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A Simplified Method for the Quantitative Determinations on Free Pregnanediol Excretion in Pregnancy.

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Venning and Browne[1] described a method for the isolation of sodium pregnanediol glucuronidate in the urine and theorized that this chemical substance represented the end-product in the metabolism of progesterone. Further studies confirmed the relationship of pregnanediol excretion to the luteal phase of the ovarian cycle and to normal pregnancy. Furthermore, a correlation between the administration of progesterone and the excretion of pregnanediol has been established although in the non-pregnant individual only a small fraction of progesterone administered can be recovered as sodium pregnanediol glucuronidate.

The gravimetric method of Venning for the quantitative estimation of pregnanediol is long and tedious and it has the disadvantage that a number of factors other than progesterone influence the excretion of glucuronidate. Astwood and Jones[2] described a method for the determination of free pregnanediol utilizing the hydrolysis of glucuronidate. Talbot *et al.*[3] made use of the color reaction produced by pure sulphuric acid to provide a colorimetric method for the quantitative determination of pregnanediol. Guterman[4] modified the Astwood-Jones method to provide for the more rapid qualitative determination of pregnanediol and made it more applicable to clinical use.

The quantitative method described here has the advantage of simplicity thereby making it possible to follow patients over long periods of time by serial determinations permitting the study of the complications of pregnancy and the evaluation of their therapy. The determination of free pregnanediol rather than the conjugated sodium pregnanediol glucuronidate avoids the danger of loss incurred during the urine collection period. Furthermore, other substances than pregnanediol are found in the urine as glucuronidates interfering with the accuracy of the determinations.

During the past year we have carried out over 1500 determinations of pregnanediol in the urine in about 100 patients. Some of these women had normal pregnancies and serial determinations were made throughout their pregnancies and for a week following their deliveries. These women served as normal controls to evaluate the method and to establish basic curves of pregnanediol excretion. The majority of the women had pregnancy complications in whom it was desired to follow the pregnanediol excretion in order to determine any variations from the normal curves in an attempt to establish the role of progesterone metabolism in these complications. In many of these patients daily determinations were possible but in the majority the collections of urine were made 2 and 3 times a week. All of the determinations have been made in duplicate to decrease the likelihood of error in the method.

Method. The women studied for the most part were out-patients who visited this clinic. They were given standard containers for urine collection and were carefully instructed to insure complete 24 hour samples. The first morning specimen on the day of collection was discarded and all urine throughout the day and

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1 Venning, E. H., *J. Biol. Chem.*, 1937, **119**, 473.

2 Astwood, E. B., and Jones, G. E. S., *J. Biol. Chem.*, 1941, **137**, 397.

3 Talbot, N. D., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K., *J. Clin. Endocrinol.*, 1941, **1**, 668.

4 Guterman, H. S., *J. Clin. Endocrinol.*, 1945, **5**, 407.

including the first morning specimen the next day were pooled.

The completed collection was brought to the laboratory the same morning and the determination started immediately. All examinations were made the same day they were received. No preservatives were used. In some cases patients were brought into the hospital and urine collections made largely by the patients themselves. It has been our experience that much more accurate 24 hour samples were made by cooperative patients than when collections were left to an already overworked nursing staff. Patients were carefully instructed to report any loss of part of the collections. Samples which for some reason were not complete were discarded. Determinations were made at weekly, biweekly and in some of the more interesting cases, daily intervals.

The chemical procedure employed is essentialy that of Astwood and Jones[2] as modified by Guterman.[4] We have in addition made a few changes of our own for the purpose of increasing the accuracy of the quantitative determination. The technique briefly is as follows:

1. One hundred cubic centimeters of urine, 50 cc of C.P.Toluene, 10 cc of conc. HCl and a few glass beads are added to a 500 cc flat bottomed Florence flask. The flask is connected by a one-holed cork stopper to a vertical reflux condenser and the contents boiled vigorously for 15 minutes on an electric hot plate.

2. The flask it then cooled under tap water to room temperature and its contents transferred to a 500 cc separatory funnel. The lower urine-acid layer is drawn off. The urine-acid layer is shaken twice with 10-15 cc volumes of fresh toluene and returned to the separatory funnel, the urine-acid layer being drawn off between each shaking.

3. The toluene emulsion layer in the separatory funnel is then washed twice with 15 cc portions of 0.1 N NaOH followed by 2 washings with 15 cc portions of distilled H_2O.

4. The washed toluene and toluene water emulsion layers are transferred to a 125 cc Erlenmayer flask. A few glass beads are added. The separatory funnel is rinsed with fresh toluene and the rinsings are added to the flask.

5. The mixture is boiled on an electric hot plate under a hood and when the emulsion layer has disappeared and the toluene is boiling smoothly 10 cc of freshly prepared 2% NaOH in absolute methanol are added slowly. The mixture is boiled until a granular precipitate is obtained and the solution has a yellow or greenish yellow appearance.

6. The toluene mixture is then filtered through fritted glass filters of medium porosity using slight suction. The precipitate is washed with the fresh hot toluene used to rinse out the flask.

7. The combined filtrates are evaporated in a hood utilizing a hot plate. The last traces of toluene are eliminated by means of an air stream. This avoids charring the residue.

8. Five cc of acetone are then added to the residue and the measure gently heated until solution is complete. Twenty cubic centimeters of 0.1 N NaOH are slowly added while the flask is still on the hot plate. When boiling occurs the flask is placed in the refrigerator overnight.

9. The precipitate that forms is collected by filtering through a fritted glass filter and washed with the rinsings of the flask using distilled H_2O. The precipitate is then washed with 10 cc of petroleum ether.

10. The receiving flask is changed and the precipitate dissolved by passing 10 cc of hot absolute ethanol through the funnel. If the precipitate shows any discoloration of a reddish or yellowish tint it is reprecipitated by adding 40 cc of distilled H_2O to the alcohol solution and heated to boiling. This last step is repeated until the precipitate is white.

11. The alcoholic filtrate is evaporated to dryness on a hot plate utilizing an air jet to remove the last few cubic centimeters.

12. Ten cc of C.P. H_2SO_4 are added to the dried white precipitate and allowed to stand one hour for full color development. After proper dilution (usually 1-10) the solution is read in a Coleman model 11 Spectrophotometer at a wave length of 420μ. The readings are interpolated on a curve made by using pure pregnanediol.

In order to test the accuracy of our quan-

TABLE I.
Comparison of Venning Gravimetric Method and the Modified Guterman Method.

No.	Pregnanediol	NaPG as Pregnanediol
1	78.1	78.3
2	68.4	60.1
3	65.7	67.9
4	18.2	16.3
5	9.1	8.1

titative results a few determinations using our technique and the original Venning method were run on the same samples. Fig. 1 shows the close agreement between the two methods.

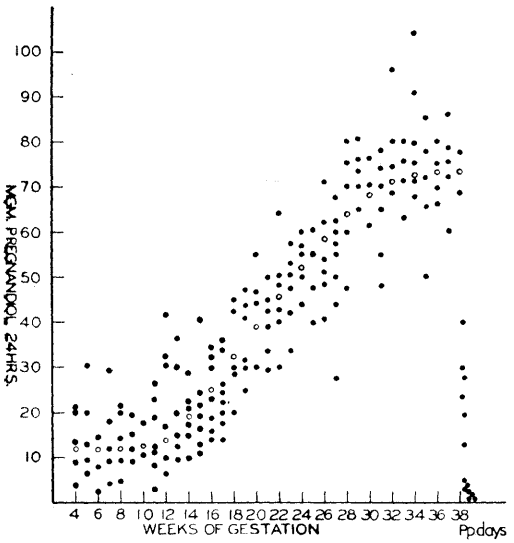

FIG. 1.
A composite curve demonstrating the excretion of pregnandiol in a group of patients during normal pregnancy and the immediate postpartum period.

Normal Pregnancy Curve. There is considerable variation in the daily output of pregnanediol in the individual with a normal pregnancy but the curve follows a typical pattern. During the first 12 to 14 weeks of the gestation the daily excretion will vary from a low of 5 or 6 mg to a high of 20 mg. Some individuals excrete more pregnanediol throughout the pregnancy than do others. The daily output increases slowly during the second trimester of pregnancy leveling off at about the twenty-eighth week of the gestation. The level remains fairly constant during the last 10 weeks of the pregnancy, varying from 70 to 90 mg per day. Individual daily amounts may fluctuate rather widely reaching as high as 120 mg or as low as 50 or 60 mg. Serial determinations on the same patient are extremely important for only in this way can one recognize sudden changes in the normal pattern. No sudden drop in the level of free pregnanediol prior to the onset of labor has been recognized in our curves although there may be some recognizable decrease in the daily output during the last few weeks prior to the onset of labor.

Following delivery there is a sudden drop in the excretion of pregnanediol. The first 24 hour urine collection may contain as little as half the amount present on the previous day. No more than 10 to 20 mg are eliminated during the second 24 hours. Small amounts varying from 2 to 3 mg may be present for an additional day or two, following which little is excreted. We have collected all urine in our postpartum patients by means of an inlying catheter to provide for complete samples. (Fig. 1)

Most authors agree that pregnanediol is the urinary metabolite of progesterone. During the latter half of the menstrual cycle and the early part of pregnancy, the corpus luteum is the principal source of this hormone. However, with the development of the placenta this organ becomes the principal source of the progestational hormone. It may serve as the only source for in 2 instances in our series as well as in the experience of others (Seegar and Delfs)[5] the removal of the ovary containing the corpus luteum did not alter appreciably the excretion of pregnanediol.

Placental functions must be directly related to the quantitative production of progesterone and the output of the biologically inert steroid pregnanediol. In that the placenta is primarily a circulatory organ providing the circulation link between mother and fetus, the excretion of pregnanediol depends indirectly on the efficiency of placental circulation. Complications of pregnancy associated with disturbances of placental function must invariably be reflected in disturbed pregnanediol excretion. It is not surprising that decreased amounts have been reported in patients who

[5] Seegar, G. E., and Delfs, E., *J. A. M. A.*, 1940, **115**, 1267.

threaten to abort, in the toxemias of pregnancy, in premature labor, in late death of the fetus. Before intelligent therapy of these complications can be developed, the relationship of progesterone metabolism to these conditions must be understood.

Summary.—A rapid, accurate colorimetric method for the quantitative determination of pregnanediol based on the methods of Venning, Talbot and Guterman is described. Serial determinations in normal pregnancy and pregnancy complications have been carried out in over 100 patients. Pregnanediol excretion in the last 28 weeks of the gestation can be used as a quantitative measure of uteroplacental circulation. During this period the placenta is the chief source of this urinary metabolite for corpus luteum activity wanes rapidly after the first trimester. Serial determinations in normal pregnancy and the complications of pregnancy may throw light on the adequacy of the placenta as the essential organ for the survival of the fetus.

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Symmetrical Patterns of Bacteriophage Production.

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In a previous note[1] it was shown that the problem of how bacteriophages are produced can be directly approached through the electron microscopy of metal shadowed "replicas" of the surfaces of agar cultures on which bacteria and bacteriophage are growing together. Such studies of several different bacteriophages are showing many of the phenomena involved in the production of these virus-like objects. The processes are complex and depend not only on the type of bacterium and the strain of bacteriophage but also on such factors as the rate and the duration of growth of the culture. Detailed evidence furnished by the electron microscope will be described elsewhere.

One of the most impressive aspects of the development of bacteriophage from bacterial protoplasm is its completeness, and in certain instances its regularities. With the T3 bacteriophage against the colon bacillus, the pattern of this conversion shows a symmetry as perfect as that of the molecular particles in a crystalline array[2] (Fig. 1). As in this figure the pattern often covers the entire surface of a bacterium; but it is also to be seen spreading throughout extruded protoplasm (Fig. 2). It extends into and includes the thick masses which were bipolar bodies in the original cells. When most clearly visible, the pattern is one of concavities but in many places the separate indentations are filled with spherical bodies having the size of free bacteriophage particles. Photographs have been obtained of this honeycomb structure starting to form within cells that otherwise seem normal; but it is often difficult to be sure whether these indentations are places where fully formed particles have escaped or where relatively immature particles are just beginning to form.

Correspondingly complete conversion to bacteriophage has been observed with other strains but highly symmetrical nets of particles have not been seen following infection with any of the "tailed" bacteriophages.

The many electron micrographs already made suggest far-reaching speculations into the nature of bacteriophages and of any viruses that may exhibit similar mechanisms of increase; but the techniques being used offer opportunities for gaining further information that make discussions of this nature seem premature.

1 Edwards, O. F., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 16.

2 Price, W. C., and Wyckoff, R. W. G., *Nature*, 1946, **157**, 764; Markham, R., Smith, K. M., and Wyckoff, R. W. G., *ibid.*, 1947, **159**, in press.