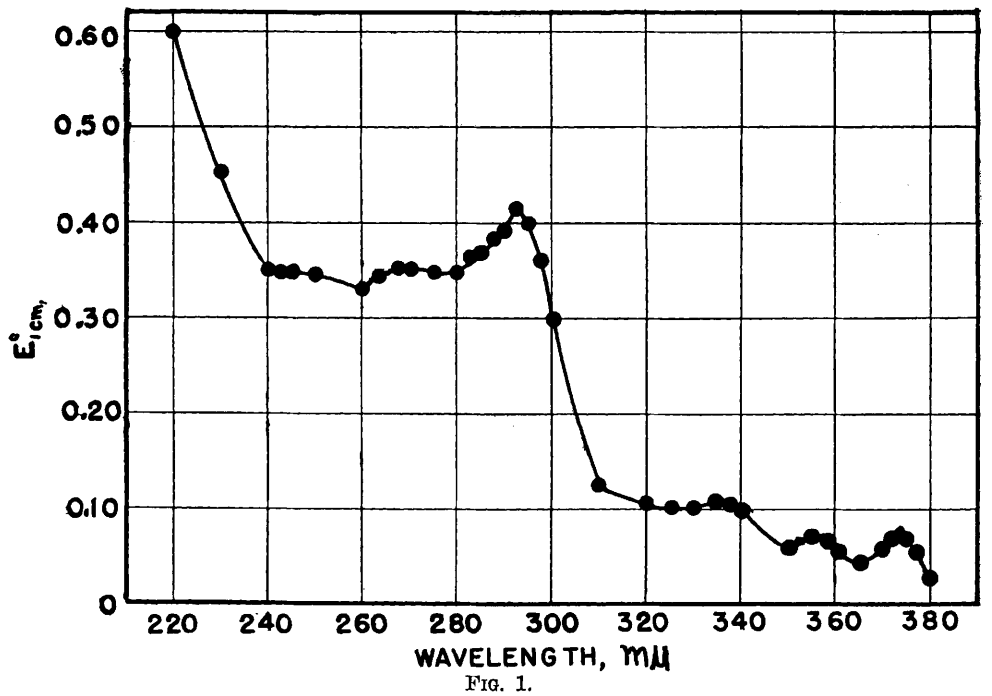

FIG. 1.
Absorption Spectrum of Fradicin.
c = 0.0174 g/l in absolute methanol.
E^c_{1cm} = extinction at conc. c.

in a number of organic solvents and has a characteristic absorption spectrum which differentiates it from other antibiotics.

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Does the Excretion of Diethylstilbestrol Glucuronide Influence the Venning Determination for Urinary Pregnanediol? (17688)

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An apparent increase in the excretion of pregnan-3(α), 20(α)-diol as a result of the prophylactic administration of diethylstilbestrol to a pregnant woman was first reported in 1946(1). This observation has been amply confirmed in this laboratory(2,3,4) and by other investigators(5), provided the pregnanediol is measured as the sodium glucuronide (NaPG) by the Venning procedure(6).

1. Smith, O. W., Smith, G. V. S., and Hurwitz, D., *Am. J. Obst. and Gynec.*, 1946, v51, 411.

2. Smith, O. W., and Smith, G. V. S., *West. J. Surg.*, 1947, v55, 313.

3. Smith, O. W., *Am. J. Obst. and Gynec.*, 1948, v56, 821.

4. Smith, O. W., to be published.

5. Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 436.

6. Venning, E. H., *J. Biol. Chem.*, 1937, v119, 473.

It has also been reported, however (and confirmed in this laboratory)(4), that pregnanediol measured by colorimetric assay on acid hydrolyzed urine usually shows no increase following stilbestrol administration(5,7,8). In fact, an apparent drop has been observed in short time experiments by Marrian and Clayton upon the initiation of therapy or an increase in dosage(8). This discrepancy in results, apparent even when aliquots of the same specimens are analyzed by the two methods(4,5), introduces some question as to the validity of the interpretation of the original observation, namely, that the apparent increase in pregnanediol excretion reflects higher levels of circulating progesterone as a result of the administered diethylstilbestrol.

It is now known that recrystallized NaPG prepared from human urine by the Venning method may contain the sodium glucuronides of substances other than pregnanediol. This statement is based upon the identification of three compounds, pregnane-3(α), 17, 20-triol(9), pregnane-3(α), 17-diol-20-one(10), and pregnane-3(α)ol-20-one(11) as glucuronides in "purified" NaPG complex from human urines. The latter is present in considerable amounts in pregnancy urine(11) and has been shown to be an excretory product of progesterone(12). No evidence has been presented that diethylstilbestrol glucuronide is recovered by the Venning procedure but the possibility has naturally arisen(5,8) since, from the urines of both rabbits(13) and women(14,15) to whom stilbestrol has been administered, diethylstilbestrol glucuronide has been obtained in quantities amounting to 6 to 35% of the stilbestrol given. If the glucuronide of diethylstilbestrol were recovered by the Venning procedure, therefore, its presence in the NaPG crystals might account for the apparent effect of stilbestrol administration upon pregnanediol excretion. In this communication evidence is presented which eliminates this possibility. Some other explanation must be sought, therefore, for the discrepancy in results for "pregnanediol" excretion, and investigations along this line are in progress(16).

Estrogenic activity of NaPG from the urine of pregnant women taking diethylstilbestrol by mouth. Four samples of crystalline NaPG recovered by the Venning procedure from the urines of 4 women, normally pregnant at weeks 34 to 38, were tested for estrogenic activity. All of the women had been on oral therapy with gradually increasing dosages of stilbestrol from early in pregnancy. At the time when these samples of NaPG were recovered they were taking 125 to 150 mg of the drug daily.

The first sample (m. p. 282°C) was dissolved in isotonic buffer solution and tested directly, without any hydrolysis or extraction. In 10 spayed female rats, standardized for their responsiveness to crystalline estrogens, the vaginal smears were completely negative when testing for 10 rat units in 15 mg. By our assay 2.5 μg of diethylstilbestrol glucuronide is equivalent to one rat unit. It may be assumed, therefore, that this sample contained less than 0.08 mg of diethylstilbestrol glucuronide in the 45.8 mg of NaPG recovered, an amount of estrogenic impurity well below the degree of accuracy of the Venning method.

The other 3 samples were dissolved either in concentrated phosphate buffer solution or postmenopausal urine, subjected to acid hydrolysis and the phenolic fractions recovered by ether extraction and partition out of toluene with normal sodium hydroxide. The method of extraction used has been shown to

7. Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 283.

8. Marrian, G. F., and Clayton, B. E., *The Lancet*, April 23, 1949, p. 680.

9. Mason, H. L., and Kepler, E. J., *J. Biol. Chem.*, 1945, v161, 235.

10. Mason, H. L., and Strickler, H. S., *J. Biol. Chem.*, 1947, v171, 543.

11. Marrian, G. F., and Gough, N., *Biochem. J.*, 1946, v40, 376.

12. Dorfman, R. J., Ross, E., and Shipley, R. A., *Endocrinol.*, 1948, v42, 77.

13. Mazur, A., and Shorr, E., *J. Biol. Chem.*, 1942, v144, 283.

14. Dodson, K. S., and Williams, R. T., *Nature*, 1948, v161, 604.

15. Teague, R. S., Wingard, C., and Brown, A. E., *Fed. Proc.*, 1949, v8, 337.

16. Smith, O. W., *J. Clin. Endocrinol.*, in press.

give a quantitative recovery of crystalline diethylstilbestrol, but the method of hydrolysis (10 minute boiling with 15 volumes percent of concentrated hydrochloric acid) accomplishes only 50% hydrolysis of diethylstilbestrol glucuronide. In calculating from the estrogenic activity recovered the possible contamination of the NaPG with stilbestrol glucuronide, therefore, a 50% loss was assumed.

An 80 mg sample (m. p. 268 to 275°C) was found to contain 50 to 67 r.u. of estrogenic activity after hydrolysis and extraction. By our assay, 0.15 μg of diethylstilbestrol is one rat unit. This sample, therefore, could have contained no more than 0.038 mg of sodium diethylstilbestrol glucuronide, too little to influence the gravimetric analysis.

A 55 mg sample (m. p. 275 to 276°C) treated in the same manner yielded 22 to 30 r.u. of estrogen. It could have contained no more than 0.017 mg of sodium diethylstilbestrol glucuronide, too little to be detected gravimetrically.

A 60 mg sample (m. p. 282°C) was subjected to our modification of the Sommerville, Gough and Marrian method for the hydrolysis and extraction of urinary pregnanediol(16). According to the estrogenic activity recovered from the phenolic fraction, the highest possible concentration of sodium diethylstilbestrol glucuronide in this NaPG was 0.022 mg, again too little to measure with gravimetric accuracy.

Failure to recover NaPG from the urine during the administration of diethylstilbestrol. Results have been previously reported(2) showing that the oral administration of 75 to 125 mg of stilbestrol daily to patients already showing the clinical signs of late pregnancy toxemia causes no increase in pregnanediol excretion (as measured by the Venning procedure). This observation has been repeatedly confirmed in this laboratory. A recent case specifically illustrates the point. The patient was 26 weeks pregnant with fulminating severe pre-eclampsia. Three specimens of urine collected on 3 consecutive days before stilbestrol was started were found to contain 8.2 to 11.0 mg of pregnanediol as NaPG per 24 hour volume. During the following 10 days she was on 100 mg of diethylstilbestrol daily by mouth. Four urines were analyzed and found to contain 9.3 to 11.7 mg of pregnanediol as NaPG per 24 hour volume, no increase over the pretreatment level. In 3 unpublished experiments it was also found that 125 to 200 mg of stilbestrol daily would not prevent the drop in NaPG that precedes normal labor at term.

An obese diabetic woman thought to be pregnant by her obstetrician was put on 35 mg of diethylstilbestrol daily at the seventeenth week from her last menstrual period. Her urines were analyzed for pregnanediol by the Venning procedure at weekly intervals. No glucuronide was recovered in any of 7 specimens, the last one having been collected at 25 weeks when 75 mg of stilbestrol were being ingested daily. At this time the obstetrician confirmed our opinion that the patient was not pregnant.

To another non-pregnant woman 100 mg of diethylstilbestrol was administered experimentally. No NaPG was recovered by the Venning procedure from the butyl alcohol extract of all of the urine collected during the following 72 hours. A mature female rabbit was injected with 200 mg of crystalline diethylstilbestrol and the urine for the next 72 hours extracted by the Venning procedure. No NaPG was recovered.

Recovery experiment with diethylstilbestrol glucuronide added to urine. A sample of diethylstilbestrol glucuronide was prepared by one of us (S. S.) from the urine of a rabbit that had received 1.5 g of crystalline diethylstilbestrol by injection over a 7 day period. The procedure described by Mazur and Shorr (13) was followed. Repeated recrystallization was resulting in so much loss of material that complete purification was not attempted. The sample used crystallized in white needles, softened at 91 to 96°C and melted with decomposition at 132 to 170°C. Tests for estrogenic activity before and after hydrolysis indicated that this was practically pure diethylstilbestrol glucuronide. Prior to hydrolysis it assayed at 1 rat unit to 2.5 μg. An aliquot was hydrolyzed according to the method of Teague(15), after which the activity was increased to 1 rat unit to 0.45 mg, *i.e.*, 1 rat

unit to 0.25 μg of free diethylstilbestrol, the same assay value acquired from a sample of crystalline diethylstilbestrol which was simultaneously submitted to the same hydrolysis procedure.

Forty mg of this sample were dissolved in 200 cc of postmenopausal urine and extracted with butyl alcohol for the recovery of NaPG by the Venning method. No precipitate whatsoever settled out of the aqueous acetone solution. Estrogenic assay of the various fractions revealed that half of the stilbestrol glucuronide had been left in the tenth normal NaOH solution after being re-extracted with butyl alcohol. The other half was present in the residue of the final butyl alcohol extract but, unlike pregnanediol glucuronide, was not precipitated out of 5 cc of water by the addition of 95 cc of acetone. It is quite possible that when considerable amounts of NaPG are present, traces of stilbestrol glucuronide may be carried down by adsorption and even persist through the second precipitation out of water with acetone. That no more than traces are present, however, is apparent from the very slight estrogenic potency of NaPG recovered from pregnant women taking large doses of stilbestrol (v.s). In this connection it may be mentioned that estrogenic activity has also been found by us in hydrolyzed NaPG recovered from the urine of pregnant women who were not taking diethylstilbestrol. The probable explanation is that traces of estriol glucuronide, large amounts of which are present in late pregnancy urine, may also be carried down by adsorption upon the precipitated NaPG. Here again the amount present, although readily demonstrable by estrogenic assay, is too little to influence the figures for pregnanediol recovered as NaPG.

Conclusion. Diethylstilbestrol glucuronide is recovered in no more than trace amounts from human urine by the gravimetric Venning procedure for the determination of sodium pregnanediol glucuronide. In pregnant patients taking large amounts of stilbestrol the quantitative values for urinary pregnanediol were not influenced by this possible source of error. Some other explanation must be found for the fact that values acquired by the Venning method on the urines of such patients do not agree with colorimetric assays for "freed" pregnanediol upon hydrolyzed specimens.

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Activity of Trypsin in Solution Through Membranes.* (17689)

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The concept of specific long range forces of certain biologically active materials has been promulgated by Rothen(1-7). His experiments employing films of antigen and antibody, or films of enzyme and subtrate, separated by inert film barriers indicate the possible existence of such forces. The evidence thus far presented is the increase in thickness of the layered films when homologous antigen-antibody systems are allowed to interact through the barriers. Similarly, trypsin acting through a barrier on bovine albumin prevents an increase in thickness when an antiserum to bovine albumin is subsequently added. Rothen also finds that increasing the number of layers of barrier be-

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1. Rothen, A., *J. Biol. Chem.*, 1944, v152, 679.
2. Rothen, A., *Rev. Scient. Instruments*, 1945, v16, 26.
3. Rothen, A., *Science*, 1945, v102, 446.
4. Rothen, A., *J. Biol. Chem.*, 1946, v163, 345.
5. Rothen, A., *J. Biol. Chem.*, 1947, v167, 299.
6. Rothen, A., *J. Biol. Chem.*, 1947, v168, 75.
7. Rothen, A., *J. Am. Chem. Soc.*, 1948, v70, 2732.