 }	
A	0·2	2·8	0·10	1·3	48
	,,	,,	0·05	0·71	26
	,,	,,	0·06	0·88	32
B	,,	3·6	0·17	3·1	87
	,,	,,	0·11	1·9	53
	,,	,,	0·19	3·4	94
B	0·3	5·4	0·31	5·5	102
	,,	,,	0·25	4·5	83
	,,	,,	0·28	5·2	94
A	0·4	5·6	0·28	3·9	70
	,,	,,	0·24	3·3	60
	,,	,,	0·34	4·7	85
B	0·4	7·2	0·35	6·3	88
	,,	,,	0·34	6·1	85
	,,	,,	0·39	7·0	98
A	0·6	8·4	0·53	7·5	89
	,,	,,	0·47	6·6	86
	,,	,,	0·51	7·1	85
B	0·5	9·0	0·41	7·3	82
	,,	,,	0·43	7·7	86
	,,	,,	0·45	8·1	90

will be likely to give fictitiously high results when applied to urine containing an abnormally high amount of cholesterol or of neutral 17-ketosteroids. This method is only recommended for routine diagnostic purposes where a high degree of accuracy and great sensitivity are not essential; for quantitative studies of pregnanediol excretion during the menstrual cycle or early pregnancy the longer method of Sommerville et al. (1948) would be preferable.

METHOD

Into a 500 ml. flask fitted with an interchangeable ground-glass joint and a reflux condenser is placed 100 ml. of a 24-hour urine specimen, the volume of which is measured, and 50 ml. of toluene ("sulphur-free," redistilled). The contents are heated to boiling and, after the addition of 10 ml. of concentrated hydrochloric acid (A.R. quality), the boiling is continued for exactly 10 min. The mixture is cooled, transferred to a 250 ml. separating funnel, and shaken. After standing for about 5 min, the lower urine layer is run off and discarded, and the upper toluene emulsion layer is filtered through a Buchner funnel with gentle suction, a Whatman no. 1 paper being used to break the emulsion. The filtrate is then transferred back into the separating funnel, and the lower urine layer that separates from the emulsion is run off and discarded.

The toluene layer is washed in the funnel twice with 15 ml. lots of *N*/1 NaOH and twice with 15 ml. lots of water. It is then transferred to a 200 ml. round-bottomed flask fitted with a bent ground-glass "socket adapter" (Quickfit and Quartz Ltd.) and evaporated to dryness under reduced pressure in a boiling water-bath.

The residue in the flask is quantitatively transferred with warm ethyl alcohol to a test-tube (1 in. diam.) which has a graduation mark at the 5 ml. level. The solution is then evaporated to 5 ml. in a warm water-bath under a gentle stream of air. After the tube has been placed in a beaker of water at 75°C, 20 ml. of *N*/10 NaOH is added slowly from a burette over a period of 3 min. and stirred gently with a glass rod. After a further minute the beaker and tube are transferred to an incubator at 37°C and left for 2 hours.

The contents of the tube are then filtered through a sintered glass funnel (3 cm. diam. plate; average por. diam. 20–30 μ), the tube and filter being subsequently washed liberally with water to remove all traces of alkali from the precipitate. The funnel is removed from the filter flask and fitted to a test-tube (1 in. diam.) with a side-arm. The precipitate is then washed through into the tube with three 5 ml. lots of boiling alcohol under gentle suction.

To the filtrate in the filter-tube is added about 1–2 mg. of 'Norit' charcoal, and the mixture is heated in a water-bath for 2 min. and filtered through a Whatman no. 1 paper in a conical filter (2 in. diam.) into a test-tube (1 in. diam.). The filtrate is finally evaporated to dryness in a water-bath under a stream of air.

To the dry residue in the tube is added 10 ml. of concentrated sulphuric acid (A.R. quality). The tube is placed in a constant temperature bath at 25°C and left for 20 min. with occasional shaking. The intensity of the yellow colour produced is then measured in a 'Spekker' photo-electric absorptiometer using a 'spectrum violet' no. 601 light filter. The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-3(α), 20α-diol, and the final result is expressed as mg. of pregnanediol excreted per 24 hours. The calibration curve should be checked at short intervals.

RESULTS

Urine Blanks.—Blank determinations in triplicate were carried out on eight 100 ml. samples from 24-hour urine specimens collected from seven different normal men. The values for "apparent pregnanediol" excreted per 24 hours varied from 0·13 to 0·61 mg. Though pregnanediol has been reported to be present in small amounts in men's urine (Westphal 1944), there are reasons for believing that these small urine blank values are largely due to chromogenic substances other than pregnanediol which are incompletely removed in the precipitation procedure. However, the highest of these blank values is so much smaller than the lowest amount of pregnanediol that can be estimated with any degree of accuracy that errors due to this cause may be neglected.

Recovery Experiments.—Two 24-hour urine specimens (A and B) were collected on successive days from the same normal man. Twelve 100 ml. samples were taken from specimen A and fifteen from B. After hydrolysis of these with hydrochloric acid, amounts of pure pregnane-3(α), 20α-diol varying from 0·2 to 0·6 mg. were added in alcoholic solution, three samples from each specimen being reserved as triplicate blanks. The pregnanediol in each sample was then determined by the method described, and the recoveries calculated after correcting for the appropriate blank. The results (see table) show that satisfactory recoveries were obtained with amounts of pregnanediol corresponding to more than about 5 mg. per 24 hours.

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EFFECT OF DIETHYLSTILBŒSTROL ON URINARY EXCRETION OF PREGNANEDIOL AND ENDOGENOUS ŒSTROGEN DURING PREGNANCY

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THE proposal of Smith et al. (1946) to give diethylstilbœstrol by mouth as a prophylactic to pregnant women with histories of repeated accidents in pregnancy has attracted widespread interest, particularly in connexion with the high fœtal mortality associated with maternal diabetes. This proposal is based on the suppositions that many accidents of late pregnancy are referable to a deficiency in the secretion of progesterone and other sex steroids by the placenta, and that the secretion of these steroids can be stimulated with diethylstilbœstrol. The second of these suppositions is based on the interesting theories concerning the interdependence of œstrogen and progesterone metabolism, developed by the Smiths in a long series of papers published from 1931 onwards and recently reviewed (Smith and Smith 1948).

The Smiths believe (1) that the œstrogenic hormone (œstradiol⇌œstrone) is converted in the body not only by facilitating the metabolism of œstradiol and œstrone by the alternative pathway to œstriol, and conversely that the formation of the inactivation products is enhanced when there is a deficient secretion of progesterone; and (3) that the œstrogen inactivation products play an important physiological rôle as stimulators of the secretion of progesterone and other "sex steroids."

These views led Smith et al. (1944) to suggest that massive doses of the œstrogenic hormone during mid-pregnancy might forestall an incipient premature deficiency in the secretion of progesterone and œstrogen from the placenta by raising the concentration in the body of these œstrogen inactivation products.

Later Smith et al. (1946) suggested that the artificial œstrogen, diethylstilbœstrol, might be more effective in stimulating the placental secretion of steroids, and they tried to test this suggestion by estimating the urinary pregnanediol excretion of a pregnant diabetic woman during intermittent treatment with this œstrogen. The results seemed to show conclusively that the excretion of pregnanediol had been increased by the diethylstilbœstrol,* and it was concluded that this increase was the result of a greater placental secretion of progesterone.

More recently some doubt on their findings has been cast by Davis and Fugo (1947), who claimed that the excretion of pregnanediol by non-diabetic women in early pregnancy was unchanged by treatment with diethylstilbœstrol. This claim calls for some comment. The published data show that no pronounced and progressive rise in the excretion of pregnanediol, like that observed by Smith et al. (1946), resulted from the treatment; but, since the pregnanediol excretion levels before treatment with the œstrogen were not established with certainty, the statement that the pregnanediol excretion was unchanged hardly seems justifiable. In any case, as Smith and Smith (1948) pointed out, the doses of diethylstilbœstrol used by Davis and Fugo were greatly in excess of those used by themselves; hence the two sets of experiments are hardly comparable.

We report here the results of a reinvestigation of the effect of diethylstilbœstrol on the urinary excretion of pregnanediol during pregnancy. Since the Smiths' theories imply that diethylstilbœstrol stimulates the secretion of œstrogen as well as of progesterone by the placenta, the urinary excretion of endogenous œstrogen has also been studied in these experiments.

METHODS

Daily 24-hour specimens of urine were collected in containers cooled with "dry ice" from all the cases studied. In most instances the completeness of the collection of urine was checked by determining the creatinine content by the method of Folin (1914). All determinations were carried out in duplicate on aliquot portions of the 24-hour specimens of urine.

Urinary pregnanediol was determined by the method of Sommerville et al. (1948).

Total urinary endogenous œstrogen (œstradiol+œstrone+œstriol) was determined by the method of Stevenson and Marrian (1947) using the Kober colour reaction, which is unaffected by the presence of diethylstilbœstrol in the urine. This method, though not so reliable as could be desired, is sufficiently accurate to detect any pronounced changes in the excretion of endogenous œstrogen.

RESULTS

One non-diabetic pregnant woman and three diabetic pregnant women were studied. The essential clinical points of the diabetics, for which we are indebted to Prof. D. M. Dunlop, were as follows:

Case	*Age (yr.)*	*Duration of diabetes*	*History of previous pregnancies*
1	30	Since childhood	—
2	30	2 years	Intra-uterine death 3 days antepartum
3	30	11 years	(i) Intra-uterine death 5 days antepartum (ii) Miscarriage at 19th week

Doses of diethylstilbœstrol greater than, less than, and the same as, those recommended by Smith et al. (1946) were given by mouth intermittently. Smith et al. recommended a dosage of 30 mg. daily at the beginning of the 16th week of pregnancy, increasing by 5 mg. a day at weekly intervals to the 35th week.

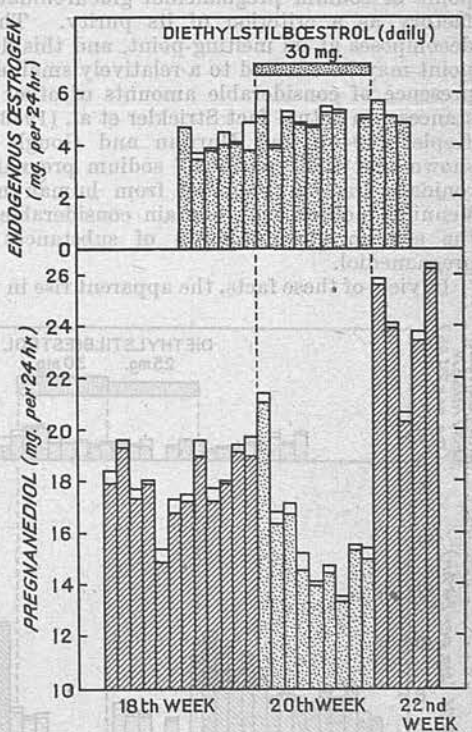

Fig. 3—Effect of diethylstilbœstrol on excretion of pregnanediol and endogenous œstrogen in a diabetic pregnant woman (case 2).

Details of the diethylstilbœstrol dosages and of the effects on the excretion of pregnanediol and endogenous œstrogen are shown in figs. 1–5,† from which it will be seen that the effect of diethylstilbœstrol on the urinary excretion of pregnanediol is unequivocal. In every instance

* Dr. O. W. Smith has privately informed one of us (G. F. M.) that she has obtained similar results with other pregnant diabetic women treated with diethylstilbœstrol.

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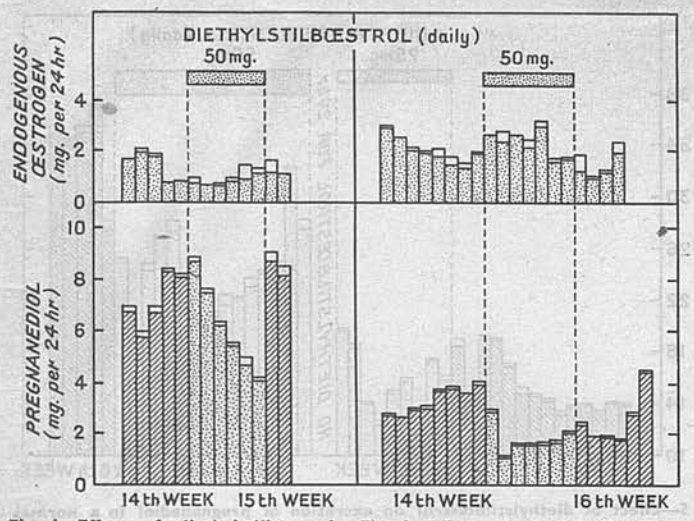

Fig. 1—Effect of diethylstilbœstrol on excretion of pregnanediol and endogenous œstrogen in a normal pregnant woman.

Fig. 2—Effect of diethylstilbœstrol on excretion of pregnanediol and endogenous œstrogen in a diabetic pregnant woman (case 1).

into œstriol but also into certain œstrogenically inactive oxidation products, which, however, have yet to be isolated and described; (2) that progesterone depresses the formation of these "œstrogen inactivation products" by facilitating the metabolism of œstradiol and œstrone by the alternative pathway to œstriol, and conversely that the formation of the inactivation products is enhanced when there is a deficient secretion of progesterone; and (3) that the œstrogen inactivation products play an important physiological rôle as stimulators of the secretion of progesterone and other "sex steroids."

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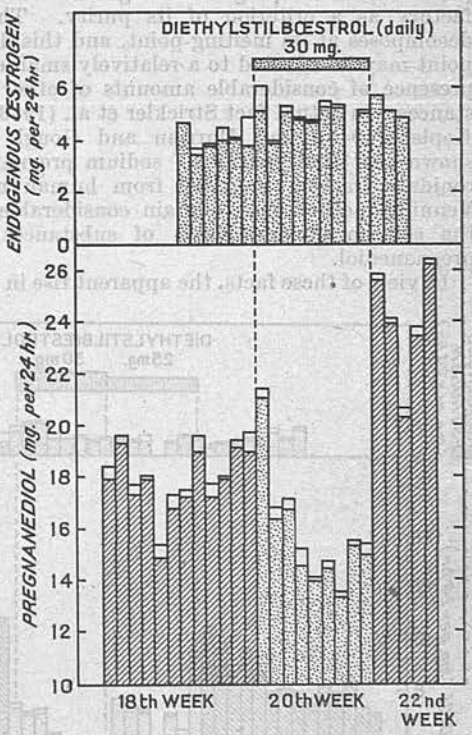

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the giving of diethylstilbœstrol caused a sharp fall in the excretion of pregnanediol, whereas after withdrawal of the œstrogen the excretion rose either immediately or after a short delay.

The figures for the excretion of total endogenous œstrogen are unfortunately less clear-cut owing possibly to the known unreliability of the method of estimating œstrogen. It can be safely stated, however, that in these experiments the giving of diethylstilbœstrol caused no gross change in the excretion of total endogenous œstrogen.

DISCUSSION

The discrepancy between the results reported by Smith et al. (1946) and those reported here may possibly be due to the different methods used for the estimation of pregnanediol. The method of Sommerville et al. (1948), used in the present work, gives satisfactorily accurate and reproducible results when about 2 mg. of pregnanediol is excreted per 24 hours, and there is good reason to believe that it is reasonably specific for pregnanediol and *allo*pregnanediol. There can be little doubt, therefore, that the reduction in pregnanediol excretion following treatment with diethylstilbœstrol reported here was genuine.

Smith et al., on the other hand, used the original Venning (1937, 1938) method, which, it is now known, is not highly specific for pregnanediol. In this method sodium pregnanediol glucuronidate is extracted from the urine and weighed after "purification," the purity of the final product being checked by determining its melting-point. Unfortunately, however, the melting-point of sodium pregnanediol glucuronidate is unsatisfactory as a criterion of its purity. The compound decomposes at its melting-point, and this decomposition point may be affected to a relatively small extent by the presence of considerable amounts of other related substances. In actual fact Strickler et al. (1943), Mason and Kepler (1945), and Marrian and Gough (1946) have shown that the "purified" sodium pregnanediol glucuronidate fraction prepared from human urine by the Venning method may contain considerable amounts of the sodium glucuronidates of substances other than pregnanediol.

In view of these facts, the apparent rise in pregnanediol excretion following the administration of diethylstilbœstrol reported by Smith et al. (1946) may well have been due to the excretion of substances related to, but not identical with, sodium pregnanediol glucuronidate. In this connexion it should be pointed out that Dodgson and Williams (1948) have demonstrated the presence of a glucuronide of diethylstilbœstrol in human urine after the administration of diethylstilbœstrol by mouth. The possibility cannot be dismissed, therefore, that the results of Smith et al. were affected by the contamination of the sodium pregnanediol glucuronidate isolated by sodium diethylstilbœstrol glucuronidate.

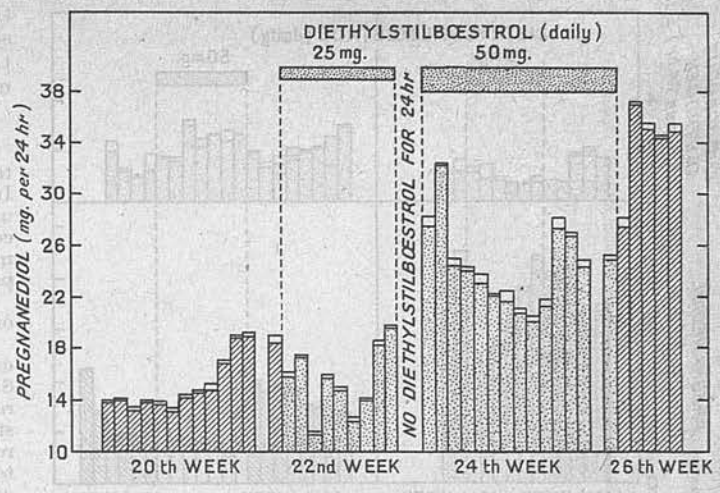

Fig. 5—Effect of diethylstilbœstrol on excretion of pregnanediol in a normal pregnant woman.

Knowledge of the metabolism of progesterone is as yet so incomplete that it should not be assumed that changes in the excretion of pregnanediol in the urine necessarily reflect changes in the secretion of endogenous progesterone.

SUMMARY

The administration of diethylstilbœstrol by mouth to diabetic and normal pregnant women causes a sharp fall in the urinary excretion of pregnanediol. Withdrawal of the diethylstilbœstrol results in a rise in pregnanediol excretion, either immediately or after a short delay. These results differ from those reported by Smith et al. (1946).

The administration of diethylstilbœstrol causes no gross change in the excretion of endogenous œstrogen in diabetic and normal pregnant women.

We are indebted to the Medical Research Council for a personal grant to one of us (I. F. S.) and for a grant (to G. F. M.) out of which part of the expenses of this work was defrayed. We are also greatly indebted to Prof. D. M. Dunlop, Prof. R. J. Kellar, Dr. G. D. Matthew, and Dr. J. A. L. Gilbert for advice and coöperation; to Messrs. W. L. Aikman and H. A. F. Blair for technical assistance; and to Dr. Olive W. Smith, of the Fearing Research Laboratory, Boston, Mass., for so generously providing one of us (G. F. M.) with the opportunity of discussing with her the present results and those of her own investigations. This investigation was undertaken at the request of the committee of management of the Clinical Endocrinology Research Unit (Medical Research Council) in the University of Edinburgh.

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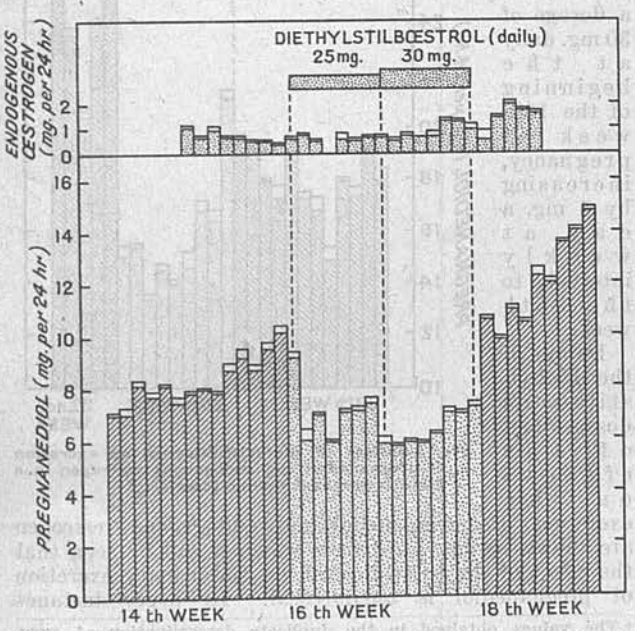

Fig. 4—Effect of diethylstilbœstrol on excretion of pregnanediol and endogenous œstrogen in a diabetic pregnant woman (case 3).

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