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PROGESTERONE SECRETION AND METABOLISM IN THE HUMAN MALE AND CASTRATE FEMALE.

by

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Thesis submitted for Degree of
Doctor of Medicine, University
of Glasgow.

SEPTEMBER. 1963.

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Summary.

A radio-isotope tracer technique for estimation of progesterone secretion rates is established and is proved specific, accurate, and reproduceable. The theory of calculation of secretion rates is discussed and the validity of this aspect of the method confirmed. Normal male subjects and surgically castrate female subjects are studied after intravenous injection of $7H^3$-progesterone and significant differences in the metabolism of progesterone are noted. The normal males excrete significantly less total radioactivity in urine, significantly less radioactivity as urinary pregnanediol but significantly more radioactivity as peak X than do the surgically castrate females. The males excrete significantly more urinary pregnanediol and have a significantly greater progesterone secretion rate than the castrate females. The progesterone secretion rate for the surgically castrate females is an estimation

of the secretion rate of progesterone by the intact female adrenal gland and is in vivo proof that the human female adrenal gland secretes progesterone in the normal steady state. A collaborative study estimates the volumes of distribution, metabolism rate constants and metabolic clearance rate of progesterone and pertinent aspects of this work are described and discussed. Mean plasma progesterone levels are calculated from the knowledge of the progesterone secretion rate and metabolic clearance rate and the level is shown to be higher in normal males than surgically castrate females. The total body content of progesterone is calculated from the knowledge of the mean plasma progesterone level and the final volume of distribution of the injected tracer dose of tritiated progesterone and the progesterone content of normal males is noted to be significantly greater than that of the surgically castrate females.

Introduction.

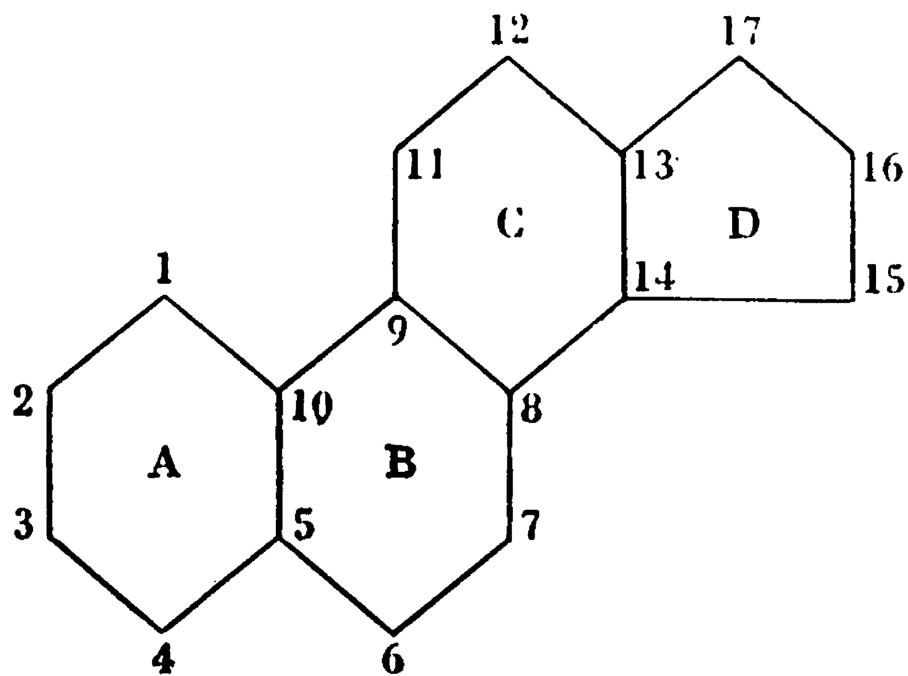
In the beginning of this century Fraenkel (1903) and Loeb (1908, 1909) demonstrated the dominant role of the corpus luteum in the preparation of the uterus for the implantation of the fertilized ovum. About twenty-five years later Corner and Allen (1929) first prepared the active principle of the gland in an impure form and established a method of bioassay which made possible further purification of the corpus luteum hormone. In 1934 this hormone, now known as progesterone, was isolated as a chemically pure substance by four different groups of workers almost simultaneously (Wintersteiner and Allen 1934 a & b; Butendant Westphal and Hohlweg, 1934; Slotta, Ruschig, and Fels, 1934; Hartmann and Wettstein, 1934). Very shortly after this the structure of the hormone was elucidated by Butendant and Schmidt (1934) and by Fernholtz (1934).

Five years prior to the synthesis of progesterone, Marrian (1929) isolated an inactive steroid from human pregnancy urine which Butendant (1930, 1931) identified, characterised and named pregnanediol. The similarity in the structure of this compound to progesterone suggested the possibility of a metabolic relationship between the hormone and the urinary steroid. Two years later Venning and Brown (1936) reported the excretion of pregnanediol in the urine in the form of a water soluble conjugate with sodium glucosiduronidate. Subsequently the in vivo conversion of exogenous progesterone to pregnanediol diglucuronide has been described by many investigators (Sommerville and Marrian, 1950 a & b; Davis, Plotz, Le Roy, Gould and Werbin, 1956).

Progesterone (pregn - 4 ene - 3, 20 dione), like the other steroid hormones, has the basic cyclopentanoperhydrophenanthrene

nucleus (Fig. 1), and has the structure shown in Fig. 2. The hormone is secreted by the adrenal gland (Short 1960), the ovary and placenta (Zander 1959) and is synthesized in the testis (Slaunwhite and Samuels 1956). The hormone is synthesized along the pathway from acetate→cholesterol→pregnenolone→progesterone. Progesterone has a central role in steroid production (Fig. 3). It is a precursor of aldosterone (Grant 1960). 17-hydroxylation is a necessary step in the biosynthesis of cortisol. The biosynthesis of androgens and ostrogens depends on the cleavage of the side chain of progesterone and a step which renders the side chain unstable is 17-hydroxylation of progesterone to form 17-hydroxyprogesterone.

Metabolism of steroid hormones takes place in many tissues but the chief site is the liver (Samuels and Weist, 1952). There are three main classes of biologically inactive metabolic reduction products of



Cyclopentanoperhydrophenanthrene.

FIG. I.

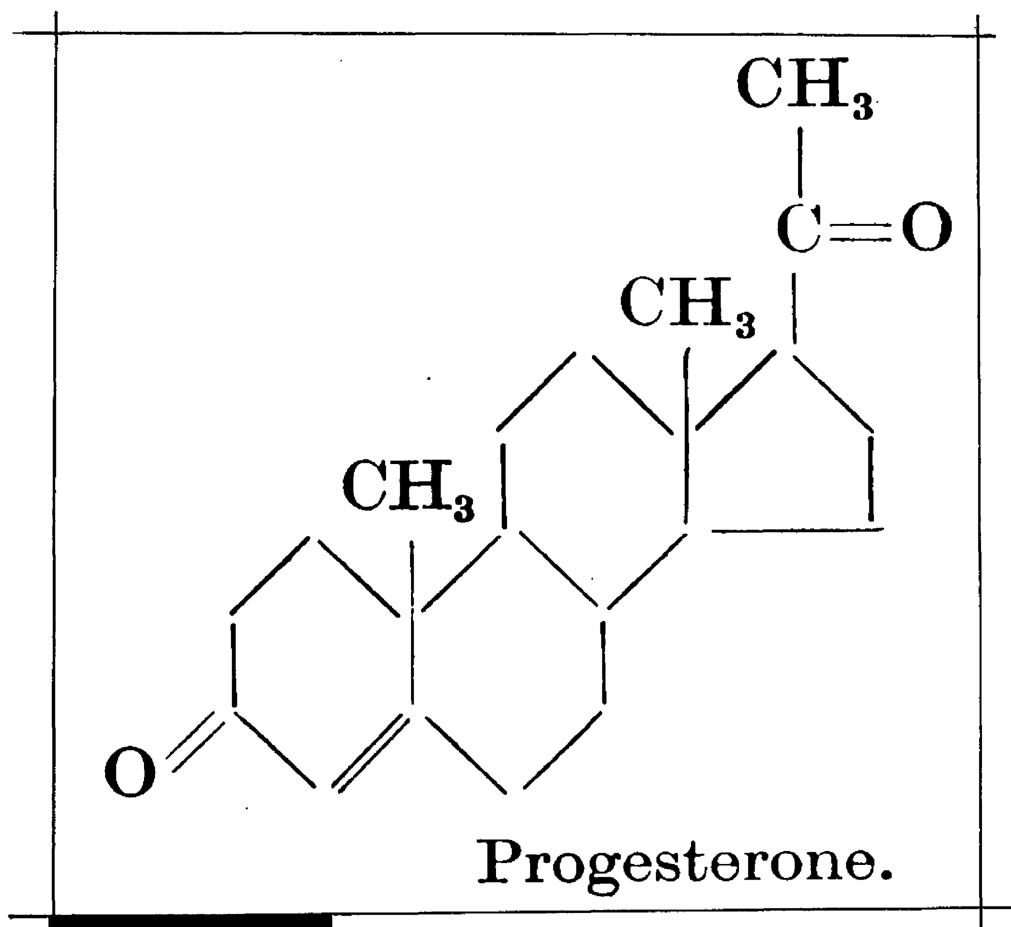


FIG. 2.

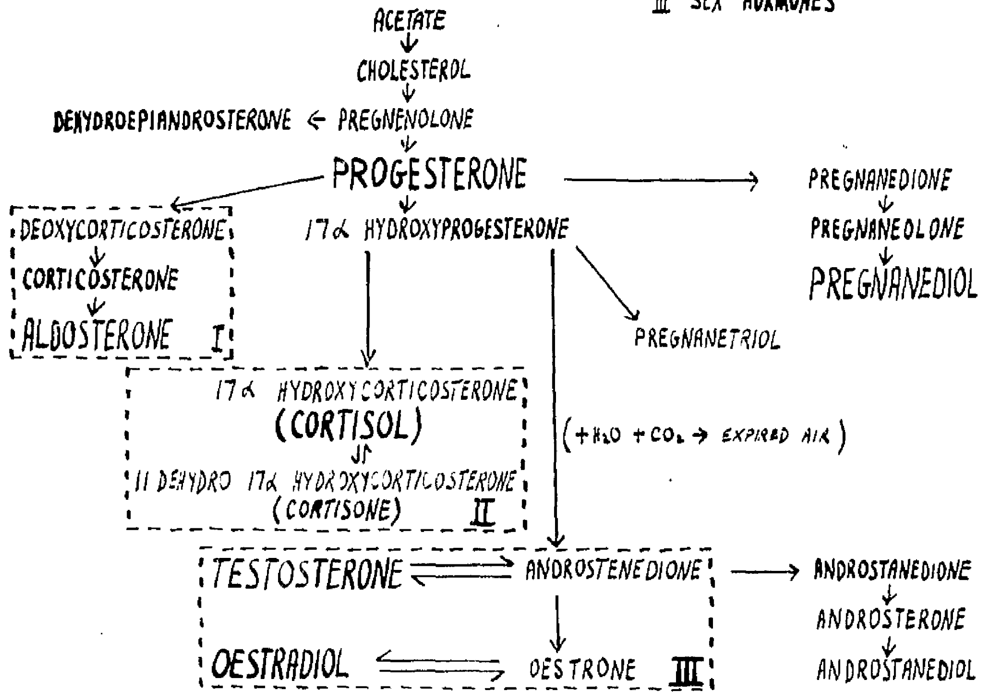


FIG. 3.

progesterone 1) Pregnanediones, 11) Pregnane-olones, 111) Pregnanediols. Pregnanediol (5β pregnane- 3α, 20α, - diol : Fig. 4) is the principal metabolite of progesterone (Ungar, Dorfman, Stecher and Vignos, 1951). The only other naturally occurring steroid that is known to be metabolised to pregnanediol is deoxycorticosterone. However since little if any deoxycorticosterone is normally secreted by the adrenal, and as only a small proportion of it is metabolised to pregnanediol, excretion of pregnanediol has come to be accepted as a measure of progesterone production (Short 1960).

Two biologically active metabolites of progesterone (Fig. 5) have been recently demonstrated in human tissues, pregn-4 ene - 20α, ol - 3 one and pregn - 4 ene - 20β, ol - 3 one (Zander, Forbes, Von Munstermann and Neher 1958), the 20α epimere being found in higher concentration in human beings and the 20β epimere being more

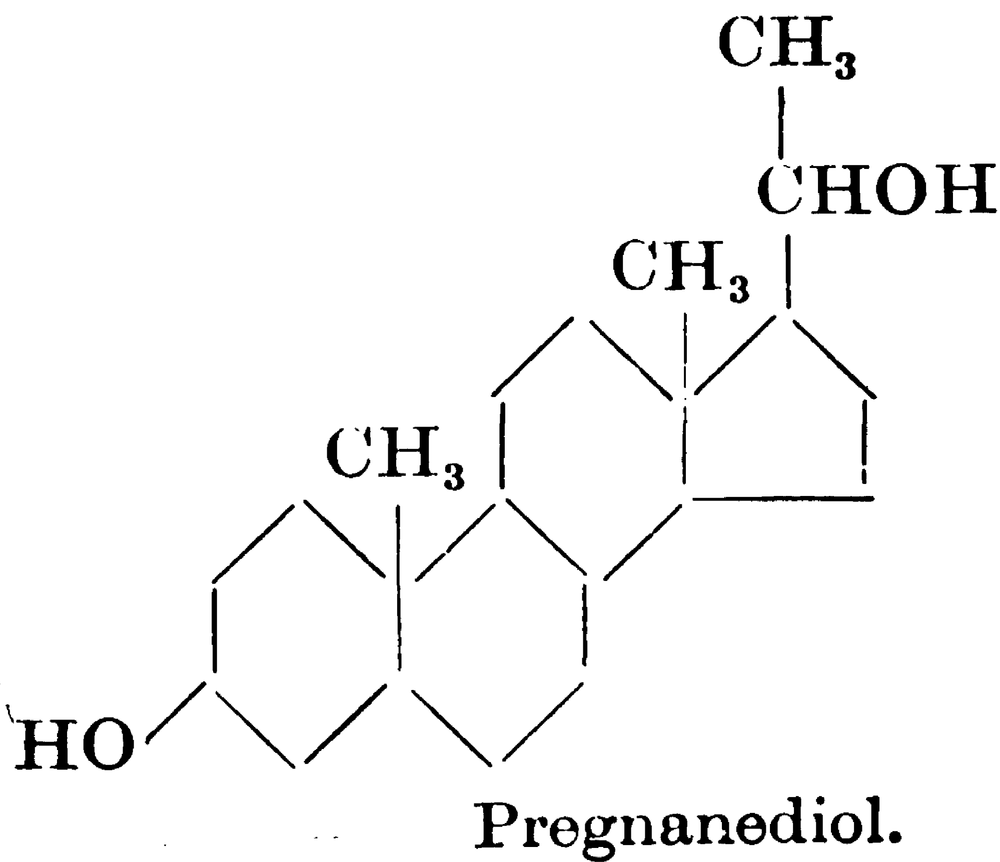


FIG. 4.

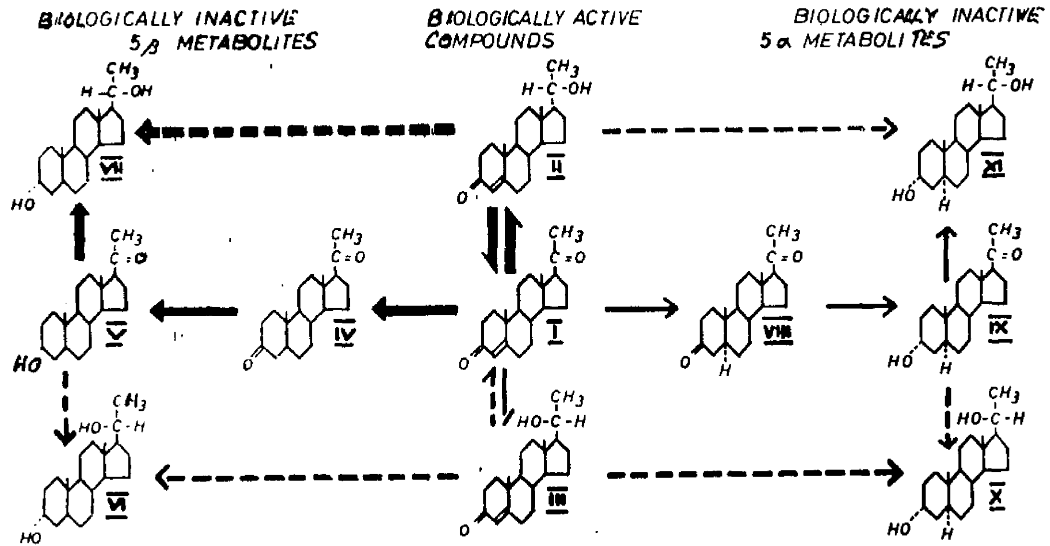


FIG. 5.

I Progesterone.
II Pregn - 4,ene - 20α,ol - 3,one.
III Pregn - 4,ene - 20β,ol - 3,one.
IV Pregnane - 3,20, diol.
V Pregnane - 3α,ol - 20,one. (Pregnanolone)
VI Pregnane - 3α,20β, diol.
VII Pregnane - 3α, 20α, diol. (Pregnanediol)
VIII Allopregnane - 3,20, dione.
IX Allopregnane - 3α,ol - 20,one.
X Allopregnane - 3α, 20β, diol.
XI Allopregnane - 3α, 20,α, diol.

effective in the Hooker and Forbes (1947) bioassay for progesterone than progesterone itself.

There are three main excretory pathways of progesterone metabolites 1) Urine (Venning and Browne 1937, 1940), 2) Faeces and 3) expired air (Davis and Plotz, 1958). The excretion of metabolites by the skin is minimal. In contrast to the large amount of progesterone metabolites in urine, faeces and expired air, the concentration in the blood plasma is very low. Chemical determinations by Zander (1954) and Zander and Simmer (1954), and Zander, Von Munstermann and Marx (1955) reveal an average level of 0.142 gamma per millilitre of plasma during the fourth to ninth month of gestation. Thus there is a rapid disappearance of the free steroid from the circulation due to 1) a speedy metabolism of the steroid molecule 2) a rapid diffusion into the body tissues, particularly into

the fat compartment where it can be retained for considerable length of time. (Fig. 6). Zander (1961) also excludes the possibility that progesterone is present in the target tissues in higher concentration than in the blood.

At the present time there is no definite knowledge of the actual mechanisms by which a steroid hormone exerts its effect on the target organ. It is possible that progesterone, originally secreted by an endocrine gland, is metabolised to other compounds which finally produce the so called progesterone effect on the target organ at very low concentration. Many investigators now believe that the mechanism of action of steroid hormones is related to cytoplasmic enzyme-substrate reactions (Coutinho 1961). This is in keeping with the fact that progestational effects are not highly specific and not only produced by progesterone. Other steroids such as testosterone and desoxycortone under

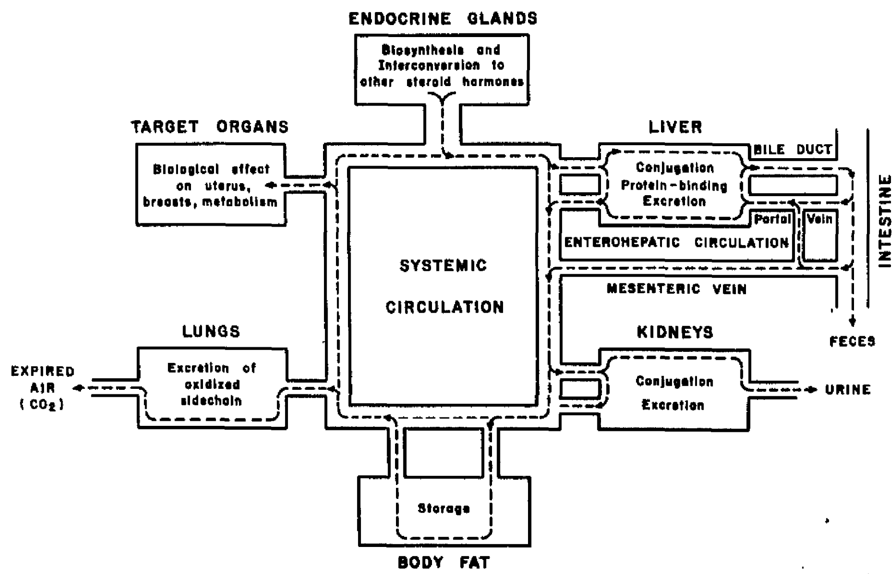


FIG. 6.

certain circumstances can have progesterone-like activity. Furthermore many new synthetic compounds have been produced with progestational activity.

The physiological effects of progesterone are varied. It is likelythat progesterone promotes the postovulatory or secretory changes in the endometrium but only after the endometrium has been prepared or primed by oestrogen; stimulates the decidual reaction in the stromal cells of the endometrium; and maintains the integrity of the embedded ovum early in pregnancy. Oestrogen and progesterone are considered to be responsible for the local changes in the generative tract and breasts in pregnancy. In this respect, oestrogen may be regarded, as the hormone of growth and increased blood supply, and progesterone as the hormone of function. During pregnancy the placenta produces large amounts of progesterone and appears to take

over from the corpus luteum of pregnancy as the chief source of supply of progesterone. Probably the most characteristic effect of progesterone on the myometrium is a block – the progesterone block (CSAPO 1961) i.e. a suppression of the propagation of membrane activity from one uterine portion to another. This effect is best observed on a uterus where activity has evolved as a result of oestrogen action – notably pregnancy. The relationship of progesterone blood levels to the onset of labour, and the importance of progesterone concentration in the physiology of uterine action are still undecided.

The roles played by progesterone in pathological states are not fully understood. The real significance of variations in progesterone production in certain disorders of pregnancy (e.g. abortion, pre-eclampsia, diabetes, inco-ordinate uterine action) is not known. The progesterone pattern in

disorders of the endometrium is only now being studied satisfactorily. The clinical effects of abnormalities in steroid synthesis and metabolism are still to be completely elucidated. For example, it is now known that congenital adrenal hyperplasia results in pseudohermaphroditism because the abnormal gland is unable to convert 17-hydroxyprogesterone to 17-hydroxycorticostrone. Accummulation of 17-hydroxyprogesterone, which is a precursor of the androgens, may account for the virilising changes and causes the abnormal urinary excretion of pregnanetriol in this condition. However, accurate study of the steroid status of female patients with hirsutism is only now becoming possible. The specific abnormality in hormone producing tumours in general is a field that has still to be adequately investigated. Full investigations of steroid secretion in patients with chromasomal abnormalities and intersex has still to be undertaken.

Rapid developments in steroid chemistry and the introduction of ingenious experimental techniques such as the perfusion of isolated endocrine glands and the placenta have helped to elucidate the biosynthesis and metabolism of hormones. The introduction of radioactive tracers into medicine, however, has provided new tools for the study of biological processes hitherto impossible to pursue accurately. Unlike concentration studies, this dynamic approach is particularly desirable for the investigation of the complicated metabolism of steroids and the various inter-relations between endocrine glands. There are limitations placed on such studies by the possible radioactive hazard but much basic information can be achieved within the limits of safety.

Many of the current ideas on the physiology of progesterone or its pathological significance have been based upon conjecture or on the results of inadequate research

technique, because, in any study of this hormone, the research worker, hitherto, has come up against a fundamental problem which virtually prevented any further advance. This problem was the simple straightforward one of the amount of progesterone present in the circulation or in the body at any one time and in any given state, the progesterone level being so low and the available assay methods insufficiently sensitive or specific that accuracy reliability and reproducibility were impossible to attain.

The advent of 1) reliable chemical methods for estimation of steroids, 2) the use of isotopically labelled steroids as a means of studying hormone metabolism, 3) and labelled reagent methods of steroid estimation, have made possible accurate techniques for measuring the amount of steroids produced by the body and present in the body fluids.

In view of the major unsolved problems

relating to the physiology of progesterone it seemed worthwhile to attempt to devise a method for the study of the metabolism of this hormone. The aim of this thesis, therefore, has been

A. to establish a method for the estimation of

1) The progesterone secretion rate - i.e. the amount of progesterone produced in the body per day.

11) The transport and metabolism constants, volumes of distribution, and metabolic clearance rate of progesterone.

111) The mean plasma progesterone level.

B. to apply the method to a group of normal males and a group of surgically castrate females as a preliminary to the application of the method to further groups of patients.

The study of the disappearance of radioactivity in plasma after the injection of isotopically labelled progesterone would elucidate the transport and metabolism of

the steroid in man. In particular, this would allow calculation of the metabolic clearance rate in various clinical conditions. This value together with a concomitant determination of the secretion rate estimated from the specific activity of a urinary metabolite (pregnanediol) would enable the mean plasma progesterone level to be calculated. This was particularly important as the direct analysis of progesterone in peripheral blood in the above two groups had so far been impracticable because of the very small amount present in the blood. Furthermore the estimation of progesterone secretion rates directly relates the pregnanediol output in the urine to the actual progesterone production of the intact organism without requiring correction for losses in the chemical processes involved in the method. This gives an accurate value for progesterone production which will allow comparison of the quantities produced by

various groups of patients in different clinical states.

Materials and Methods.

A research programme was devised to achieve a simultaneous study of the progesterone secretion rates and the disappearance of radioactive progesterone from the plasma of human subjects. To achieve this, chemical techniques for the estimation of progesterone secretion, and for the study of plasma radioactive progesterone levels had to be established.

The study of the progesterone secretion rates was carried out by the author of this thesis and this work is described in full. The collaborative study of plasma radioactive progesterone was the responsibility of Dr. Brian Little and his colleagues. An account of this latter portion of the research programme is included because the mean plasma progesterone level of the individual research subjects can be calculated from the knowledge of the metabolic clearance rate (which can be calculated from the disappearance curve of

plasma radioactive progesterone) and the secretion rate.

The method for the estimation of the progesterone secretion rate from the specific activity of urinary pregnanediol following a single intravenous injection of radioactive progesterone was proved to be applicable, specific, reproducable and efficient. The technique was applied to thirteen individuals to study their progesterone metabolism.

The list of chemicals and apparatus used in this research work is given in Appendix I.

The various stages of the experimental techniques are described in the following order -

1. Experimental subjects and their management. (Appendix I).
2. Extraction of pregnanediol from urine. (Appendix II).
3. Partition column chromatography. (Appendix III).

4. Scintillation spectrometry.
   (Appendix IV).
5. Absorption spectrophotometry.
   (Appendix V).
6. Proof of purity of pregnanediol recovered by the method. (Appendix VI).
   a. Absorption spectrum
   b. Acetylation.
   c. Counter current distribution.
   d. Infrared analysis before and after acetylation.
7. Plasma radioactive progesterone estimation.
   (Appendix VIII).
8. Constant infusion experiment.
   (Appendix VIII).

This is not the order in which the work was actually performed but it develops a logical sequential description of the method. The following explanation of the research processes is a simplified account so that a ready appreciation of the experimental approach to the problem can be achieved. A more

detailed discussion of the individual techniques, their function, capabilities and application in the research is contained in the appropriate appendices.

Experimental Subjects.

Two groups of patients were studied. The first consisted of 9 normal healthy adult males between the ages of 26 and 33 years. The second group consisted of 4 females between 37 and 48 years of age who had recently undergone total hysterectomy and bilateral salpingo oophorectomy and are henceforth referred to as castrate females.

The normal male was selected for the initial study because -

I. This was the first time the method had been applied in a human experiment.

II. By choosing a class of patient without a monthly cyclical change in progesterone production, any variation in results due to faulty technique would be more readily apparent.

III. The low level of progesterone production expected in the male would be an indication of the sensitivity of the method.

IV. No recorded results for progesterone secretion rates in males were known.

V. The secretion rate so measured would be the sum of the secretion of progesterone by the testes and the suprarenal glands.

The castrate female was taken for the second study group.

I. To further test the sensitivity of the method since this class of patient could be expected to have a lower progesterone production than the male.

II. To measure the progesterone secretion of the suprarenal gland, the ovaries having been eliminated by surgical castration.

III. To indicate the magnitude of testicular progesterone production by a comparison of the results from the normal males with those from the castrate females.

The details of the individual experimental

subjects and their exact management during the experiments are recorded in Appendix I. The injection programme was identical for all the patients except for the final experiment which utilised a constant infusion technique. This alteration did not affect the secretion rate study of this patient. The constant infusion experiment is fully recorded in Appendix VIII and its result and significance is discussed later.

The patient was fasted over night and the injection of radioactive progesterone arranged for 8 a.m. This was to ensure that the plasma lipids were at a low level so that the plasma samples obtained subsequent to the injection would be easier to handle during the progesterone extraction. The patient passed urine to empty the bladder just prior to the commencement of the experiment. At 8 a.m. a single intravenous injection of a known amount of tritiated progesterone was administered.

Thereafter venous blood samples were withdrawn at predetermined intervals and immediately taken for extraction.

The patient collected all urine passed for each of the next four days. Each daily urine output was collected in a two litre Winchester bottle containing 1.25 ml. Toluene as a preservative, and during this time it was kept in a refrigerator. When the output for a day (8 a.m. to 8 a.m.) was completed, the Winchester was transferred to a deep freeze and the urine frozen solid to await pregnanediol extraction.

Measuring of Pregnanediol in Urine.

Many methods have been devised to measure pregnanediol in the urine both as the sodium glucuronidate (Venning 1937, Allen & Viergiver 1941) and as the free alcohol obtained by acid (Astwood & Jones 1941, Talbot, Berman, MacLachlan & Wolfe 1941, Sommerville, Gough & Marrian 1948,

Chaney, McKee, Fischer & McColgan 1952) or enzyme (Goldfine & Cohen 1953) hydrolysis. In an attempt to improve the specificity of earlier methods, de Watteville (1951) and Stimmel, Randolph & Conn (1952) introduced alumina column chromatography of the crude urinary extracts. Klopper, Michie & Brown (1955) reported a still more elaborate procedure which employs column chromatography before and after acetylation of the extracts and the reliability and sensitivity of their method was confirmed in an exhaustive study by Coyle, Michell & Russell (1956). Bongiovanni and Clayton (1954) reported a double column chromatographic method which was used successfully with minor modification by Ronan, Parsons Namiot & Wotiz (1960). In 1958 Ebarlein and Bongiovanni described a new paper chromatography technique for pregnanediol assay. New methods continue to appear in the literature. Most recently Staub, Gaitan & Dingman (1962) have devised

a method using glass fibre paper chromatography.

The experimental work in this thesis employed enzyme hydrolysis of the urine and separation of the crude pregnanediol extract by ether extraction and benzene-alkali partition. The crude extract was purified by partition column chromatography. The pure pregnanediol sample was assayed for radioactivity by scintillation spectrometry, and measured quantitatively by absorption spectrophotometry. (Fig. 7).

Extraction of Pregnanediol from Urine.

The extraction procedure utilised a short β- glucuronidase incubation since pregnanediol is conjugated as a glucuronide which is split more rapidly by β-glucuronidase than are some other glucuronides. A 500 ml. aliquot of a 24 hour urine collection was incubated with 50 ml. β- glucuronidase at 37°C. with shaking for a period of about 16 hours (overnight). (Fig. 8).

The urine was then extracted with

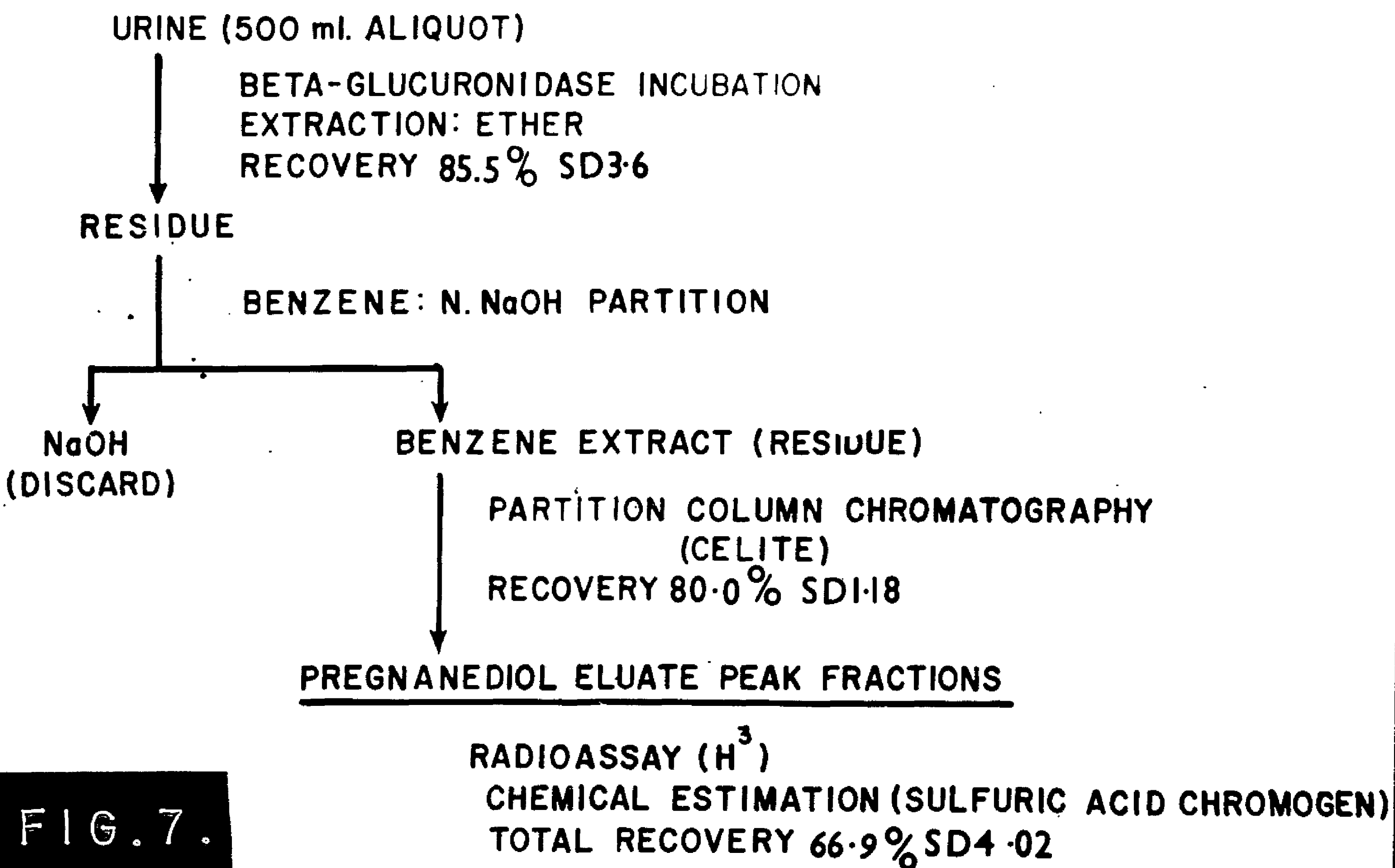


FIG. 7.

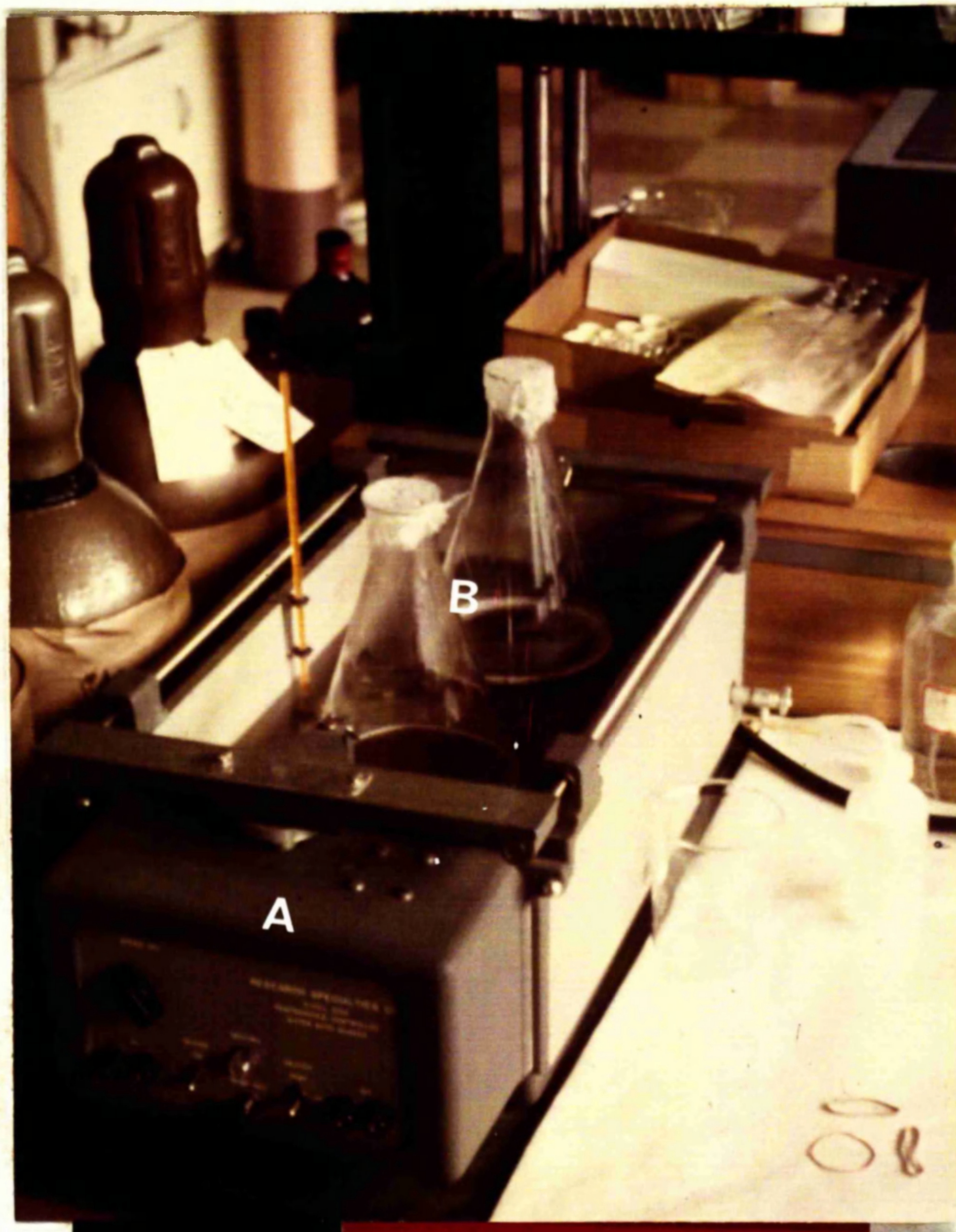


FIG.8. A. WATER BATH.
B. FLASKS WITH URINE.

peroxide free ether and the ether extract brought to dryness. The dry ether extract was partitioned between benzene and normal sodium hydroxide. This strong alkali wash helps to clean the extract, removes the phenolic compounds, and does not affect the pregnanediol.

The benzene extract was taken to dryness and was then ready for partition column chromatography. (Fig. 9).

Complete details of the steps involved in this extraction procedure, which is basically the one reported by Pearlman (1957, b), are given in Appendix II.

Partition Column Chromatography.

Since the greater part of any steroid hormone secreted is excreted in the form of metabolites chiefly in the urine, the secretion rate of the hormone can be evaluated if a metabolite can be specifically measured. One of the chief obstacles to this procedure is that any given metabolite

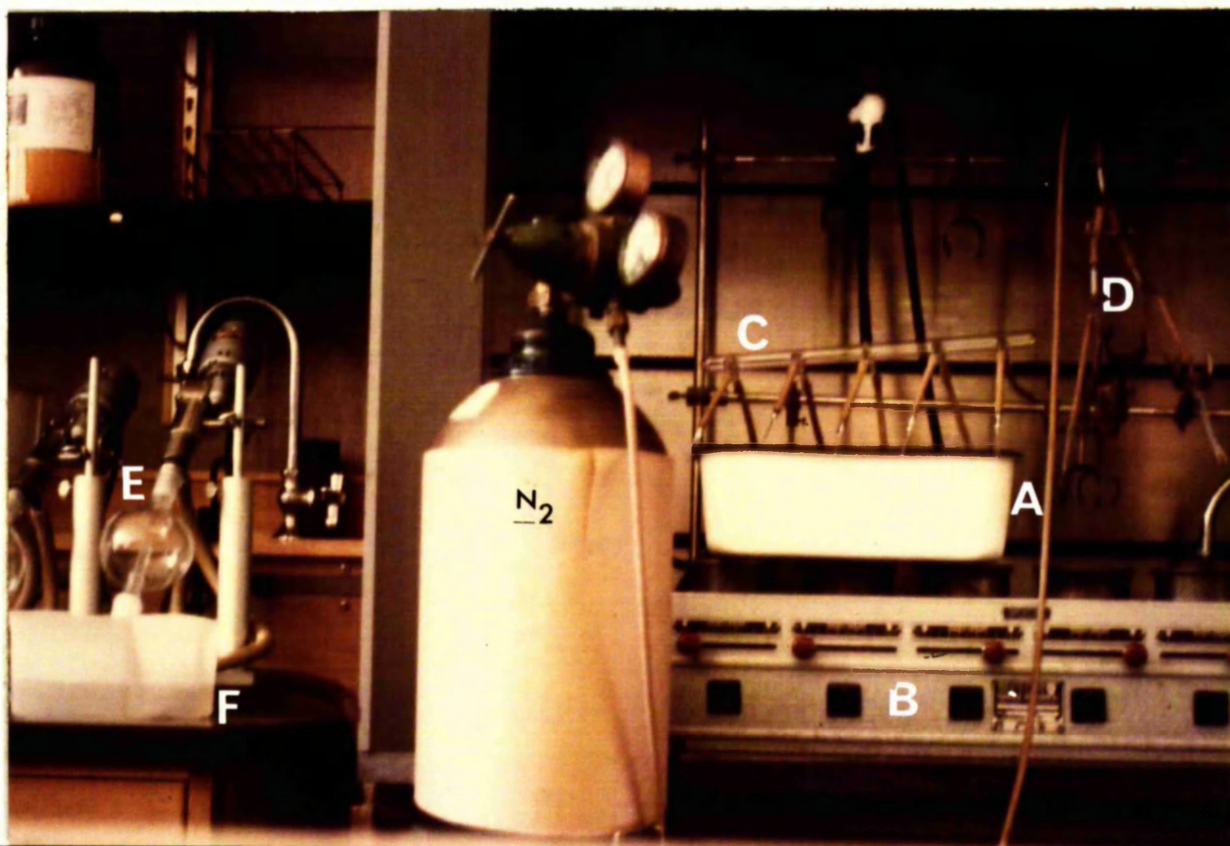


FIG.9. A.WATER BATH.

B.TEMPERATURE CONTROLLED HEATER.

C.AIR JETS FOR DRYING DOWN TUBES.

D.NITROGEN FOR DRYING DOWN TUBES.

E.ROTATING EXHAUST COUPLING.

F.FLASK WITH EXTRACT IN WATER BATH.

of one hormone may give the same chemical test as other metabolites of that hormone or metabolites of another hormone. This difficulty can be overcome by partition chromatography.

The value of chromatography lies in its ability to separate from each other, compounds which cannot be separated by chemical means. Mixtures of steroids may be separated chemically into several categories. The chemical separation depends on the reaction of functional groups in the molecule. For example oestrogens can be separated from neutral steroids because their phenolic hydroxyl groups form sodium salts, and ketones can be separated from non-ketones by means of their water soluble "Girard" hydrazones, but these procedures still leave mixtures of chemically similar compounds.

However, substances which have similar chemical properties may have quite different

physical properties since physical properties reflect the nature of the whole molecule. For example fatty acids all have one carboxyl group but differ progressively in solubility as their molecular weight increases. Chromatography is a physical process which exploits differences in a physical property among a series of chemically similar substances. This property can be termed "relative solubility in two immiscible solvents".

A chromatographic column is simply a modified glass tube containing a column of finely divided solid polar material which will adsorb and tightly hold one of the immiscible solvents and so constitute the stationary phase. By applying a crude extract to the top of this column and allowing the other immiscible solvent (the mobile phase) to flow down the column, the various components of the extract are eluted from the column at different rates in proportion

to their relative solubilities in the two phases. By collecting the eluate in serial volumes from an appropriate column system, a particular component of an applied extract can be obtained in pure form in a specific volume of the eluate.

The column constructed for this research work was made longer and more retentive towards less polar steroids to obtain better separation and maximum purity of pregnanediol. The column was 60 cm. in length and contained approximately 30 G of celite as the adsorbent material. The solvent system described by Eberlein and Bongiovanni (1955) was used. This consisted of -

Stationary phase	80% Methanol.
Mobile phase	2-2-4- Trimethylpentane 9 parts. Toluene 11 parts.

A discussion of the application of chromatography in this research and a detailed description of the construction and

use of the column is given in Appendix III.

The crude urine extract was applied to the column and the mobile phase allowed to run through it. The eluate was collected in 5 ml. measured portions into tubes by means of a Fraction Collector. Pregnanediol was recovered in the range of tubes No. 10 to 14. The rate of flow was about 5 ml. per hour so that the column required to run overnight. Tubes 1 - 16 were then accurately labelled and taken for further study.

<u>Scintillation Spectrometry.</u>

Scintillation Spectrometry.

The tritium labelled Progesterone (7-H^3 Progesterone) utilised in this research emits β particles from the unstable tritium atom attached to Carbon atom 7 on the steroid nucleus. The urinary pregnanediol produced from this radioactive progesterone retains the tritium on Carbon 7 and so is itself radioactive. The pregnanediol obtained after urine extraction and column

chromatography is the result of the metabolism of the injected tagged progesterone and of endogenous progesterone secreted by the patient. The radioactivity which this pregnanediol will therefore contain, can be detected by liquid scintillation spectrometry.

If a substance containing a β emitting isotope is dissolved in a dilute solution of a fluorescent compound in an aromatic solvent, the scintillor or fluor will convert the β particle energy to Light Quanta. The Packard Tri-Carb Scintillation Spectrometer (Fig. 10) detects, analyses and counts each light emission which results from the disintegration of a radioactive atom. The radioactivity of the sample is thus measured as counts per minute which can be corrected to disintegrations per minute if the efficiency of the system is known.

The sample prepared for scintillation counting was as follows -

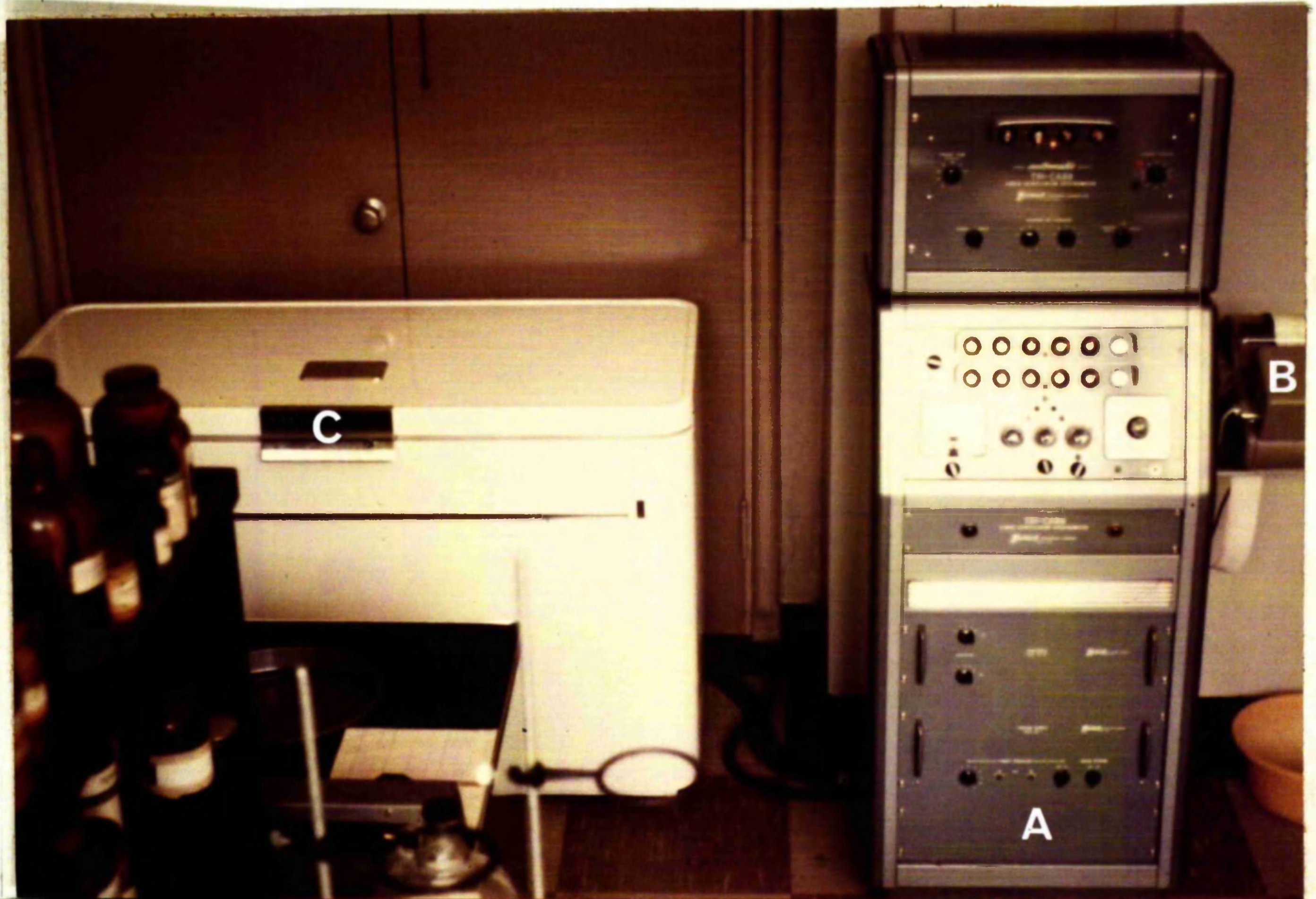


FIG. 10. A. SCINTILLATION SPECTROMETER.
B. AUTOMATIC COUNT RECORDER.
C. FREEZER AND SAMPLE CHANGER.

I) Solvent - Toluene.
Ethanol.

II) Fluor - Primary: 2.5-diphenyloxazole (P.P.O)

Secondary: 1.4-bis. 2-(5-phenyloxazolyl)-benzene (P.O.P.O.P.)

III) Vial - 22 ml. glass vial with silver foil lined plastic screw caps.

IV) Radioactive material - H^3 Progesterone.
H^3 Pregnanediol.
C^{14} Acetate.
Urine containing tritiated metabolites of progesterone.

The scintillation fluid was -

P.P.O. 4.0 G

P.O.P.O.P. 0.1 G

Toluene to 1 litre.

Ethanol added to give 2%.

Urine because of its water content, was not soluble in the toluene. Ethanol therefore had to be added to the scintillation fluid to hold the urine in solution. (6 ml. ethanol to 10 ml. scintillation fluid for 0.3 to

0.6 ml. urine).

Counting Experimental Pregnanediol.

The first 16 tubes of eluate from the chromatographic column were labelled in numerical order. Scintillation vials were similarly labelled from 1 to 16. Equal aliquots of 0.5 ml. were transferred from each tube to the corresponding vial and thereafter 10 ml. of scintillation fluid was added to each vial and the screw caps applied.

The vials were placed in order in the Scintillation Counter and counted for one minute each. The vials containing significant counts were then kept aside for a longer period of Counting.

Radioactive decay being a random process the measurement of radioactivity by recording disintegrations or counts per minute requires the numbers involved to be statistically significant. Furthermore there is a background level of radioactivity

which is always present. This has to be measured and a suitable correction for this applied to the results obtained from the machine. This is achieved by including a number of standard samples with the experimental series being counted. These standards include one containing no radioactivity so that counting it will record the background level of radiation. Another contains a known amount of radioactivity so that the counts per minute recorded by the Scintillation Counter can be correlated with the actual disintegrations per minute known to be present in that standard sample. From this the efficiency of the Scintillation Counter can be calculated and a check kept on the accuracy of the mechanism which must be maintained throughout each experiment and each series of experiments. Finally a standard sample of tritiated pregnanediol is counted so that the continued efficiency of the spectrometer

in estimating the experimental radioisotope can be confirmed. Thus each group of results from the Scintillation Counter must contain figures for the standard samples counted in series with the experimental samples.

Another factor of significance in liquid scintillation counting is quenching. Quenching is a process occurring in the sample which reduces the efficiency of the system so that fewer counts are recorded for any given radioactivity present in the sample. For example, should the solvent tend to absorb the β particle energy before it can cause fluorescence, this is "Thermal" quenching since the β particle energy is dissipated as heat: should the solvent tend to absorb the light emitted by the fluor before the light can be detected by the photomultipliers of the machine, this is "Colour" quenching. Quenching in the counting of the column samples was 2 to 3

per cent. Correction is made for this quenching factor (which was a constant throughout the experiments) in the final calculation for secretion rates.

When Ethanol was added to the urine samples, a considerable amount of quenching resulted (some 70 to 75 per cent). Thus the Counting efficiency for each sample had to be measured. This was achieved by the use of an internal standard. By this method a known amount of a standard of the same isotope (tritiated pregnanediol) is added to the sample after it has been counted. The sample is then recounted and the increment in count noted. This increment is then related to the counts obtained from an unquenched sample of the standard isotope, the quenching factor calculated, and each result suitably corrected.

A discussion of the function, application and efficiency of the liquid scintillation spectrometer with full details

of the methods in this research are contained in Appendix IV.

Absorption Spectrophotometry.

Absorption spectrophotometry is based on the observation and comparison of absorption spectra. The absorption spectrum - a curve showing the amount of radiant energy absorbed at each wavelength - is a characteristic property of chemical compounds: and each such chemical compound has its own absorption spectrum. Basically the spectrophotometer provides absorption data to assist the chemist in quantitative and qualitative analysis.

Problems involving the identification of organic compounds can be solved by spectrophotometric techniques through the use of the "matching fingerprints" method. If the spectroscopist has an extensive library of absorbance curves of known compounds, positive identification of the unknown is established if the unknown

spectrum matches any of the known spectra.

Usually in quantitative work the constituents of the sample are known and it is desired to determine the concentration of one or more of them. Quantitative spectrophotometry is based upon the fact that the absorbance of an absorbing material is dependent on its concentration. By comparing the absorbance of an unknown amount of pregnanediol with the absorbance of a known amount, the weight of the unknown amount of pregnanediol can be calculated. As the absorbance is not strictly directly proportional to the concentration, the system does not completely obey Beer's Law. A simple arithmetical comparison of absorbance and concentration cannot, therefore, be accepted and a correction factor has to be used (Allen 1950).

The only colorimetric assay presently available for pregnanediol is the yellow colour it gives with concentrated sulphuric acid. This is obviously very non specific

so that it is most important to effect sufficient purification of the pregnanediol from other substances giving the same test.

Talbot, Berman, MacLachlan and Wolfe, (1941) were the first investigators to apply this reaction specifically to the determination of pregnanediol although it had long been recognised that a variety of steroids develop yellow colour upon exposure to sulphuric acid. This lack of specificity is a serious disadvantage since the reliability of any method which employs the reaction must depend upon the degree of purity in which the steroid reactant has been isolated. The pregnanediol isolated during this research work has been proved to be of the required degree of purity as will be shown later.

Klopper (1956), Klopper & Michie (1956) and Klopper, Strong & Cook (1957) have thoroughly studied the characteristics of the reaction. Contamination of the sulphuric acid with traces of oxidising

agents exerts a considerable influence upon the chromogenicity. In order to obviate this source of error Klopper has advocated inclusion of a small amount of reducing agent, such as sodium sulphite, in the reaction mixture. Moreover he has emphasised the influence of solvent residues upon colour development and has advised that standard samples be prepared in identical solvents used for the steroid extracts.

Comparatively little is known about the mechanism of the colour development but it is likely that it involves dehydration of reactive groups such as hydroxl groups (Gold & Tye 1952). A variety of steroid hormones constitute potential sources of interference in estimation of pregnanediol by the sulphuric acid colour reaction (Sommerville, Marrian & Keller 1948) and acid hydrolysis contributes to this (Bongiovanni & Clayton 1954). Dehydroepiendrosterone and pregnanetriol produce strong colour

reactions; on the other hand pregnaneolone and allopregnaneolone possess negligible chromogenicity. A broad spectrum of non steroid metabolites and drugs may cause artefactitious elevations (Gold & Tye 1952).

The colour reaction in this research work has been carried out by the method described by Eberlein & Bongiovanni (1958) with minimal modification. 100-110 G of sodium bisulphite is added to 400 ml. concentrated sulphuric acid and this "bisulphite sulphuric acid" is what is used to develop the colour. The tubes of column eluate containing the significant pregnanediol radioactivity detected by scintillation counting are taken and 2 ml. aliquots are transferred to appropriately labelled boiling tubes. 2 ml. aliquots of pure column solvent were also added to tubes containing known amounts of pure pregnanediol. All the tubes were dried

down under a stream of air. Thereafter they were placed in a vacuum dessicator and a vacuum established with a vacuum pump. They were left under the vacuum overnight to make sure they were absolutely free from water.

Next day 4 ml. of bisulphite sulphuric acid wereadded to each tube and the tubes placed in a boiling water bath for 4 minutes. They were then removed and allowed to cool at room temperature and in about 20 minutes they were ready for reading in the Beckman D.U. Spectrophotometer. (Fig. 11).

A further account of the application of spectrophotometry in this research with a detailed description of the complete technique is contained in Appendix V.

Proof of Purity of Pregnanediol Recovered by the Method.

This was the final proof of the specificity and reliability of the method. An accepted method of pregnanediol extraction

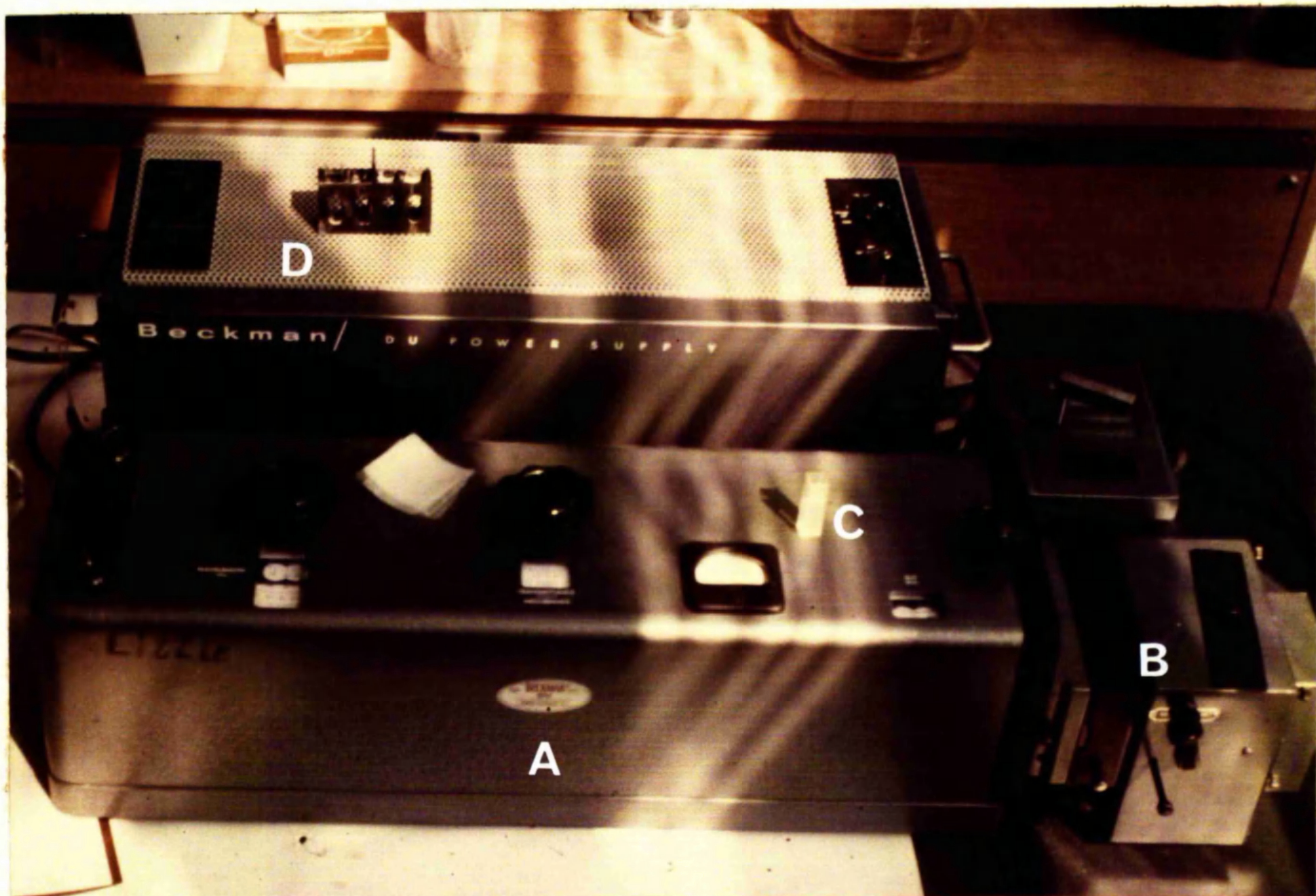


FIG. II. A. BECKMAN SPECTROPHOTOMETER.
B. CUVETTE CARRIER CHAMBER.
C. 4 ML QUARTZ CUVETTE.
D. CUVETTE CARRIER.

from urine was used (see Appendix II). The Scintillation Counter was appropriately set to detect the radioactivity of the tritium labelled compounds (see Appendix IV). A reliable method of pregnanediol estimation by absorption spectrophotometry was established (see Appendix V). A chromatography column (Appendix III) was built which proved efficient in recovering pregnanediol as did the method as a whole (see Results - Proof of Method).

Now it had to be shown that the substance obtained from the column during an experiment was pure pregnanediol. This was confirmed in four ways, full details of which are contained in Appendix VI.

1) Absorption Spectrum:

The absorption spectrum of an experimental sample of pregnanediol was compared with that of a known pure sample of pregnanediol and found to be similar.

II) Acetylation:

Pregnanediol diacetate can be formed from pregnanediol by incubating pregnanediol with acetic anhydride in the presence of pyridine. If the specific activity of the pregnanediol and the pregnanediol diacetate are constant this is a further proof of the purity of the pregnanediol.

An experimental sample of tritiated pregnanediol was acetylated with tagged acetic anhydride (1 - C^{14} - acetic anhydride) and a pure known standard sample of pregnanediol was similarily and simultaneously acetylated as a control.

The pregnanediol diacetate was separated from any residual pregnanediol by paper chromatography (see Appendix III for discussion on chromatography).

The pregnanediol diacetate radioactivity was measured by scintillation spectrometry and the diacetate estimated quantitatively by absorption spectrophotometry. The specific

activity of the experimental pregnanediol and its derivative, pregnanediol diacetate, were shown to be similar.

III) Counter Current Distribution of pregnanediol diacetate.

This is another method of chromatography (see Appendix III). A simple eight tube Counter Current System was used with petroleum ether and 90 per cent methanol as the Solvent System.

The control pregnanediol C^{14} diacetate and the experimental $7H^3$ pregnanediol C^{14} diacetate was submitted to this Counter Current distribution. The distribution of the radioactivity of the control Carbon 14, and the experimental Tritium and Carbon 14 atoms were compared and shown to be similar confirming the purity of the experimental pregnanediol through the purity of its diacetate derivative.

IV) Infrared Analysis:

Independent proof of the purity of

pregnanediol obtained from the method was obtained by submitting an experimental sample to Dr. Lewis L. Engel, Ph.D., Associate Professor of Biological Chemistry, Harvard Medical School, for infrared analysis before and after acetylation.

Because of the small quantities of pregnanediol produced by the experimental subjects, urine samples from two pregnant patients near term were extracted to obtain sufficient pregnanediol. Tritiated pregnanediol was added as an internal standard. Because the extracts contained more pregnanediol than the columns could handle, each extract was divided into three before column chromatography.

The pregnanediol recovered from these experiments was assayed by Scintillation Spectrometry and Absorption Spectrometry to check the efficiency of the method for pregnant urine. The pregnanediol samples from the column were pooled, taken to

dryness under a stream of air and then redissolved in methanol.

The pregnanediol was crystallised out from solution in methanol by adding distilled water. This was repeated three times before the sample was submitted for infrared analysis. Dr. Engel found that the infrared spectra of the sample before and after acetylation agreed in all respects with the reference samples. His report is included in Appendix VI.

Plasma Radioactive Progesterone Estimation.

Venous blood samples were taken from the experimental subject 2.5, 5, 7.5, 10, 15, 20, 30, minutes after injection as a rule but on occasion later samples were taken at 50 and 70 minutes. Enough blood was withdrawn to obtain 5, 5, 10, 10, 20, 20, 20 ml. plasma respectively. If 50 and 70 minute samples were taken, 40 and 100 ml. plasma were required.

The blood samples were centrifuged

and the plasma separated. C^{14} progesterone was added to each sample as internal standard and cold progesterone also added as carrier.

The progesterone was separated by ethyl acetate extraction. The extract was taken to dryness and placed on a 60 cm., 30 G Celite Column. The Solvent system was 80% methanol and 224 trimethylpentane.

The progesterone came off in three tubes (11 12 13 as a rule) which were pooled and counted for radioactivity in the Scintillation Counter. The internal standard enabled the samples to be corrected for loss during the extraction and chromatography. (Fig. 12).

Full details of the method and the settings of the Counter for assaying the mixture of 4 C^{14} and $7H^3$ progesterone are given in Appendix VIII.

Constant Infusion Experiment.

At the steady state when the endogenous progesterone production rate, P, and the

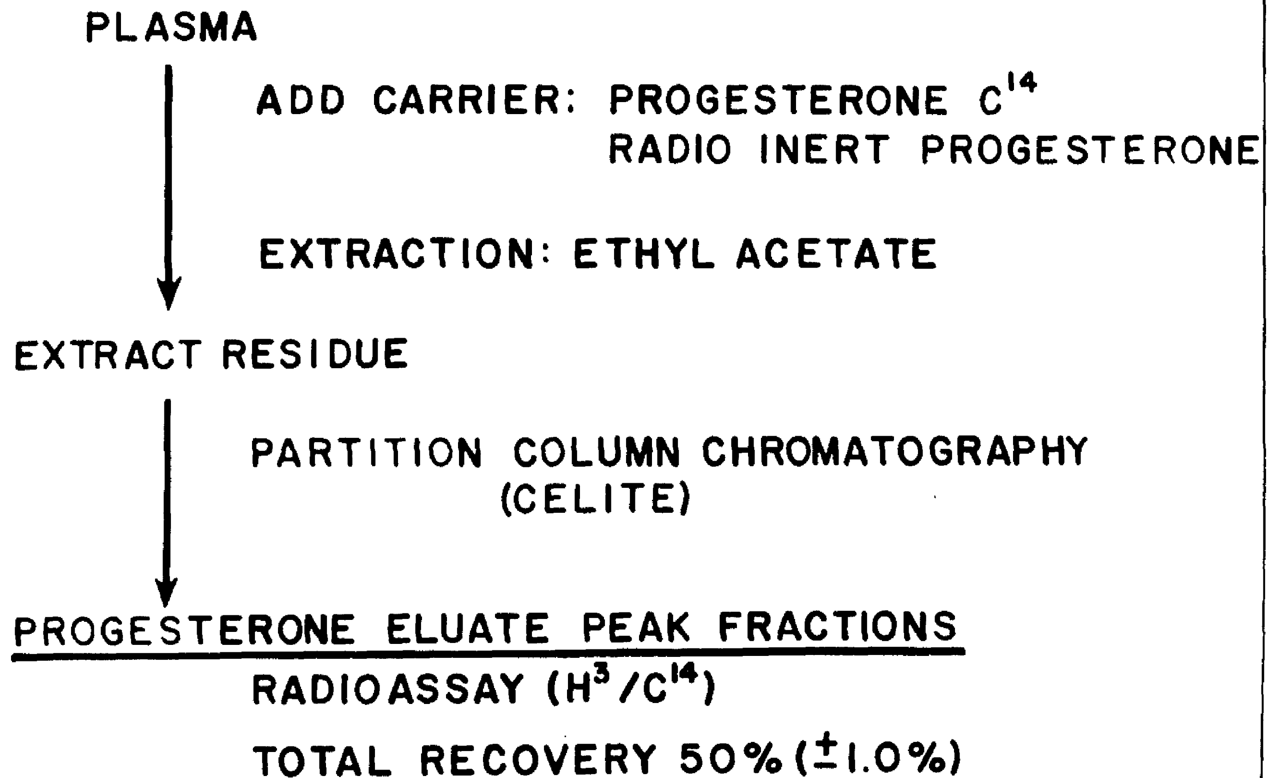


FIG. 12.

total amount, A, of circulating hormone are constant, $\frac{A}{P}$ = the turnover time, i.e. the time required for the complete replacement of the circulating hormone by a fresh supply from the endocrine glands. Since A is the product of the blood hormone concentration i and the blood volume v, the turnover time = $\frac{i \times v}{P}$.

The turnover rate constant TR is the fraction $\frac{P}{A}$ of the total amount of hormone replaced per unit time, i.e. the reciprocal of the turnover time TR = $\frac{P}{i \times v}$ with the dimension of Time $^{-1}$.

The endogenous progesterone production rate P (Secretion rate) = TR x v x i (After Pearlman 1957 a).

Therefore P = M x i where M is the metabolic clearance rate i.e. the volume of plasma cleared of progesterone per unit time. Thus, in the mathematical treatment applied to this research work, metabolic clearance rate has the dimension of Volume ÷ Time.

Consequently the mean plasma progesterone level $i = \frac{P}{M}$, the progesterone secretion rate being estimated from the urinary pregnanediol excretion and the metabolic clearance rate from the plasma radioactive progesterone disappearance curve. (Fig. 13).

Having obtained a mean value for the metabolic clearance rate M from a series of experiments on male subjects it is possible to calculate the amount of radioactive progesterone which has to be injected over a period of time as a constant infusion after an initial priming dose so that the infusion is equal to the rate of metabolism of radioactive steroid. If the experimental method is accurate then this should results in the plasma radioactive progesterone disappearance curve becoming a straight line during the time of infusion. Such an experiment was successfully carried out. This confirms the validity of the plasma method and the calculation of the mean plasma

ENDOGENOUS SECRETION RATE

$$= \frac{\text{RADIOACTIVITY OF PROGESTERONE INJECTED}}{\text{SPECIFIC ACTIVITY OF URINARY PREGNANE } 3\alpha\, 20\alpha \text{ DIOL}} \left(\begin{array}{c}\text{CORRECTED FOR}\\ \text{DAYS OF URINE}\\ \text{COLLECTION}\end{array}\right)$$

$$= \text{mg. PROGESTERONE SECRETED/DAY}$$

METABOLIC CLEARANCE RATE

$$= \frac{\text{SECRETION RATE}}{\text{BLOOD CONCENTRATION}} = \text{LITERS OF PLASMA (BLOOD) CLEARED OF PROGESTERONE/DAY}$$

FIG. 13.

progesterone levels. Full details of this experiment are contained in Appendix VIII.

The theory of secretion rate calculation is fully discussed in Appendix VII and the mathematical treatment of metabolic clearance rate studies by single injection and constant infusion techniques is discussed in Appendix IX.

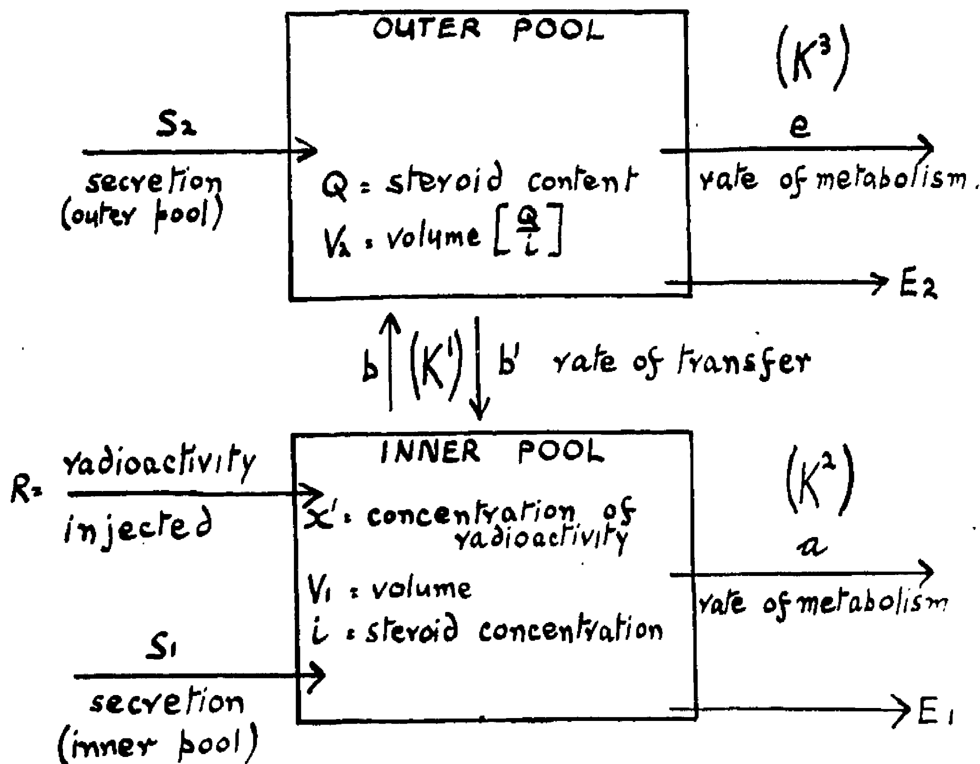
However it is pertinent at this juncture to present a summary of the theory and mathematical treatment applied to the calculation of the results of this research work.

For the purpose of describing the metabolism and transport of progesterone, or of any steroid whose plasma disappearance curve after a single injection can be described adequately by two exponential functions, let the body be represented by two compartments. The radioactive steroid is introduced into the inner pool which includes the plasma. (See Figure 40).

MODEL FOR STEROID METABOLISM

$$M = \frac{S_1 + S_2}{i}$$

$$= \frac{\text{total secretion rate}}{\text{plasma concentration of steroid}}$$



$$f = \frac{1}{1 - \frac{S_2 e}{S(S_2 + b)}} \qquad w = \frac{1 + \left(\frac{a}{a+e}\right)\frac{S_1}{b}}{1 + \left(\frac{E_1}{E_1 + E_2}\right)\frac{S_2}{b}}$$

E_1 and E_2 = rate of excretion of a particular metabolite

DUPLICATE OF FIG. 40.

The whole of the inner pool is assumed to be in rapid equilibrium with the plasma. Transport of steroid into the other outer pool, is relatively slow. These pools are not necessarily well defined anatomically. (In pregnancy however, the possibility must be considered that the foetus is part of the outer pool, and that secretion, or metabolism of the hormone, or both may occur there). Metabolism and secretion of the hormone are therefore assumed, in the general case, to occur in both pools. (See Fig. 40).

The mathematical proof contained in Appendix VII (after Laumas, Tait & Tait 1961) and Appendix IX (after Tait, Tait, Little & Laumas, 1961) can be summarised as follows (after Tait, Little, Tait & Flood, 1962).

Let -

S_1 = Secretion rate into inner pool.

S_2 = Secretion rate into outer pool.

$P = S_1 + S_2$ = total secretion rate.

I = Steroid content of inner pool.

Q = Steroid content of outer pool.

i = Steroid concentration in inner pool (including plasma).

a = Steroid metabolised in inner pool in unit time.

e = Steroid metabolised in outer pool in unit time.

b = Steroid transferred from inner to outer pool in unit time.

b^1 = Steroid transferred from outer to inner pool on unit time.

θ_1 = Fraction of steroid in inner pool transferred to outer pool in unit time $(\frac{b}{I})$

θ_2 = Fraction of steroid in outer pool transferred to inner pool in unit time $(\frac{b^1}{Q})$

θ_3 = Fraction of steroid in inner pool metabolised in unit time $(\frac{a}{I})$

θ_4 = Fraction of steroid in outer pool metabolised in unit time $(\frac{e}{Q})$

x = Fraction of radioactivity (measured specifically as the hormone) in inner pool at time t

z = Fraction of radioactivity (measured specifically as the hormone) in the outer pool at time t.

x^1 = Fraction of injected radioactivity as concentration (equal to that in plasma) at time t.

V_1 = Volume of the inner pool $\left(\frac{I}{i}\right)$

Dynamic equations are

$$-\frac{dx}{dt} = \theta_1 x - \theta_2 z + \theta_3 x \qquad [1]$$

and $$\frac{dz}{dt} = \theta_1 x - \theta_2 z - \theta_4 z \qquad [2]$$

assuming first order individual reactions, homogenicity of the two pools and no recirculation of isotope after metabolism. Steady state requirements are

$$\theta_4 Q + \theta_3 I = S_1 + S_2 = P \qquad [3]$$

since total rates of metabolism and secretion must be equal. Also, for no net accummulation or disappearance in pool,

$$(\theta_2 + \theta_4)\ Q - \theta_1 I = S_2 \qquad [4]$$

$$(\theta_3 + \theta_1)\ I - \theta_2 Q = S_1 \qquad [5]$$

$$S_1 + b^1 = a + b \qquad [6]$$

$$S_2 + b = b^1 + e \qquad [7]$$

Also

$$\frac{I}{P} = \frac{\theta_2 + \theta_4}{\theta_2\theta_3 + \theta_4\theta_3 + \theta_4\theta_1} \times \left[1 - \frac{S_2 e}{P(S_2 + b)}\right] \qquad [8]$$

Secretion Rate:

Where E_1 = rate of excretion (combined formation of metabolite and its renal clearance) of one particular metabolite from the inner pool, E_2 = rate of excretion of one particular metabolite from the outer pool, and R = total radioactivity injected, then the total radioactivity excreted as metabolite =

$$R \times \left[\frac{\frac{E_1\ (\theta_2 + \theta_4)}{I} + \frac{E_2\theta_1}{Q}}{\theta_1\theta_4 + \theta_3\theta_2 + \theta_3\theta_4}\right] \qquad [9]$$

Thus where T = time of urine collection and S.A. = Specific activity of the metabolite.

$$SA = R\left(\frac{E_1 + E_2}{a+e}\right) \left(\frac{1}{(E_1 + E_2)T}\right) \qquad [10]$$

$\frac{R}{SA}$ = Secretion rate x single injection factor (w).

$$\text{Where} \quad w = \frac{1 + \left(\frac{a}{a+e}\right) \frac{S_2}{b}}{1 + \left\{\frac{E_1}{E_1+E_2}\right\} \frac{S_2}{b}} \qquad [11]$$

(See Appendix VII).

Single injection:

$x = Ae^{-\alpha t} + Be^{-\beta t}$ is the Solution of Equations [1] and [2] and

$$\alpha + \beta = \theta_1 + \theta_2 + \theta_3 + \theta_4 \qquad [12]$$

$$\alpha\beta = \theta_2\theta_3 + \theta_4\theta_3 + \theta_4\theta_1 \qquad [13]$$

$$A\alpha + B\beta = \theta_1 + \theta_2 \qquad [14]$$

$$\text{and} \quad A\beta + B\alpha = \theta_2 + \theta_4 \qquad [15]$$

As pointed out by Tait, Tait, Little and Laumas (1961) these equations cannot be solved for θ_1, θ_2, θ_3, or θ_4, nor for $\frac{I + Q}{i}$ = the final volume of distribution, unless θ_3 or θ_4, metabolism in either pool, is made equal to zero.

In all cases, however

$$V_1 = \frac{1}{A^1 + B^1} \qquad [16]$$

being the reciprocal of the plasma concentration at zero time. x^1 (the plasma concentration at time t)

$$= A^1 e^{-\alpha t} + B^1 e^{-\beta t}$$

$$\frac{A^1\alpha + B^1\beta}{A^1 + B^1} = \theta_1 + \theta_3$$ (from [8] and [13]). [17]

and $$\frac{A^1\beta + B^1\alpha}{A^1 + B^1} = \theta_2 + \theta_4$$ (from [12] and [13]) [18]

A^1, B^1, α and β are the experimentally determined quantities. (See Appendix IX).

Then MCR = $\frac{\text{total secretion rate}}{\text{concentration of nonisotopic steroid in plasma}}$

$= \frac{P}{I}$ by definition

As before

$$\frac{I}{P} = \left[\frac{\theta_2 + \theta_4}{\theta_2\theta_3 + \theta_4\theta_3 + \theta_4\theta_1}\right] \times \left[1 - \frac{S_2 e}{P\,(S_2 + b)}\right] \qquad [8]$$

and from equations 13, 15, 16, 18.

$$\frac{1}{P} = \frac{A^1\beta + B^1\alpha}{\alpha\beta} \times \left[1 - \frac{S_2 e}{P(S_2 + b)} \right]$$

$$MCR = \frac{\alpha\beta}{A^1\beta + B^1\alpha} \times \left[\frac{1}{1 - \frac{S_2 e}{P(S_2 + b)}} \right]$$

Let the estimated MCR value

$$MCR_E = \frac{\alpha\beta}{A^1\beta + B^1\alpha} \qquad [19]$$

Then true MCR = MCR_E x f and

$$f = \frac{1}{1 - \frac{S_2 e}{P(S_2 + b)}} \qquad [20]$$

Constant infusion:

Let r = rate of infusion of radioactive steroid (fraction of total infused radioactivity in unit time). Then

$$\frac{dx}{dt} = -(\theta_1 + \theta_3)x + \theta_2 z + r \qquad [21]$$

$$\text{and } \frac{dz}{dt} = \theta_1 x \quad \theta_2 Z - \theta_4 Z \qquad [22]$$

The infusion is continued until x is constant, (x_c) as determined experimentally,

when z must also be constant. Then

$$\frac{dx}{dt} = 0 = \frac{dz}{dt} \quad \text{at} \quad x_c \qquad [23]$$

$$\text{and} \quad r = \left\{ \frac{\theta_1\theta_4 + \theta_2\theta_3 + \theta_4\theta_3}{\theta_2 + \theta_4} \right\} x_c$$

from equations [18], [19], and [20].

$$\text{Then } \frac{P}{I} = \frac{r}{x_c^{\ I}} \times \left[\frac{1}{1 - \frac{S_2 e}{P\ (S_2 + b)}} \right]$$

$$\text{and} \quad \frac{P}{I} = MCR = \frac{r}{x_c^{\ I}} \times \left[\frac{1}{1 - \frac{S_2 e}{P(S_2 + b)}} \right] \qquad [24]$$

Then let estimated MCR

$$MCR_E = \frac{r}{x_c^{\ I}} \qquad [25]$$

Then the true MCR = $MCR_E \times f$ when

$$f = \frac{1}{1 - \frac{S_2 e}{P(S_2 + b)}} \qquad [26]$$

Therefore in the general case for both the Constant infusion and Single injection methods the estimates of equations [19] and [25] will give the same result.

$MCR = MCR_E \times f$ and f is the same for both equations [20] and [26]. If $f = 1$, this estimate will be valid and this will be the case when either S_2 or e is zero, i.e. when secretion or metabolism is negligible in the outer pool. It should be noted that these criteria for validity of the MCR method are similar to those for the valid calculation of secretion rates from the specific activity of a urinary metabolite. (See Appendix VII).

RESULTS.

The results are calculated from the experimental data detailed in Volume II and are collated in the following order.

A. Method Proof.

B. Proof of Purity of Experimental Pregnanediol.

C. Experimental Subjects.

D. Collaborative Work.

E. Combined Results.

Statistical analysis and comparison of results are included where relevant. The statistical methods employed are

a) The t test.

b) Calculation of the Correlation Coefficient.

c) Analysis of Variance.

The following symbols and equations are used -

Number in series: N and N'

Arithmetic mean: $\bar{x}$ and $\bar{x}'$

Standard Deviation: s or S.D.

Standard Error: S.E. $= \frac{s}{\sqrt{N}}$

Variance: s^2

Summation Sign: S

$$x + x + x + \text{----} = S\,(x)$$

Standard deviation of difference of mean : s_D

Common variance: $s^2\ xx'$

Degrees of freedom: $n = N + N' - 2$

Coefficient of variation = SD±%

$$s^2 = \frac{1}{N-1}\left(S\,(x^2) - \bar{x}\ \ S(x)\right)$$

$$s^2\ xx' = \frac{S(x^2) - \bar{x}\ S(x) + S(x'^2) - \bar{x}\ S(x')}{N + N' - 2}$$

$$s_D = \sqrt{\frac{s^2 xx'}{N} + \frac{s^2 xx'}{N'}}$$

The deviations (t) by means of which the probability (P) is obtained are calculated from $t = \frac{\bar{x} - \bar{x}'}{S_D}$

$$n = N + N' - 2$$

P is then read from statistical tables (Fisher R.A. and Yates F. 1953; Statistical Tables for Agriculture, Biological and Medical Research, pub. Oliver & Boyd, Edinburgh).

RESULTS.

A. METHOD PROOF.

a) Column Recovery of Pregnane-3α,20α-diol.

b) Column Recovery of Pregnanetriol.

c) Column Recovery of Tritiated Pregnanediol.

d) Recovery of internal standard H^3 pregnanediol from urine.

a) <u>Column recovery of pregnane - 3α,20α-diol.</u>

Techniques - Partition column chromatography (Appendix III).

Absorption Spectrophotometry (Appendix V).

Detailed Results - Volume II (pages 5-10).

Experiment.	Column.	Tubes.		% Recovery.
1	I	11,12		78.4
2	I	12,13,14,15		78.8
3	I	12,13		78.6
4	II	12,13		82.8
5	I	11,12		77.3
			Mean	79.2
			S.D.	2.11
			S.E.	0.94

b) Column recovery of pregnanetriol.

Techniques - Partition Column chromatography (Appendix III).

Absorption Spectrophotometry (Appendix V).

Detailed results - Volume II (pages 11-16).

Experiment	Isomers.	Column.	Tubes.	% Recovery.
1	Pregnane-3,/17α, 20-triol	I	24-34	72.4
2	Pregnane-3, 17α, 20-triol	II	26-36	77.8
3	Pregnane-3, 17α, 20-triol	I	28-30	66.2
4	Pregnane-3α, 17α, 20α-triol	I	32-35	74.5
5	Pregnane-3α, 17α, 20α-triol	II	36-39	66.4
			Mean	71.5
			S.D.	5.1
			S.E.	2.3

e) Column recovery of tritiated pregnanediol.

Techniques - Partition Column chromatography (Appendix III).

Scintillation Spectrometry (Appendix IV).

Absorption Spectrophotometry (Appendix V).

Detailed results - Volume II (pages 17-21).

Experiment.	Column.	Tubes.	%Recovery. Weight	%Recovery. Radioactivity.
1	I	11, 12, 13	80.5	78.7
2	II	12, 13, 14	79.0	81.7
3	III	13, 14	81.4	79.5
4	IV	8, 9	79.0	86.8
		Mean	80.0	81.7
		S.D.	1.18	3.64
		S.E.	0.59	1.82

d) Recovery of internal standard H^3 pregnanediol from urine.

Techniques - Urine extraction (Appendix II).
Partition Column Chromatography (Appendix III).
Scintillation Spectrometry (Appendix IV).
Absorption Spectrophotometry (Appendix V).

Detailed results - Volume II (pages 22-36).

Experiment.	Sex.	Column.	% Recovery Urine Extraction.	% Recovery Total Method.	Pregnanediol mgm/day corrected.
1	F	II		64.0	0.578
2	M	I	84.3	68.9	0.2682
3	M	II	90.9	70.1	0.1635
4	M	IV	83.5	61.3	0.1938
5	M	I	83.5	70.3	0.1842
	Mean		85.5	66.9	0.2024 (Exp. 2, 3, 4, & 5)
	S.D.		3.6	4.02	.036
	S.E.		1.8	1.8	.018

Calculated Column Recovery of H^3 pregnanediol = 78.2%

These results show that the components of the method and the total method for pregnanediol as applied in this research project are reliable, efficient, and reproduceable.

The chromatographic column was not so effective for the recovery of pregnanetriol which was eluted at a slow rate and over a greater number of tubes. The larger spread could be related to a mixture of isomers in three of the standard samples. The two samples of pure pregnane-3α,17α,20α-triol were eluted in four tubes. The method was applied to one sample of female urine collected during a period of amenorrhoea (see Volume II, page 37). The total output of pregnanetriol for the day was 0.825 mgm. (corrected for a recovery rate of 64% - the recovery for internal standard H^3 pregnanediol in that experiment).

Fotherby & Love (1960) report a pregnanetriol excretion in female urine of 0.1 to 3.0 mgm. per day (mean 0.9) and

Ronan, Namiot, Parsons and Wotiz (1960)
0.3 - 1.2 mgm. per day (mean 1.14).

A critical study of the method applied to pregnanetriol recovery was not undertaken at this time but such a study is merited because the results so far are encouraging and if the method proves effective a study of pregnanetriol excretion could be undertaken to investigate the metabolism of 17α hydroxyprogesterone.

RESULTS.

B. Proof of Purity of Experimental Pregnanediol.

a) Absorption spectra of pregnanediol.

b) Acetylation of Experimental pregnanediol sample.

c) Counter current distribution of experimental pregnanediol diacetate.

d) Infrared analysis of Experimental pregnanediol sample.

a) <u>Absorption spectra of pregnanediol:</u>

Technique - Absorption spectrophotometry (Appendix VI).

Detailed results - Volume II (pages 39, 40).

The absorption spectrum of an experimental sample of pregnanediol was compared with spectra of samples of known pregnane-3α,20α-diol (see Fig. 14). The spectrum of the experimental sample is similar to the spectra of standard samples of pregnanediol. Comparison of the spectrum of the experimental sample with the spectrum of the 50 gamma standard gives a Correlation Coefficient of 0.973 (For perfect Correlation the Correlation Coefficient is + 1.0).

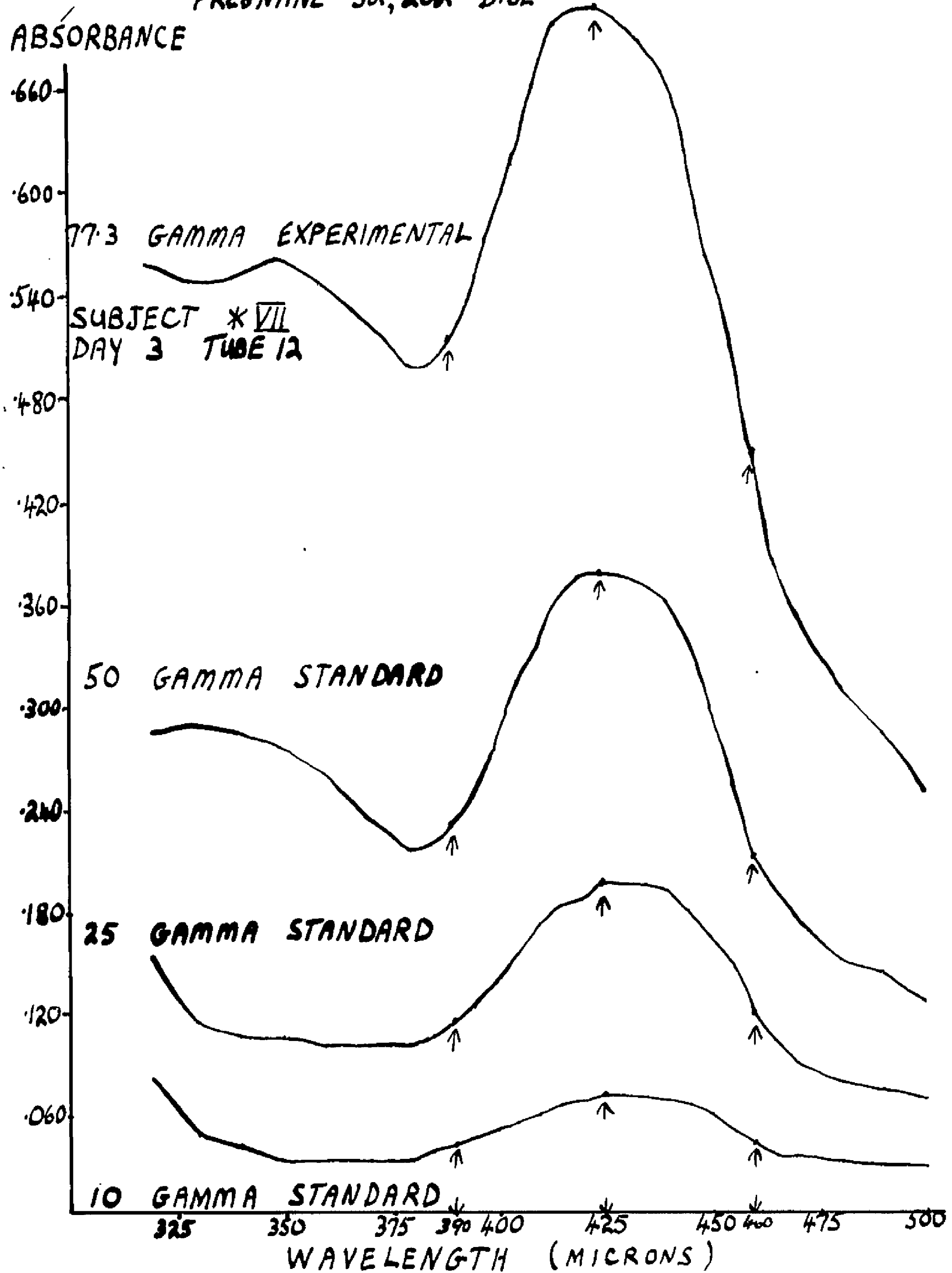


FIG. 14.

b) Acetylation of experimental pregnanediol sample:

Techniques - Paper partition chromatography (Appendix II).

Scintillation Spectrometry (Appendix IV).

Absorption Spectrophotometry (Appendix V).

Acetylation (Appendix VI).

Detailed results - Volume II (pages 41-44).

Experimental pregnanediol sample -

2.5 ml. from tube 11 and 2.5 ml. from tube 12 from Experimental Subject *I, day 1, (extraction Method 3) plus 300 gamma standard pregnanediol (not radioactive) as carrier.

Specific activities (counts per minute per milligram).

1)	Tube 11	- 252,900
2)	Tube 12	- 279,200
3)	Mean (tube 11 & 12)	- 266,050
4)	Experimental sample (before acetylation).	- 63,485
5)	Corrected for added carrier	- 219,085
6)	Experimental sample (after acetylation).	- 57,331
7)	Corrected for added carrier	- 179,906
8)	Experimental sample (after acetylation). Corrected for difference in molecular weights of pregnanediol and pregnanediol diacetate	- 72,237

9) plus correction for added carrier 249,362

(Ratio of molecular weight of pregnanediol diacetate to molecular weight of pregnanediol = 1.26. To allow comparison of specific activities, the specific activity of the diacetate has to be increased by the factor 1.26 because, with fewer molecules per milligramme, the specific activity of the diacetate will contain 1.26 more radioactivity (disintegrating atoms) than a similar specific activity of pregnanediol.

Comparison of the above specific activities gives the following deviations from the mean.

1) and 2)	± 4.9%
3) and 5)	± 9.7%
4) and 8)	± 6.4%
3) and 9)	± 3.2%

The specific activities of the experimental pregnanediol and its diacetate were constant (within ± 5 to 10% - the error of the method) so giving a second proof of the purity of the experimental pregnanediol sample.

c) <u>Counter current distribution of the acetate derivation of the experimental pregnanediol.</u>

<u>Sample:</u>

Techniques - Counter Current distribution (Appendix III - Appendix VI).

Scintillation spectrometry (Appendix IV).

Detailed results - Volume II (pages 45-48).

Since the acetic anhydride used for acetylation was radioactive (1-C^{14} - acetic anhydride) the acetate derivation was radioactive (C^{14} - diacetate).

A standard sample of pregnanediol (500 gamma) was acetylated simultaneously with the experimental sample as a control. The radioactivity contained in the tubes following the counter current distribution of the experimental sample and the control sample is compared in Figure 15. The H^3 and C^{14} radioactivity of the experimental sample coincide and are comparable with the C^{14} control pregnanediol diacetate.

Comparison of the counter current

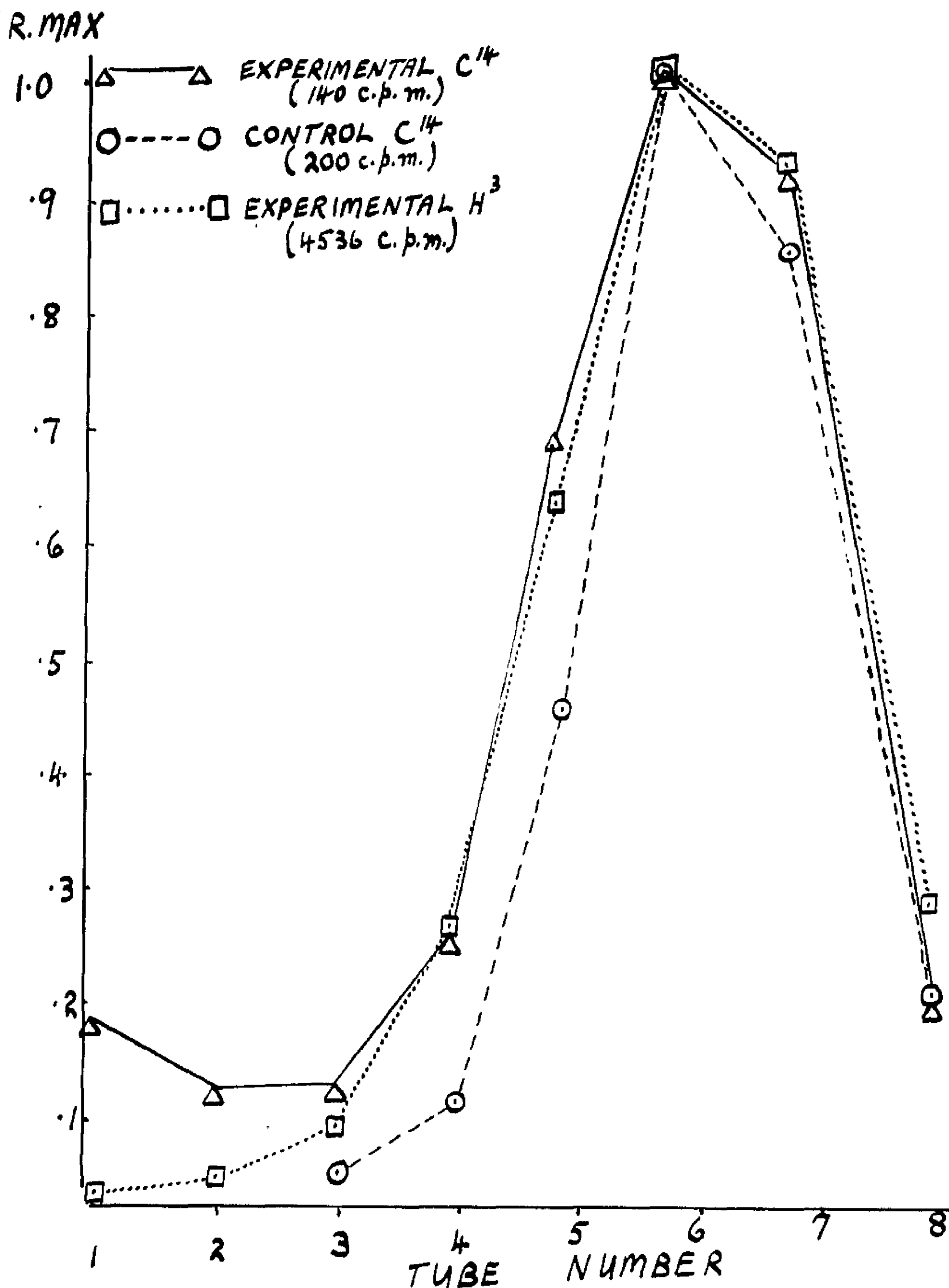


FIG. 15.

distribution curves of the isotopes from the samples gives the following Correlation Coefficient -

Experimental H^3 to Experimental C^{14} = 0.985

Experimental H^3 to Control C^{14} = 0.988

Experimental C^{14} to Control C^{14} = 0.977

The Correlation Coefficients indicate practically perfect Correlation (For perfect Correlation the Correlation Coefficient is + 1.0). This confirms that $7H^3$ pregnanediol C^{14} diacetate has resulted from acetylation of the experimental pregnanediol sample and is a third proof of the purity of the experimental sample.

d) Infrared analysis of Experimental pregnanediol sample.

A sample of experimental pregnanediol, obtained from pregnancy urine by extraction Method 4 (Appendix II) as described in Appendix VI and detailed in Volume II (pages 49-65), was submitted to Dr. Lewis L. Engel for infrared analysis who reported as follows -

The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University. July 19th. 1961.

We have just finished running and interpreting the infrared spectra on the sample you sent us. The pregnanediol was run as a micropellet in KBr and the spectrum agreed in all respects with that of our reference sample. A portion of the material was converted to the acetate and run in carbon disulphide. This spectrum too agreed in all respects with the reference spectrum of pregnane- $3\alpha, 20\alpha$ - diol diacetate.

For your reference I am giving you the spectrum numbers in case you ever want to go back and check them over. For the

pregnanediol they are Nos. 484-61, 485-61 and 486-61. For the diacetate they are Nos. 487-61, 488-61 and 489-61.

Lewis L. Engel, Ph.D.
Associate Professor of Biological Chemistry.
Harvard Medical School.

This is a fourth, and independent proof of the purity of the pregnanediol sample.

Thus the method has been proved specific for the extraction of pregnane-3α,20α-diol.

Note on Recovery of Internal Standard H^3 Pregnanediol from Pregnancy Urine.

Method IV (Appendix II).

Detailed results - Volume II (pages 49-65).

Recovery of internal standard H^3 pregnanediol.

Urine extraction (two experiments) 73.5% : 60.2%

Column recovery (six experiments) 51% S.D. 9.53 S.E. 3.9

Total procedure (six experiments) 34.2% S.D. 7.62 S.E. 3.1

These results are inferior to those for male urine which contains much less pregnanediol. This suggests that the sensitive chromatographic columns were overloaded with pregnanediol and so gave a poorer recovery and a greater spread of pregnanediol eluate fractions. Furthermore, the technique for transferring the extract to the columns was not efficient for the greater amount of pregnanediol in the extract since excessive radioactivity remained in the flask after transferring the extract to the columns as noted in the "remnant" assays. (15% for pregnancy urine compared with 1% for male

urine).

Correction of the column recovery and total procedure recovery for radioactivity lost in the remnant gives the following results.

Column recovery (six experiments) 61.5% S.D. 8.95
S.E. 3.6

Total procedures (six experiments) 41.0%
S.D. 6.6
S.E. 2.7

It is considered that these results can be considerably improved by a suitable modification of the technique and as a basis for further study of this method it is suggested that i) 250,000 units of beta glucuronidase are required to free not more than 2 milligrams of pregnanediol from conjugation with glucuronic acid, and ii) not more than 1.5 milligrams of pregnanediol (preferably 1.0 mgm.) should be in the extract put on the Columns.

RESULTS.

C. Experimental Subjects.

a) Trial of Method.

1) Subject *I
2) Subject *II
3) Paper Chromatography.

b) Normal Males. Subjects *III
*IV
*V
*VI
*VII
*IX
*XII

c) Surgically Castrate Females.
Subjects *VIII
*X
*XI
*XIII

a) Trial of Methods.

(1) Subject *I Results.

Detailed results are given in Volume II (pages 68-85). The quantity of radioactivity injected was 4 microcuries (1,580,000 cpm.) whereas in all subsequent experiments 2 microcuries were injected. Experimental injection solution of this higher radioactivity was prepared for this first experiment to give ample radioactivity so that any problems arising in the method could be more easily detected and corrected.

Experiment 1. Trial of Extraction Method I (Day 1 urine).

A peak of radioactivity occurred in tubes 12 and 13, the expected range for pregnanediol. These tubes gave readings for pregnanediol in the absorption spectrophotometer. A peak of radioactivity was also noted in tube 7. This peak was noted in subsequent experiments and is termed "Peak X". Minimal amounts of radioactivity were noted in the tube range

31-36 which was the expected range for pregnanetriol (1789 cpm. for Day 1).

Recovery of 78,795 cpm. as pregnanediol for the first day was disappointingly low and was considered to be the result of insufficient beta-glucuronidase in the incubation process.

Experiment 2. Re-extraction of Experiment 1 urine by Extraction Method II.

Recovery of 63,694 cpm. for the first day by re-extraction confirmed the inefficiency of Method I. A relative increase in the absorption spectrophotometer figures for pregnanediol recovery suggested that acid hydrolysis did have an effect on this part of the experimental method.

Experiment 3. Extraction of Day 1 urine by Extraction Method III.

This produced a recovery of radioactivity as pregnanediol of 142,078 cpm. per day which is in the range of the sum of the recoveries of experiments 1 and 2. (142,489 cpm.).

Quantitative recovery of pregnanediol was in keeping with the results of Method I after correction for the inferior efficiency of Method I recovery. Peak X was present and minimal amounts of radioactivity were present in the tube range 36-39 (3581 cpm. per day) - the pregnanetriol range.

<u>Experiment 4.</u> Re-extraction of Experiment 3 urine by Extraction Method II.

Tubes 5-17 gave a total recovery of radioactivity of 3170 cpm. for day 1 which is 2% of the recovery of radioactivity in the peak X and pregnanediol ranges by Method III. This confirms that Method III was efficient in recovering the radioactivity assayed in this research project.

Quantitative recovery of pregnanediol by absorption spectrophotometry gave 120.5 gamma for day 1 despite minute recovery of equivalent radioactivity. This confirms the inadviseability of using acid hydrolysis in this experimental method and the problems

that can arise in the application of absorption spectrophotometry to extracts obtained by acid hydrolysis.

Experiment 5. Extraction of Day 2 urine by Method III.

The results were in keeping with experiment 3. Minimal radioactivity was recovered in the tube range 32-38 (2042 cpm. per day) - the pregnanetriol range.

Experiment 6. Estimation of radioactivity excreted in urine (Day 1 and Day 2).

This method produced reasonable results but indicated that with the injection of 2 microcuries in subsequent experiments the small amounts of radioactivity to be expected in samples from Day 3 and Day 4 urine would be difficult to count statistically to obtain a reasonable standard deviation without excessively long counting times. However by this method it was considered likely that the Coefficient of variation for calculating the total radioactivity excreted during four days

would be in the region of 5 to 10 per cent when the pattern of excretion was taken into account. In fact the Coefficient of variation for the male subjects fully studied was 4.4 per cent (mean of 7) and for the castrate females it was 3.3 per cent (mean of 4).

<u>General notes on results of Subject *I Experiments.</u>

Extraction Method III proved satisfactory, pregnanediol being recovered in the expected tube range. When the radioactivity was counted statistically, the specific activities in the relevant tubes were constant (within the error of ± 5 per cent) which is further confirmation of the reliability of the method.

The effect of acid hydrolysis on subsequent absorption spectrophotometry was noted. This problem was not critically examined since enzyme hydrolysis was utilised in the research programme.

Small amounts of radioactivity were noted in the pregnanetriol tube range

(5623 cpm. total for 2 days). This amounted to 0.36% of the radioactivity injected, 3.0% of the radioactivity recovered as Peak X plus pregnanediol, and 3.2% of the radioactivity recovered as pregnanediol. Since the subsequent experiments were to involve injection of 2 microcuries, and the radioactivity in this pregnanediol range of eluate would be less and extremely difficult to analyse in detail, this aspect was not studied further. No relation was shown between this radioactivity and pregnanetriol apart from the similarity in the eluate tube range from the column.

Further information calculated from Subject *I is given with later results to allow comparison. The secretion rate result, however, is not valid because it only covers two days and not the necessary four days (see Appendix VII).

(2) Subject *II Results.

Detailed results are given in Volume II (pages 86-94). Injection of 2 microcuries proved a sufficient quantity of radioactivity for the method as applied in this research work.

Samples from the ether extract and benzene extract were assayed for radioactivity to enable comparison of the radioactivity of ether extract, benzene extract, and pregnanediol eluate.

Information from Subject *II is given with later results to allow comparison. Again the secretion rate result is not valid as only two days have been studied.

(3) Paper chromatography results.

Detailed results are given in Volume II (pages 95-103).

Experiment a). This was carried out to gain experience in paper chromatography and to test the efficiency of the column solvent system when applied to paper chromatography using the Bush System. The results were as noted in Volume II. The pregnanetriol sample was not pure pregnane-3α, 17α, 20α-triol.

Experiment b). This experiment was designed to demonstrate how Peak X radioactivity (Patient *I) was recovered from the above paper chromatography system. Elution of the Peak X sample strip recovered 85.4% of the added radioactivity from the pregnaneolone area of the chromatogram. Therefore Peak X radioactivity was related to pregnaneolone or some substance with a similar Rf.

Experiment c). This experiment demonstrated how the radioactivity of the pregnanediol eluate (Patient *II) was recovered from this

paper chromatography system. Elution of the experimental pregnanediol sample strip recovered 87% of the added radioactivity from the pregnanediol area of the chromatogram. This confirmed the efficacy of the system for use in the acetylation experiment.

Experiment d). This experiment showed the recovery of Peak X radioactivity (Patient *II) from the paper chromatography system. Elution of the pregnaneolone portion of the chromatogram recovered 87% of the added radioactivity again demonstrating that the Peak X radioactivity was related to pregnaneolone or a substance with a similar Rf.

No further investigation of the possible progesterone metabolite content of the Peak X fraction was undertaken at this time.

Results. C) Experimental Subjects.

b) Normal Males.

c) Castrate Females.

These results are presented at the same time for ease of comparison. Relevant calculations from subjects *I and *II are only included to indicate the values for these trial experiments. The detailed results are given in Volume II (pages 104-272).

The results are tabulated in the following order.

1) Per cent injected radioactivity recovered from Column as Pregnanediol.

11) Per cent injected radioactivity recovered from Column as Peak X.

111) Per cent injected radioactivity recovered from urine.

IV) Recovery ratios.

Ratio A = Ratio of $\frac{(1)}{(11)}$

Ratio B = Ratio of $\frac{(111)}{(1)}$

Ratio C = Ratio of $\frac{(111)}{(11)}$

V) Ratios of recovery of radioactivity during the experimental extractions.

$\frac{\text{Ether extract (cpm)}}{\text{Pregnanediol tubes (cpm)}}$ and

$\frac{\text{Benzene extract (cpm)}}{\text{Pregnanediol tubes (cpm)}}$ where

pregnanediol radioactivity is reduced to 1.

V1) Total pregnanediol recovered per day.

V11) Assay of injection solution and calculation of radioactivity injected.

V111) Calculation of progesterone secretion rate (P), mean for 4 days of experiment, in milligrams per day.

The equation used for this calculation is

$$P = \frac{S}{c} \frac{W}{d} \frac{a}{b} \text{ mgm. per day}$$

where S = specific activity of injected $7H^3$ progesterone.

W = weight of injected $7H^3$ progesterone.

a = correction factor for difference in moleculars weights of progesterone and pregnanediol = 0.98

c = specific activity of urinary pregnanediol.

b = correction factor for quenching of pregnanediol radioactivity (= 1.02 see Appendix IV, pages 265-6).

d = Number of days of urine collection.

Therefore P =

$$\frac{\left(\frac{\text{cpm}}{\text{mgm}}\ 7H^3 \text{ Progesterone injected}\right) \times \left(\text{mgm. } 7H^3 \text{ progesterone injected}\right) \times (0.98)}{\frac{\text{cpm}}{\text{mgm}} \text{ Urinary pregnanediol total} \times (4) \times (1.02)}$$

$$= \frac{\text{Radioactivity injected (cpm)} \times \text{mean daily pregnanediol (mgm)}}{\text{Total pregnanediol radioactivity (cpm)}} \times (0.96)$$

In this equation the experimental recovery rate, and counting efficiency of the Scintillation Counter cancel out. The calculation figures for each experiment are given in Volume II without correction with factor 0.96.

1) Per cent Injected Radioactivity Recovered from Column as Pregnanediol.

Males.	Day 1	Day 2	Day 3	Day 4	Total	$T\frac{1}{2}$ = Half time (hours).
*I	9.0	1.90			10.9	
*II	7.56	1.24			8.8	
*III	7.75	1.72	0.70	0.33	10.5	20
*IV	9.66	2.34	1.11	0.49	13.6	21
*V	11.08	0.83	0.27	0.12	12.3	17
*VI	11.56	2.95	1.11	0.48	16.1	18
*VII	12.77	2.33	0.69	0.21	16.0	14
*IX	6.51	1.50	0.57	0.22	8.8	17
*XII	9.31	2.49	1.52	0.53	13.85	22
Mean (7)	9.81	2.02	0.85	0.34	13.0	18.43
SD					2.75	2.74
Females.						
*VIII	7.58	1.88	0.68	0.46	10.6	24
*X	20.72	6.55	2.11	0.82	30.2	16
*XI	12.04	2.29	1.64	0.73	16.7	29
*XIII	13.83	2.12	1.49	0.56	18.0	26
Mean (4)	13.54	3.21	1.48	0.64	18.9	23.75
SD					8.16	5.57

An analysis of variance of the logarithms (to base 10) of the daily percent injected radioactivity recovered from the Columns as pregnanediol showed that the differences between the arithmetic mean values of the Males and Castrate Females for daily and overall recoveries were -

	Day 1	Day 2	Day 3	Day 4	Total (overall)
t value	-1.79	-2.45	-3.77	-4.66	-6.31
P	>0.05	<0.05	<0.01	<0.01	<0.01

The difference was not significant for Day 1 but was significant for Day 2 and highly significant for Day 3, Day 4 and total recovery.

When the logarithm of the per cent injected dose recovered is graphed against time in days, the points for days 2, 3 and 4 appear to form a straight line indicating that the excretion of the radioactivity as pregnanediol becomes a constant and the half time ($T\frac{1}{2}$) of disappearance of this radioactivity can be calculated and the $T\frac{1}{2}$ for each subject is noted with the results on

page 91. There is no significant difference between the half times of the males and castrate females ($P > 0.10$).

The logarithm of the mean percent injected dose recovered was graphed against time in days for the male and castrate female groups and is shown in Figure 16 where the points for days 2, 3 and 4 form a straight line with a half time of 19 hours for the males and 21 hours for the females. By extending this line to the right, the percent injected dose which would appear in the urine per day can be calculated until "total" (less than 0.06% of injected dose not yet recovered) excretion of the radioactivity as pregnanediol.

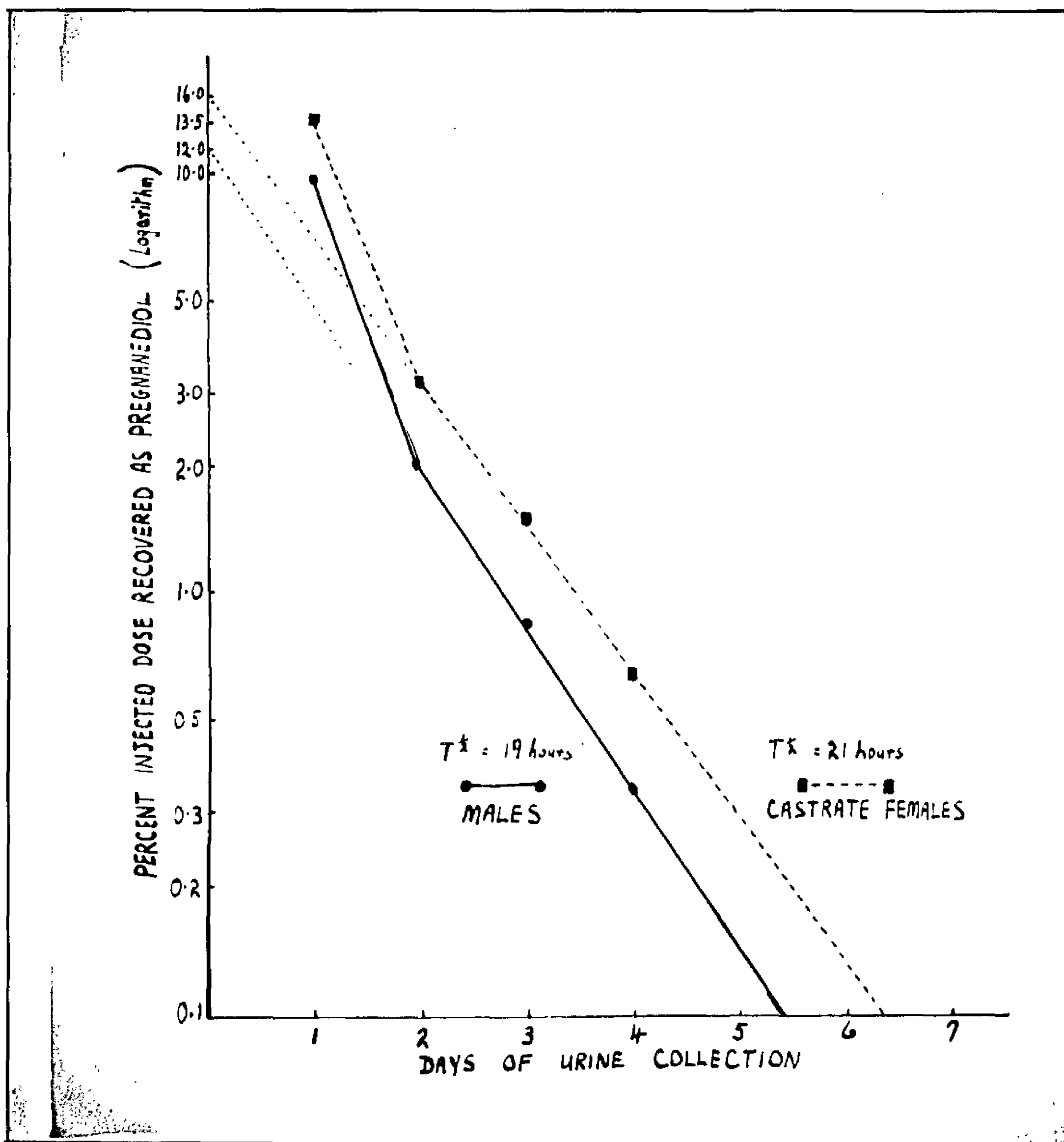


FIG. 16. Excretion of radioactivity as Urinary Pregnanediol.

Mean per cent injected dose recovered per day as pregnanediol.

	Males (7)	Females (4)
Day 1	9.81	13.54
Day 2	2.02	3.21
Day 3	0.85	1.48
Day 4	0.34	0.64
Total for 4 days	13.02	18.87
Day 5	.14	0.29
Day 6	.06	0.13
Day 7	-	0.06
Total for 7 days ("Total" excretion).	13.22	19.382

(Day 5, 6 and 7 calculated from Figure 16).

Thus the 13% four day recovery for the males is 98.9% of the total radioactivity excreted as pregnanediol, and the 18.9% recovery for the castrate females is 97.8% of the total radioactivity excreted as pregnanediol. This confirms that the method fulfils the theoretical requirement of 97% recovery of the radioactivity excreted as pregnanediol.

11) Per cent Injected Radioactivity Recovered from Column as Peak X.

Males	Day 1	Day 2	Day 3	Day 4	Total	$T\frac{1}{2}$ = half time (hours)
*I	0.76	0.12			0.88	
*II	1.76	0.23			1.99	
*III	1.54	0.32	0.11	0.04	2.01	16
*IV	1.31	0.36	0.16	0.09	1.92	24
*V	2.19	0.28	0.09	0.06	2.62	22
*VI	2.19	0.58	0.10	0.09	2.96	20
*VII	3.54	0.66	0.30	0.13	4.63	20
*IX	1.79	0.39	0.19	0.11	2.48	19
*XII	1.31	0.42	0.21	0.05	1.99	15
Mean (7)	1.98	0.43	0.17	0.08	2.66	19.43
SD					0.949	3.16
Females.						
*VIII	0.23	0.07	0.04	0.03	0.37	39
*X	1.02	0.32	0.11	0.04	1.49	16
*XI	1.36	0.28	0.24	0.08	1.96	27
*XIII	0.86	0.19	0.10	0.04	1.19	21
Mean (4)	0.87	0.22	0.12	0.05	1.25	25.75
SD					0.671	9.47

An analysis of variance of the logarithms (to base 10) of the daily percent injected radioactivity recovered from the column as peak X showed that the differences between the arithmetic mean values of the males and castrate females for daily and overall recoveries were

	Day 1	Day 2	Day 3	Day 4	Total (overall)
t value	3.49	2.93	1.62	1.96	4.99
P	<0.01	<0.01	>0.05	>0.05	<0.01

The difference was not significant for Day 3, bordering on significance for Day 4 but highly significant for Day 1 and 2 and total recovery.

When the logarithm of the per cent injected dose recovered is graphed against time in days the points for Days 2, 3 and 4 are in keeping with a straight line indicating that the excretion of radioactivity as Peak X may become a constant and therefore the half time ($T\frac{1}{2}$) of disappearance of this radioactivity may be calculated and the $T\frac{1}{2}$ for each subject is

noted with the results on page 95.
There is no significant difference between the half times of the males and castrate females for Peak X radioactivity ($P > 0.05$) or for the half times of the males and females for pregnanediol radioactivity and Peak X radioactivity ($P > 0.05$).

The logarithm of the mean per cent injected dose recovered was graphed against time in days for the male and castrate female groups and is shown in Figure 17 where the points for days 2, 3 and 4 are in keeping with a straight line with a half time of 20 hours for the males and 22 hours for the castrate females. By extending this line to the right, the per cent injected dose which would appear in the urine per day can be calculated until "total" (less than 0.004% of injected dose not yet recovered) excretion of the radioactivity as Peak X.

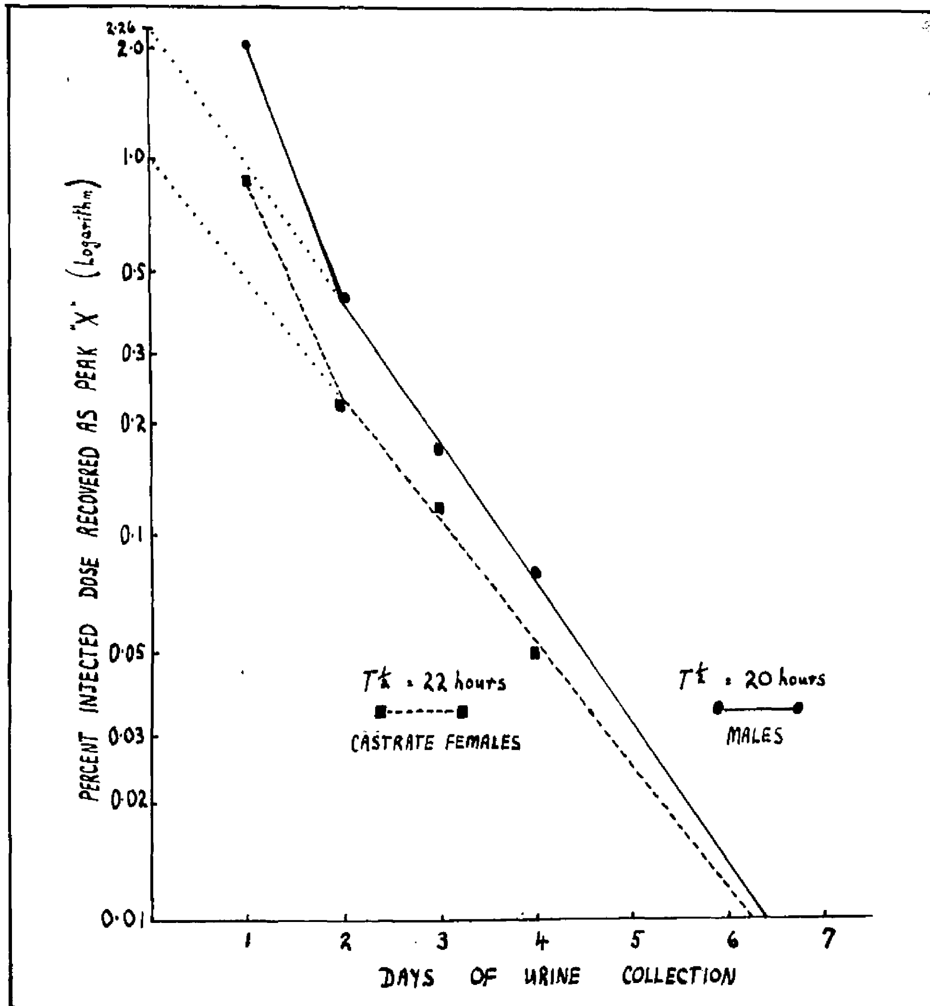


FIG. 17. Excretion of Peak "X" radioactivity in Urine.

Mean percent Injected Dose recovered per day as Peak X.

	Males (7)	Females (4)
Day 1	1.98	0.87
Day 2	0.43	0.22
Day 3	0.17	0.12
Day 4	0.08	0.05
Total for 4 days.	2.66	1.26
Day 5	0.032	0.025
Day 6	0.014	0.012
Day 7	0.004	0.004
Total for 7 days (Total Excretion)	2.71	2.301

(Days 5, 6 and 7 calculated from Figure 17)

Thus the 2.66% four day recovery for the male subjects is 98.2% of the "total" radioactivity excreted as Peak X and the 1.25% four day recovery for the castrate female subjects is 96.8% of the total recovery of radioactivity excreted as Peak X.

III. Per cent Injected Radioactivity Recovered from Urine.

Males.	Day 1	Day 2	Day 3	Day 4	Total
I	32.0	6.80			38.8
II	37.0	6.7			43.7
III	29.73	8.62	5.27	2.98	46.6
IV	31.95	9.95	5.66	5.34	52.9
V	41.34	10.35	6.37	6.24	64.3
VI	30.80	17.68	3.62	3.50	55.6
VII	36.48	13.54	6.23	3.65	59.9
IX	22.94	6.54	1.82	1.10	32.4
XIII	25.07	8.76	4.46	1.51	39.8
Mean (7)	31.19	10.78	4.78	3.47	50.2
SD					11.42
Females.					
VIII	31.91	12.33	7.64	6.02	57.9
X	53.13	14.40	9.77	6.90	84.2
XI	37.03	11.37	6.84	5.26	60.5
XIII	46.48	19.27	11.42	10.03	87.2
Mean (4)	42.14	14.34	8.92	7.05	72.5
SD					15.23

An analysis of variance of the logarithms (to base 10) of the daily per cent injected radioactivity recovered from urine showed that the differences between the arithmetic mean values of the males and castrate females for daily and overall recoveries were -

	Day 1	Day 2	Day 3	Day 4	Total (overall)
t value	2.40	2.50	5.41	6.66	8.49
P	<0.05	<0.05	<0.01	<0.01	<0.01

The difference was significant for Day 1 and Day 2 and highly significant for Day 3, Day 4 and total recovery.

IV) Recovery ratios.

$$\text{Ratio A} = \frac{\%\ \text{recovery of injected dose as pregnanediol}}{\%\ \text{recovery of injected dose as Peak X}}$$

$$\text{Ratio B} = \frac{\%\ \text{recovery of injected dose from urine}}{\%\ \text{recovery of injected dose as pregnanediol}}$$

$$\text{Ratio C} = \frac{\%\ \text{recovery of injected dose from urine}}{\%\ \text{recovery of injected dose as peak X}}$$

Males.	Ratio A	Ratio B	Ratio C
*III	5.22	4.44	23.2
*IV	7.08	3.90	27.6
*V	4.62	5.23	24.2
*VI	5.44	3.54	18.8
*VII	3.46	3.74	12.9
*IX	3.55	3.68	13.1
*XII	6.96	2.90	20.0
Mean	5.19	3.92	20.0
SD	1.46	0.733	5.49
Females.			
*VIII	28.6	5.46	156.6
*X	20.3	2.79	56.5
*XI	8.5	3.62	30.9
*XIII	15.1	4.84	73.3
Mean	18.1	4.18	79.3
SD	8.42	1.194	54.31
s_D	3.137		19.748
t	4.115		3.003
P	<0.01		<0.02

These figures compare the results of sections 1), 11) and 111) and indicate the similarities and differences, viz.

a) The males excrete less pregnanediol radioactivity and less total radioactivity than the castrate females. The proportion of pregnanediol radioactivity to total radioactivity is, however, similar in the two groups and this is indicated by the similarity of the B ratios.

b) The males excrete more peak X radioactivity than the females ($\times$ 2.128) and the females excrete more total radioactivity ($\times$ 1.444) and more pregnanediol radioactivity ($\times$ 1.454) than the males and this is indicated by the significant differences in the A ($P < 0.01$) and C ($P < 0.02$) ratios.

V) Ratios of recovery of radioactivity during experimental extraction procedures.

Subject	Ether Extract. Day 1	Ether Extract. Day 2	Benzene Extract Day 1	Benzene Extract Day 2	Pregnanediol Tubes.
II	2.08	2.07	1.64	1.76	1
III	2.06	2.02	1.88	1.79	1
IV	1.91	2.38	1.72	1.06	1
V	2.12	-	1.79	-	1
VII	-	-	1.82	1.74	1
Mean	2.04	2.16	1.77	1.59	1
SD	.09		.14		
Mean Day 1 and Day 2	2.09		1.67		
SD	.14		.24		

These results were obtained to monitor the functioning of the extraction procedure during the initial male experiments but the counting of the extract samples was not long enough to obtain acceptable standard deviations. However the figures indicate the pattern of radioactivity in the extracts. The benzene extract retains 80% of the ether extract radioactivity and the Pregnanediol tubes contain 60% of the benzene extract radioactivity.

Therefore since the total radioactivity excreted as pregnanediol is 19.4% of the injected dose (13% corrected for 67% recovery rate for the method), it can be calculated that, for the male patients, the benzene extract (Neutral extract) will contain 32.3% and the ether extract 40.4% of the injected dose while the urine contains 50.2%.

VI) Total pregnanediol recovered per day (Micrograms).

Males	Day 1	Day 2	Day 3	Day 4	Mean of 4 days
*I	335.8	113.8			
*II	196.0	218.0			
*III	278.25	305.9	158.4	297.1	260.0
*IV	421.2	688.8	644.8	665.3	605.0
*V	645.0	329.0	304.4	370.6	413.6
*VI	697.8	946.0	850.6	1262.7	939.4
*VII	434.0	456.5	579.6	417.6	471.8
*IX	188.0	220.5	147.0	148.5	176.0
*XII	394.0	333.0	470.0	284.0	345.25
Mean for series (28)					458.7
SD (28)					268
Females.					
*VIII	208	141.75	105.6	100.8	139.0
*X	227	330	364	346	314.0
*XI	97.5	163.3	232.25	204.7	174.5
*XIII	244.4	208.8	144	223	205.0
Mean for series (16)					208.1
SD (16)					70

Males. $\bar{x} = 459$
$S(x) = 12844$
$S(x^2) = 7,848,695$

Females $\bar{x} = 208$
$S(x) = 3330$
$S(x^2) = 692890$

$s_D = 70$
$t = 3.586$ ($P < 0.001$)
$n = 42$

The mean value for daily pregnanediol excretion as measured by the experimental method (without correction for loss during the method) differ significantly for the male (458.7 micrograms) and female (208.1 micrograms) groups. ($P < 0.001$). The males therefore excreted more pregnanediol but less radioactivity as pregnanediol and less total radioactivity in urine than the castrate females.

Because the specific activities of all the metabolites of progesterone will be the same in each group of subjects the weight of possible metabolite excreted as peak X (X^M micrograms for male group and X^F micrograms for female group) can be calculated since the radioactivity of peak X is known.

a) Male group.

Specific activity constant

$$\text{Specific activity} = \frac{\text{Counts per minute}}{\text{micrograms}}$$

$$\text{Specific activity of pregnanediol} = \frac{13\%}{458.7} \text{ micrograms.}$$

$$\text{Specific activity of peak X} = \frac{2.66\%}{X^M} \text{ micrograms.}$$

Therefore $\frac{13\%}{485.7} = \frac{2.66\%}{X^M}$

$$X^M = \frac{2.66}{13} \times 458.7 \text{ micrograms}$$

$$= 93.85 \text{ micrograms}$$

b) Female group.

Specific activity of pregnanediol = $\frac{18.9\%}{208.1}$ micrograms

Specific activity of peak X = $\frac{1.25\%}{X^F}$ micrograms

Therefore $\frac{18.9\%}{208.1} = \frac{1.25\%}{X^M}$

$$= \frac{1.25}{18.9} \times 208.1 \text{ micrograms}$$

$$= 13.76 \text{ micrograms}$$

Thus the male subjects excreted 93.85 micrograms of a possible progesterone metabolic product as peak X per day and the female subjects 13.76 micrograms per day. The possible metabolite content of peak X was not fully investigated. Furthermore these calculations do not consider the correction necessary for the molecular weight of the unknown metabolic substance and can only by a rough theoretical exercise indicating that in males, peak X may contain

6 to 7 times more metabolite than castrate females.

VII) Radioactivity Injected.

Subject.	cpm.
I	1580000
II	746000
III	627500
IV	664000
V	664000
VI	780000
VII	762000
IX	785000
XII	770000
Mean (7)	722000
VIII	766500
X	642500
XI	928500
XIII	655000
Mean (4)	748000

Counting efficiency 1/6

1 Microcurie = 0.370 10^6 cpm.

2 Microcurie = 740,000 cpm.

Mean 2 microcurie injection aliquot = 740,800 cpm.
(Mean of 14) SD = 82,700

VIII) Progesterone Secretion Rate. (P)

mgm. per day - mean rate for 4 days.

Males	(P)	Corrected X.96
I	(2.071)	
II	(2.341)	
III	2.477	2.378
IV	4.446	4.267
V	3.362	3.228
VI	5.826	5.593
VII	2.946	2.828
IX	2.002	1.922
XII	2.495	2.395
Mean (7)	3.365	3.230
		SD 1.29
Castrate Females.		
VIII	1.312	1.260
X	0.975	.937
XI	1.042	1.000
XIII	1.132	1.087
Mean	1.115	1.071
		SD .141

The mean value for secretion rate for the male group differs significantly from that of the castrate females. ($0.001 < P < 0.01$).

Males			Females		
	$\bar{x}$	3.230		$\bar{x}$	1.071
	$S(x)$	22.611		$S(x)$	4.284
	$S(x^2)$	83.014		$S(x^2)$	4.648

$s^2_{xx'} = 1.116$ $\qquad s_D = 0.662$

$t = 3.261$ $\qquad (0.001 < P < 0.01)$

RESULTS

D. COLLABORATIVE WORK.

Volumes of distribution, transport and metabolism rate constants and metabolic clearance rate of progesterone.

a) Single injection experiments.

b) Constant infusion experiment.

(A concomitant study on the experimental subjects by Dr. Brian Little of the Department of Obstetrics and Gynaecology, Harvard Medical School).

a) Single Injection Experiments.

a) Single Injection Experiments.

The values for radioactivity present specifically as progesterone and fully corrected for recovery in plasma taken at timed intervals after the intravenous injection of 2 microcuries of $7H^3$ progesterone were obtained for each experimental subject (See Appendix VIII).

These values were plotted as the logarithm of the per cent injected dose present per litre of plasma against time after injection (see Appendix IX). A rough example of such a graph for Subject *VII is shown in Figure 18.

From these graphs the following factors can be calculated - (the graphs being drawn from the experimental data using the method of least squares) A, B, A+B, Alpha, Beta. (Appendix IX) and they are noted in the table on page 112.

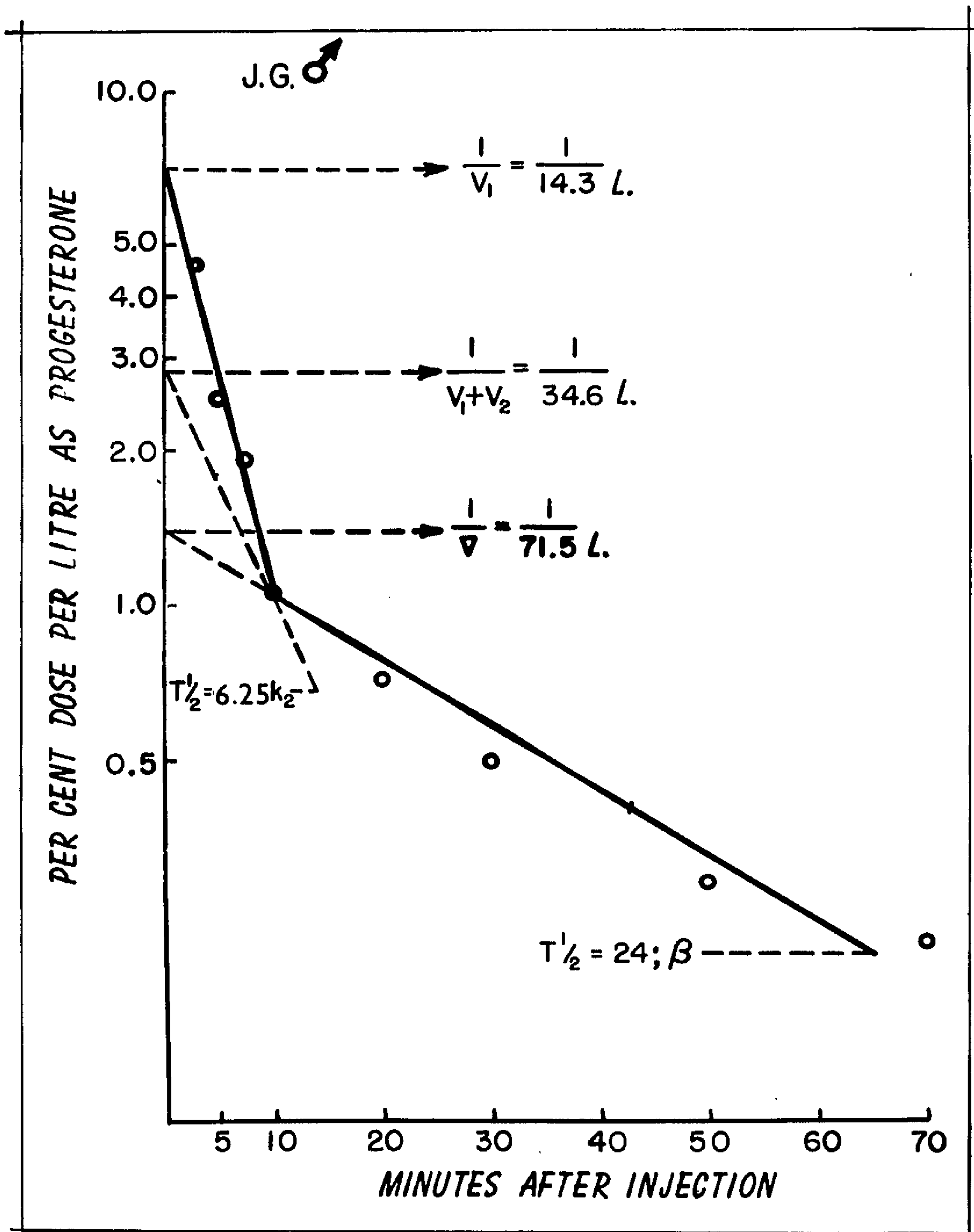


FIG. 18. Disappearance of Plasma Progesterone Radioactivity.

Collaborative Work : Experimental Results.

	% dose per litre			Units per day	
Subject.	A	B	A+B	Alpha	beta
Males.					
*VIII	6.73	1.007	7.737	686.17	80.0
*IV	1.98	2.575	4.555	715.0	106.47
*VII	16.10	3.42	19.52	1744.0	83.0
*IX	4.74	1.28	6.01	388.0	40.0
Mean	7.39	2.07	9.46	883.3	77.4
Castrate Females.					
*X	4.92	3.52	8.44	663	120
*XI	6.03	1.76	7.79	709	76
Mean	5.48	2.64	8.11	686	98

From a knowledge of these factors the following can be calculated. (Appendix IX).

K_1, K_2, V_1, $V_1 + V_2$, V_2, $\bar{V}$ and M

Since $V_1 = \frac{1}{A+B}$

$$V_1 + V_2 = \frac{\beta^2 A + B\alpha^2}{(A\beta + B\alpha)^2}$$

$$\bar{V} = \frac{1}{B}$$

$$K_1 = V_2 \quad (A\beta + B\alpha)$$

$$K_2 = \frac{\alpha\beta(A+B)}{A\beta + B\alpha}$$

$$M = V_1 K_2 = \frac{\alpha\beta}{A\beta + B\alpha}$$ = metabolic clearance rate (litres/day).

These results are given in the Table on page 114.

Collaborative Work: Data calculated from Experimental Results.

Subject	Units per day		Litres				Litres per day.
	K_1	K_2	V_1	V_1+V_2	V_2	V	M
Males.							
*III	226.70	345.46	12.92	31.36	18.44	99.3	4464
*IV	202.11	168.99	21.95	31.80	9.85	38.83	3710
*VII	1065.92	387.09	5.12	19.72	14.60	29.24	1982
*IX	172.29	135.93	16.64	35.19	18.55	78.12	2262
Mean	416.70	259.35	14.26	29.52	15.26	61.35	3104
Calculated from Mean of Table on Page	433.27	269.84	10.57	28.62	18.05	48.3	2850
Castrate Females.							
*X	206.73	229.63	11.85	18.92	7.07	28.41	2721
*XI	319.89	246.27	12.84	31.59	18.75	56.82	3158
Mean	263.31	237.95	12.35	25.26	12.91	42.62	2940
Calculated from Mean of Table on Page	263.05	231.73	12.33	23.49	11.16	37.88	2858

b) Constant Infusion Experiment.

(Experimental Male Subject *XII)

The values for radioactivity present specifically as progesterone and fully corrected for recovery in plasma taken at timed intervals during the constant infusion are obtained (see Appendix VIII). These values, calculated as micromicrocuries per litre of plasma are shown in Figure 19 plotted against time.

The values at 85, 95 and 105 minutes are constant within the error of the method with a mean value of 5.28 micromicrocuries per litre of plasma.

Since $M = \frac{r_1}{x_0}$ (Appendix IX)

$$= \frac{21.6}{.00528}$$

$$= 4091 \text{ litres per day.}$$

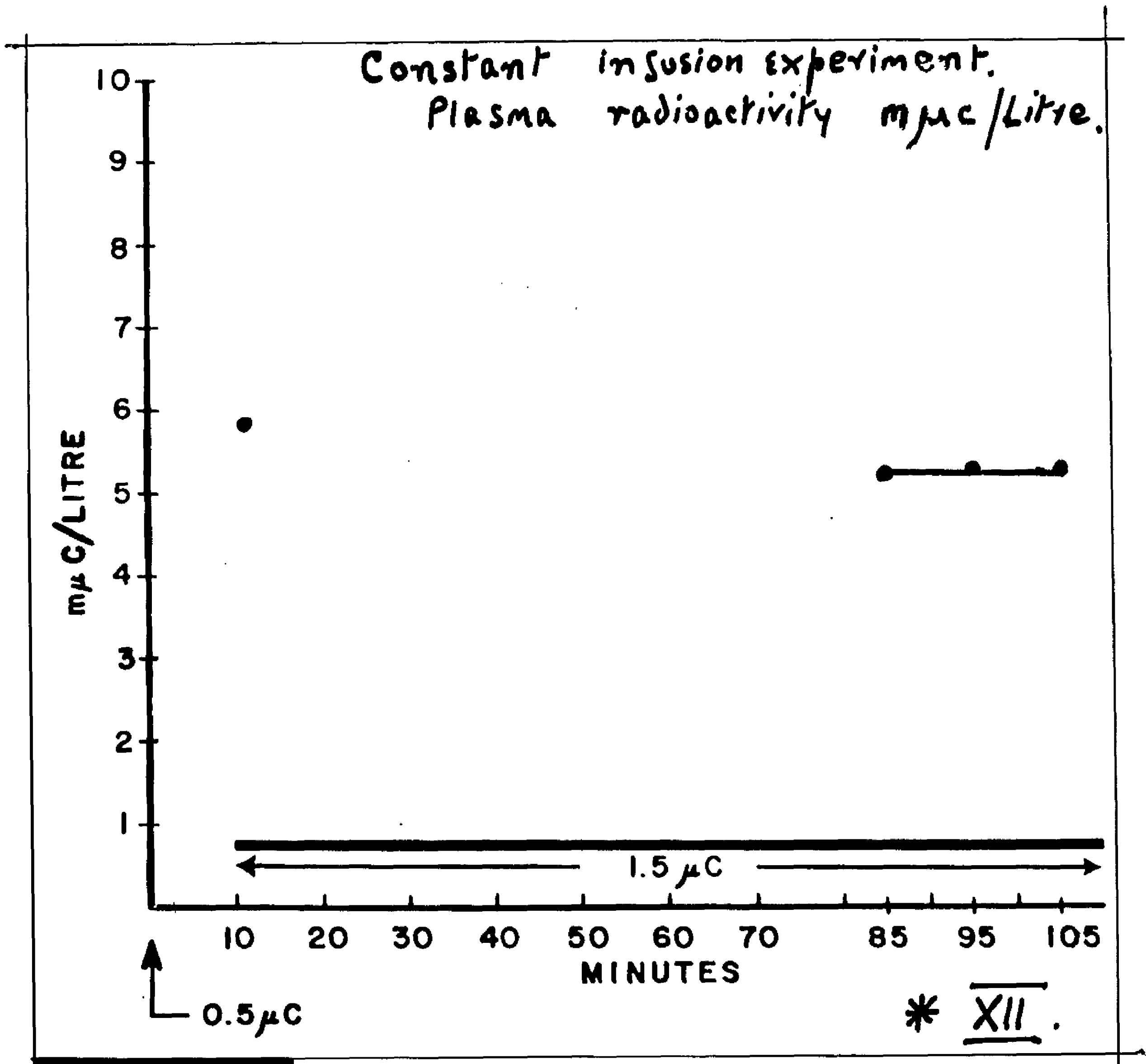


FIG. 19.

RESULTS.

E.

Results calculated from combination of the Secretion Rate Study (Results C), and the Collaborative Study (Results D).

Results calculated from combination of secretion rate study (Results C) and the collaborative study (Results D) are calculated as follows and detailed in the table on page 118.

a) i = mean plasma progesterone level

$$= \frac{\text{Progesterone Secretion Rate}}{\text{Metabolic Clearance Rate}}$$

$$= \frac{P}{M} \text{ micrograms per 100 ml. plasma}$$

b) h = total body content of non isotopic hormones

$$= i\ (V_1 + V_2) \text{ micrograms}$$

c) The turnover time (TT) can also be calculated (See Material and Methods, page 47).

$$TT = \frac{i \times V_1}{P} \quad \text{or}$$

$$= \frac{V_1}{M} \quad \text{or}$$

$$= \frac{1}{K_2}$$

Combined Results.

	Mgm. per day.	litre/day	Micrograms per 100 ml. plasma.	Micrograms.	Minutes.
Subject.	P	M	i	h	TT
Males.					
*III	2.378	4464	0.053	17.62	4.17
*IV	4.267	3710	0.115	36.57	8.52
*VII	2.828	1982	0.143	28.2	3.72
*IX	1.922	2262	0.085	29.91	10.6
*XII	2.495	4091	0.061		
Mean	2.778	3302	0.091	28.08	6.7
SD	0.894		0.038	7.81	
Castrate Females.					
*X	0.937	2721	0.034	6.43	6.27
*XI	1.000	3158	0.032	10.11	5.85
Mean	0.969	2940	0.033	8.27	6.06
SD	0.032		0.001	2.56	
s_D	0.669		0.0286	5.958	
t	2.703		2.028	3.325	
	$.02 < P < .05$		$0.05 < P < 0.10$	$.02 < P < .05$	

DISCUSSION.

A. The Method of Pregnanediol Assay.

B. The excretion of radioactivity in urine after administration of radioactive progesterone.

C. The excretion of urinary pregnanediol after the administration of progesterone.

D. Peak X.

E. The Excretion of Pregnanediol in Urine.

F. Progesterone Secretion Rates.

G. Progesterone Secretion by the Adrenal Gland.

H. Collaborative Work.

- a) Method.
- b) Results.

I. Combined Results.

- a) Mean plasma progesterone level.
- b) Turnover Time.
- c) Mean total body content of progesterone.

A. Method of Pregnanediol Assay.

The method for the extraction of pure 5 β pregnane-3α, 20α,-diol from urine which was established in this research programme has been described in detail and has been shown to be efficient, specific and reproduceable (see Results pages 61 to 75). It compares favourably with the best of the many methods for the extraction of pregnanediol from urine which have been published as noted previously (Materials and Methods, pages 21 and 22). The 60 cms. celite chromatographic column is original to this method (see Appendix III). The solvent system employed for the column was studied by Eberlein & Bongiovanni (1955) and utilised by them in a paper chromatographic method for the measurement of pregnanediol in urine (Eberlein & Bongiovanni 1958).

The glucuronidase incubation avoids the interference with subsequent spectrophotometry which can result from acid hydrolysis of the

urine and an accepted method of urine extraction was employed (See Appendix II).

The pregnanediol recovered from the urine was assayed for radioactivity in a Packard Tri-Carb Scintillation Spectrometer by an appropriate technique (Appendix IV). The pregnanediol was also measured quantitatively by absorption spectrophotometry in a Beckman DU Spectrophotometer by a method which took into consideration the various problems of the procedure (Appendix V). A critical factor in the spectrophotometry was that the pregnanediol recovered from the column was in fact pure 5β pregnane-3α, 20α,-diol. That this was so is confirmed by four proofs of purity of the experimental pregnanediol sample (See Appendix VI and Results, pages 68 to 75).

The experimental subjects were managed in a simple routine manner to ensure accurate administration of the tracer dose of tritiated progesterone and collection of plasma samples

and urine. The dose of radioactivity administered was safe and permission for the administration of 7-H^3 progesterone was obtained from the appropriate authorities. Suitable precautions for radiation hazard were taken throughout the work (See Appendix I).

The method proved to be effective when applied to the experimental subjects. The ratios of recovery of radioactivity during the experimental extraction procedures showed a steady pattern (Results page 103). The calculated mean content of radioactivity in the neutral extracts studied was 32.3 per cent of the injected dose (Results page 104), which was in keeping with the results of Pearlman (1957b) - 31 per cent, and of Romanoff (1962) - 32 per cent.

B. Excretion of Radioactivity in the Urine after administration of Radioactive Progesterone.

The recovery of total radioactivity from the urine following administration of isotopically labelled progesterone represents

the excretion of several metabolites. It may also include the excretion products of steroids which have progesterone as a step in their biosynthetic pathway (see Figure 3, page 3). Therefore the logarithm of the per cent injected dose recovered per day graphed against time in days need not be a straight time for days 2, 3 and 4 as would be expected for the radioactivity excreted as a single metabolite in the steady state.

The urine radioactivity will also depend on the position of the labelled atom in the steroid nucleus. If 21-C^{14} progesterone is used, for example, some 18.6 per cent of the injected radioactivity will be excreted in the expired air (Davis & Plotz 1958) due to metabolism of the side chain (see Figure 3, page 3) and this will reduce the radioactivity available for excretion by urine and faeces.

Bradlow & Gallacher (1955), studying the metabolism of progesterone in man, recovered from urine and faeces 61 per cent of

radioactivity injected as 4-C^{14} progesterone. Weist, Fujimoto and Sandberg (1955) obtained from the first 24 hour collection of urine 22.4 per cent of radioactivity injected as 4-C^{14} progesterone into a female subject.

Davis & Plotz (1958) recovered an average of 34.1 per cent of radioactivity injected as 4-C^{14} progesterone into non-pregnant women and found no relation between the amounts excreted and ovarian function. Plotz (1961) similarly recovered 39.6 per cent of radioactivity from the urine of pregnant patients and found no statistical difference in the recoveries from pregnant and non-pregnant women. These figures are in keeping with a 35.5 per cent recovery (only 3 days urine collected) by Zander (1959) and 30 to 69 per cent by Langmade, Notrica, Demetriou and Ware (1962). There is, however, a wide variation in the total recovery values in urine which points to an individual excretion pattern of progesterone metabolites which is not influenced

by the presence or absence of luteal function or pregnancy.

Sandburg & Slaunwhite (1958) recovered 50 per cent of radioactivity from urine after injection of 4-C^{14} progesterone into male and female subjects and Romanoff (1962) similarly recovered 54 per cent from urine of male patients. The recovery of radioactivity from male urine recorded in this thesis was 32.4 to 64.3 per cent (mean 50.2).

There is an increased excretion of radioactivity in the urine from the castrate females (57.9 to 84.2 per cent: mean 72.5) when compared with the recovery from the normal males as noted previously (mean 50.2 per cent) and the difference is statistically significant (Analysis of variance: $P < 0.01$; overall). This difference could be attributed to the action of the testis in the male subjects if it can be assumed that the male adrenal gland, at least as far as progesterone is concerned, has a basic

metabolism similar to that of the female gland. This could be confirmed by a study of castrate male subjects. The difference may also be present when castrate females are compared with normal females as is suggested by the results of the studies of normal females by the other workers quoted above. This may be taken as an indication of the difference in the pattern of progesterone metabolism in the testis and ovary from that in the adrenal gland as would be expected from the different biosynthetic pathways for steroid metabolism in these endocrine glands.

On the other hand the variation shown in this thesis may reflect a basic sex difference in progesterone metabolism between male and females which is not readily apparent from the comparison of the results of different workers studying different sex groups. It has to be remembered that, whereas the main site of progesterone metabolism is the liver (Samuels & Waist 1952), Forbes,

Coulombre & Coulombre (1961) have shown that cellular fractions of rat kidney as well as liver can inactivate progesterone in vitro, Berliner & Weist (1956) provided evidence that extrahepatic metabolism of progesterone occurs in rats and that reduction of the 20-Keto group is possible without the liver, and Cooke & Taylor (1962) demonstrated sex differences in the metabolism of progesterone by rat liver in vitro.

C. Excretion of Urinary Pregnanediol after the administration of Progesterone.

Somerville & Marrian (1950) administered some 60 mgm. of progesterone daily for two days to men and post-menopausal women and recovered pregnanediol, calculated as per cent administered progesterone, in the range of 12 to 16 per cent for men and 9 to 16 per cent for post-menopausal women. Klopper & Michie (1956) after administering similarly large doses of progesterone to men and post-menopausal women recovered from the urine

an average of 12.9 per cent of the administered dose. The results published by Klopper & Michie (1956) further demonstrated that by continuing the administration of progesterone, a significant difference in the excretion of the administered progesterone as urinary pregnanediol appeared, the postmenopausal females excreting more than the males, and analysis of their results shows that the difference is statistically significant (t test: $P < 0.001$).

These experiments were not physiological because the quantities of progesterone administered to the patient were 10 to 40 times the daily progesterone production of these patients and could alter the normal steady state of progesterone transport and metabolism. However, the recovery of radioactivity as urinary pregnanediol after the administration of isotopically labelled progesterone in tracer dosage is a physiological method of measuring the excretion of

pregnanediol in the urine as a percentage of administered progesterone. The total urinary pregnanediol (discounting the pregnanediol from the tracer amount of radioactive progesterone) therefore bears a similar relation to the total endogenous progesterone metabolism.

Pearlman (1957b) recovered radioactivity as urinary pregnanediol, measured as per cent injected dose of 16-H^3 progesterone, with an average of 11.7 per cent for pregnant women and of 20.5 per cent for surgically castrate females. Romanoff (1962) administered 4-C^{14} progesterone to normal males and recovered an average of 14 per cent of the injected dose as urinary pregnanediol. The results in this thesis (see results page 91) for recovery of radioactivity as urinary pregnanediol measured as per cent injected dose of 7-H^3 progesterone are 10.5 to 16.1 per cent (Mean 13.0) for normal males and 10.6 to 30.2 per cent (Mean 18.9) for

castrate females. The difference is statistically significant (Analysis of variance: $P < 0.01$; overall). Furthermore the excretion of radioactivity as urinary pregnanediol is directly proportional to the excretion of total radioactivity in the urine for both groups (See results page 102).

The results for the males in the thesis agree with Romanoff (1962) and the results for the castrate females are in keeping with the figures given by Pearlman (1957b). Also the results of Klopper & Michie (1956) discussed above indicate that post-menopausal women can convert a greater quantity of administered progesterone to pregnanediol than normal males and this is confirmed by the results in this thesis.

When the logarithm of the per cent injected dose recovered as urinary pregnanediol per day (mean values for 7 males and 4 castrate females) is graphed against time in days, the curve suggests a straight line for

days 2, 3 and 4 (Figure 16, page 93).
Allowing Day 1 for equilibration, this graph is in keeping with the assumption that the patients were in a steady state of progesterone metabolism as indicated by a constant rate of excretion of labelled pregnanediol. There is a significant difference in the quantity of radioactivity excreted but the pattern of excretion, i.e. the renal clearance, (the slope of the line for days 2, 3 and 4 in the two groups) shows no significant difference (Mean $T\frac{1}{2}$: 7 males = 18.43 hours; 4 females = 23.75 hours; $P > 0.05$).
Thus the difference in progesterone metabolism between normal males and castrate females is reflected in the rates of conversion of progesterone to pregnanediol. As with the difference in excretion of total radioactivity in the urine, the difference in pregnanediol excretion may reflect either the action of the testis in the male group, or a basic sex difference in progesterone metabolism.

D. Peak X.

This is the other peak of radioactivity detected in the column eluate from the patients studied. The major peak of radioactivity was pregnanediol in the range of tubes number 10 to 14 (usually in a peak of 2 tubes). Peak X usually appeared in tubes 6 to 7.

The percent injected radioactivity recovered as Peak X was 1.92 to 4.63 (mean 2.66) for the males and 0.37 to 1.96 (mean 1.25) for the castrate females and the difference is statistically significant (Analysis of variance: $P < 0.01$; overall). This difference in excretion of progesterone metabolite is greater than these figures indicate because both the radioactivity excreted as pregnanediol and the total radioactivity excreted in the urine is significantly greater in castrate females than in normal males whereas the radioactivity excreted as peak X is significantly greater

in normal males than in castrate females. When the logarithm of the mean daily per cent injected dose recovered from urine as peak X (mean values for 7 males and 4 castrate females) is graphed against time in days (Figure 17, page 97) the result is reasonably in keeping with a straight line for days 2, 3 and 4, so that the radioactivity appears to follow the pattern of a metabolite of progesterone excreted by subjects in a steady state of metabolism. The graphs indicate that there is a significant difference in the quantity of radioactivity excreted but the pattern of excretion, i.e. renal clearance (the slope of the line for days 2, 3 and 4) shows no significant difference (mean $T\frac{1}{2}$: 7 males = 19.43 hours; 4 females = 25.75 hours; $P > 0.05$).

The actual metabolic content of peak X was not identified but assuming it to be a single pure metabolite the quantity of such a metabolite excreted as peak X can be

calculated (see Results page 106) to be some seven times greater for the male group than for the castrate female group, a much greater difference than exists for pregnanediol (see later). The differing rates of progesterone metabolism in this direction indicate a significant difference in the pattern of progesterone metabolism between normal males and surgically castrate females. That this difference could be due to the activities of the testis in the male group rather than a more basic sex difference in progesterone metabolism is supported by the following argument.

Whereas pregnanediol is the major metabolite of progesterone, the second largest metabolite of progesterone is pregnaneolone. Van der Molen (1962) studied the excretion of pregnanediol and pregnaneolone in normal males and women with amenorrhoea. This worker reported 1) that the ratio of excretion of pregnanediol to pregnaneolone was 3 to 4/1

for males and 5 to 6/1 for women with amenorrhoea 2) Adrenocorticotrophic hormone stimulation of these subjects resulted in an increase in 17 hydroxycorticoid and pregnanediol excretion in both groups but no detectable increase in pregnaneolone excretion which suggests that pregnaneolone is less related to adrenal gland metabolism than pregnanediol. Contractor & Pearlman (1960) also reported results of a study of pregnaneolone excretion in castrate females. From their figures for pregnaneolone radioactivity recovered following injection of a known amount of 16 H^3 progesterone and the mean progesterone secretion rate for castrate females reported in this thesis (1.25 mgm. per day) it can be calculated that the weight of pregnaneolone recovered was some 25 micrograms (Calculation similar to that for Peak X; see Results, page 106). It is therefore tempting to postulate that the progesterone metabolite in Peak X is

pregnaneolone because a) Peak X is the next most prominent peak of excretion of radioactivity to that of pregnanediol and pregnaneolone is the next most prominent metabolite of progesterone. b) the ratio of pregnanediol to the theoretical metabolite content of peak X for normal males in this thesis (4.9/1) is reasonably in keeping with Van der Molen's figures for the ratio of pregnanediol to pregnaneolone (3 to 4/1). c) the ratio of pregnanediol to the theoretical metabolite content of peak X for the castrate females in the thesis is 15/1 and the content of possible metabolite in peak X is much smaller in the castrate female group than in the normal males indicating that this metabolite, like pregnaneolone, was less related to adrenal gland metabolism and more to testicular metabolism of progesterone. d) the weight of possible peak X metabolite for castrate females was calculated to be some 13.7

micrograms which is within reasonable range of the value of 25 micrograms calculated as the possible recovery of pregnaneolone from castrate female urine by Contractor & Pearlman (1960). e) the paper chromatography experiments (Results, pages 86 and 87) show that peak X radioactivity has the same Rf as standard pregnaneolone.

E. The amount of Pregnanediol excreted in the Urine.

Venning & Brown (1936) were the first to report the isolation of pregnanediol from human pregnancy urine and to study the excretion of pregnanediol in urine during the menstrual cycle (Venning & Brown 1937).

Pregnanediol was first recovered from male urine by Buxton & Westphal (1939) who demonstrated it in urine from patients to whom progesterone had previously been administered. Engel, Thorn & Lewis (1941) recovered $3\alpha, 20\alpha$-pregnanediol from the urine of a normal male (0.06 mgm. per litre:

0.12 mgm. per day). Daily excretion of pregnanediol in male urine has been reported as follows; Klopper, Strong & Cook (1957) - 1.11 mgm. (SD 0.29): Bongiovanni & Eberlein (1958) - under six years of age, less than 0.02 mgm; 7 to 16 years, 0.3 to 1.1 mgm. (mean 0.6); over 16 years, 0.2 to 3.5 mgm. (mean 1.53): Van der Molen (1962) - 1.36 to 2.1 mgm.: Romanoff (1962) - 0.3 to 0.9 mgm. (mean 0.6): This thesis - 0.147 to 1.26 mgm. (mean 0.459 mgm; mean corrected for loss by method, 0.685 mgm. see Results page 105).

The efficiency of the methods employed and the purity of the pregnanediol recovered will account for some of the variation in these figures. Furthermore there is a great variation in the quantity of urinary pregnanediol in the patients studied in this thesis so that a random collection of groups of males will result in a variation in the means of the samples around the mean of the

population as a whole.

Klopper, Strong & Cook (1957) reported the recovery of pregnanediol from the urine of post-menopausal women to be 0.6 mgm. per day (SD 0.22) which was significantly different from their comparable recovery rate from normal male urine (1.11 mgm. SD 0.29) and from urine from women in the proliferative phase of the menstrual cycle (1.0 mgm. SD 0.1). Pregnanediol produced by the post-menopausal female is likely to be the result of the metabolism of progesterone secreted by the adrenal gland and the post-menopausal ovary and there is no evidence that the post-menopausal ovary does not produce progesterone although a comparative study of progesterone metabolism in normal post-menopausal women and surgically castrate women would clarify this point. However, Klopper, Strong & Cook (1957) showed that after administration of adrenocorticotrophic hormone to their post-menopausal female subjects, the excretion of

urinary pregnanediol was increased indicating an adrenal component in the progesterone production of post-menopausal women.

In this thesis the recovery of pregnanediol from the urine of surgically castrate women (see Results page 105) was 0.098 to 0.364 mgm. per day (mean 0.208 mgm.) which differs significantly from the recovery from the normal male subjects studied as noted above (t test: $0.001 < P < 0.01$). The pregnanediol produced by the post menopausal women is the result of metabolism of progesterone secreted by the adrenal glands since the ovaries have been removed and no other source of progesterone production is known.

F. Progesterone Secretion Rates.

The daily hormone production rate or secretion rate can be estimated in four ways.

1) A known amount of hormone is injected into the patient and the urinary recovery as the original hormone or its metabolite is determined. A knowledge of the urinary steroid

excretion rate in the pre-injection period enables the rate of endogenous hormone production to be calculated. This is not a physiological procedure because the relatively large amounts of progesterone which have to be injected are likely to disturb the normal steady state of hormone metabolism.

2) The instantaneous rate of hormone secretion may be calculated from the difference in arteriovenous concentration of the hormone at the endocrine glands in question and the rate of blood flow through the glands. This is a technically difficult procedure and is rarely practicable.

3) Intravenous injection of a known trace amount of isiotopically labelled hormone followed by estimation of the metabolic clearance rate and simultaneous estimation of the plasma concentration of the hormone permits calculation of the secretion rate. (See page 28). However no direct method for estimation of the low plasma levels of progesterone

present in males and castrate females was known when this research work was commenced.

4) A reliable and accurate estimate of the secretion rate can be obtained by injecting a known trace amount of isotopically labelled hormone intravenously and estimating the specific activity of a urinary excretion product. This was the method followed in the present study. The theoretical implications of this technique are fully discussed in Appendix VII where it is shown that the present study fulfils all the stipulations required to make the calculation valid. The secretion rate so estimated is the mean secretion rate for the period of urine collection (4 days).

Pearlman (1957b) was the first to apply a radioactive tracer technique for study of progesterone secretion rates when he studied pregnant women and castrate females and calculated comparable secretion rates for pregnant women from the specific activity of urinary pregnanediol and pregnaneolone

(Contractor & Pearlman 1960). Zander (1961) reported results for pregnant women similar to those of Pearlman as did Solomon, Watanabe, Dominguez, Grey, Meeker & Sims in 1962 (188 to 563 mgm. per day, mean 322 mgm. per day).

Dominguez, Francois & Watanabe (1962) estimated the progesterone secretion rates for normal males - 3.9 to 6.8 mgm. per day; for normal females in the follicular phase of the menstrual cycle - 2.3 to 5.4 mgm. per day: for normal females in the luteal phase of the menstrual cycle - 22 to 43 mgm. per day. Romanoff (1962) calculated that the secretion rate for normal males was 3.2 to 5.6 mgm. per day (Average 4.5 mgm.).

The progesterone secretion rates in this thesis (see Results page 109) are, for normal males 2.0 to 5.8 mgm. per day (mean 3.23 mgm.) and for castrate females 0.94 to 1.26 mgm. per day (mean 1.071 mgm.) and the difference is significant (t test $0.001<P<0.01$).

The secretion rate for normal males is within the range of the rate for normal females in the follicular phase of the menstrual cycle as quoted above. It is therefore reasonable to assume that the progesterone secretion rate of normal females in the follicular phase will also differ significantly from the secretion rate of castrate females. This suggests that the normal ovary secretes progesterone in the follicular phase of the menstrual cycle in the absence of an active corpus luteum.

If it can be assumed that the normal male adrenal gland functions in a similar fashion to the normal female adrenal gland, at least as far as progesterone production is concerned, then the progesterone production rate of castrate males should differ significantly from the secretion rate of normal males and so support the hypothesis that the normal testis secretes progesterone. This is in keeping with the results of

Slaunwhite & Samuels (1956) who report progesterone as a precursor of testicular androgens in the rat. A study of progesterone secretion rates in castrate males would clarify this point.

G. Secretion of Progesterone by the Adrenal Gland.

In 1938 Beal & Reichstein isolated progesterone from extracts of adrenal glands from the ox. Balfour, Comline & Short (1957) demonstrated that the adrenal secretes progesterone by isolating this steroid from blood from the adrenal vein of cow and sheep. Klopper, Strong & Cook (1957) demonstrated that ACTH stimulation increased the urinary pregnanediol excretion in human subjects indicating an adrenal contribution to progesterone production. In 1960 Short reported the recovery of progesterone from adrenal venous blood obtained from an ACTH stimulated female patient and estimated that the adrenal production of progesterone in

this subject was 0.6 mgm. per day.

The study of castrate females in this thesis produced a progesterone secretion rate of 0.94 to 1.26 mgm. per day (mean 1.071 mgm.) which was a measurement of the production of progesterone by the intact female adrenal glands and the first time that this has been achieved.

H. Collaborative Work.

a) Method. The collaborative work will not be discussed in detail as it is to be published elsewhere. The method for the extraction of progesterone from plasma is described in Appendix IX. The concept of the 60 cm. celite column was original to the method.

Assay of circulating progesterone by a chemical technique was described by Haskins in 1950. Butt, Morris, Morris & Williams (1951), Zander (1954), Zander, von Munstermann & Marx (1955), Sommerville (1957), Sommerville & Desphande (1958), Short (1958),

Short & Eton (1959) and Oertel, Weiss & Eik-Ness (1959) developed methods for estimation of progesterone in plasma and tissue fluids. As with the method described in Appendix IX, these methods had a maximum sensitivity in the range of some 2 to 4 micrograms of progesterone per 100 ml. of plasma. The progesterone content of plasma from pregnant women was within the capability of these methods but the levels to be expected on the plasma of normal males and castrate females could not be measured.

However by measuring the plasma disappearance curve of injected radioactive progesterone (Appendix VIII) administered by a single injection or constant infusion technique, the transport and metabolism rate constants, volumes of distribution, and metabolic clearance rate of progesterone can be calculated as described in Appendix IX. If the progesterone secretion rate is measured at the same time as the metabolic clearance

rate, the mean plasma progesterone level can be calculated (see page 28).

The choice between a single injection or constant infusion technique is indicated by the theoretical considerations. If the initial and total volumes of distribution and also the individual metabolic and transport constants are required then the single injection method will give the information and not the constant infusion method.

If the metabolic clearance rate is required then the infusion method is likely to give the most reliable and precise results. When the disappearance of a steroid in plasma after a single injection is very rapid as for progesterone there are considerable difficulties in accurate sampling at known time intervals in order to obtain the characters of the first exponential ($Ae^{-\alpha t}$). Of more importance, because of the low plasma radioactive concentration, the character

of the second exponential ($Be^{-\beta t}$) is also difficult to estimate and it is impossible to test critically whether the final slope (Potted semilogarithomically, Fig. 18, page 111) is a straight line. With the constant infusion method the plasma radioactivity can be measured for a much longer period of time (with the same total administration of radioactivity) and the criterion for equilibrium (i.e. the constant plasma radioactive concentration) can be rigorously tested by comparing these values at intervals during the latter part of the infusion.

b) Results. The results of the collaborative work will not be discussed in full as this will be reported elsewhere. However, some comment is necessary. Figure 20 compares the plasma metabolite clearance rates for progesterone obtained by single injection and constant infusion methods. These subjects

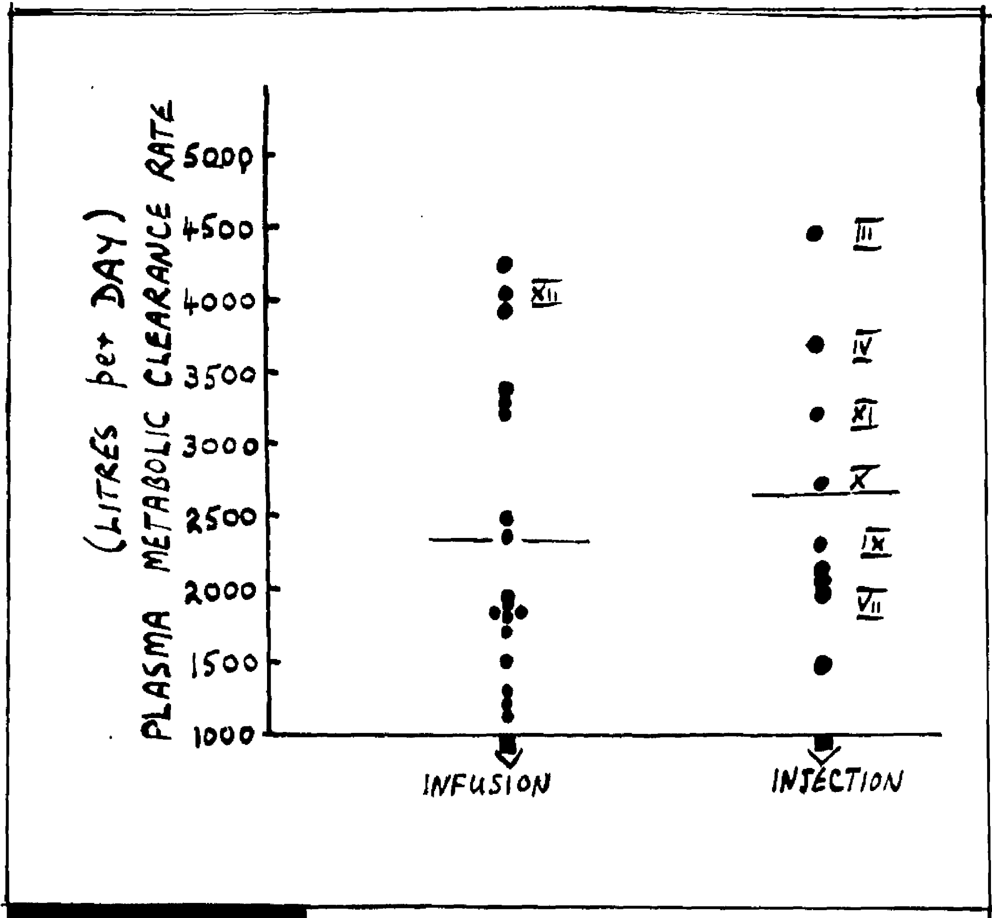


FIG. 20.

include normal males, castrate females and ovariectomised and adrenalectomized females. (27 subjects composed of the 7 subjects detailed in the collaboration study and 21 subsequent similar experiments). No significant difference in metabolic clearance rates has as yet been found among these different groups. Again there is no significant difference in the mean estimates for the metabolic clearance rates by the two methods. However, the co-efficient of variation for estimation by both methods is so large that the comparison is not a critical one.

Tait, Little, Tait, Black, Riondel & Gut (1962) have suggested that it may be useful to express the metabolic clearance rate of a steroid (whole blood) as

M = HBF x HE x EHCR where

M = Metabolic clearance rate.

HBF = hepatic blood flow.

EHCR = extra hepatic clearance rate.

If HBF (which can be determined by the usual methods) and HE (which could be determined by hepatic venous sampling of radioactive steroid after infusion) are known, then the proportion of extra hepatic metabolism may be quantitatively expressed as $\frac{EHCR}{M} = \frac{M-HBF \times HE}{M}$

It should be noted that this expression will only apply if the radioactive steroid, as for the steroid secreted by the normal gland, is mixed with the total blood flow before entering the liver. The relationship between peripheral blood concentration and secretion rate is clearly not applicable in general for oral or direct hepatic arterial administration.

The whole blood metabolic clearance rate for progesterone is about 5000 litres per day (Plasma M = 3000 L/day: progesterone blood/plasma ratio = 1.7). There is as yet no information on the hepatic extraction of progesterone but its rate of metabolism by hepatic tissues in vitro suggest that, unless its high binding to albumen prevents its

transport into liver cells in vivo, its extraction will be 100 per cent. Taking a figure of 2250 litres per day as a reasonable mean value for hepatic blood flow (Tait, Little, Tait, & Flood 1962) and accepting the hepatic extraction of progesterone as 100 per cent, then the proportion of extra hepatic metabolism of progesterone is

$$\frac{EHCR}{M} = \frac{M-HBF \times HE}{M} = \frac{5000-2250 \times 100\%}{5000} = 55\%$$

If the hepatic extraction of progesterone is less than 100 per cent then this figure will be greater than 55 per cent. However, subsequent studies reported by Tait, Little, Tait, Black, Riondel & Gut (1962), and Little, Tait, Black & Tait (1962) indicate that the mean value for metabolic clearance rate calculated from the study of larger numbers of subject is lower than 3000 litres per day, (some 2200 to 2300 litres per day), so that their estimation of the proportion of extrahepatic metabolism of progesterone is

somewhat less than 55 per cent. This relatively high value for extra hepatic metabolism of this steroid corresponds with its known rapid metabolism by extra hepatic tissues (Berliner & Wiest 1956). Figure 21 compares the whole blood metabolic clearance rate for various steroids.

Study of the transport and metabolic rate constants and volumes of distribution of progesterone will allow comparison of these values for progesterone with those of other steroids and those of progesterone in patients in various clinical conditions. Figure 22 compares the disappearance curve after a single injection of radioactive progesterone, aldosterone and cortisol, and Figure 23 compares the final volume of distribution ($V_1 + V_2$) of progesterone, aldosterone and cortisol.

<u>I. Combined Results.</u>

<u>a) Mean plasma progesterone level (i).</u>

Where M = metabolic clearance rate and

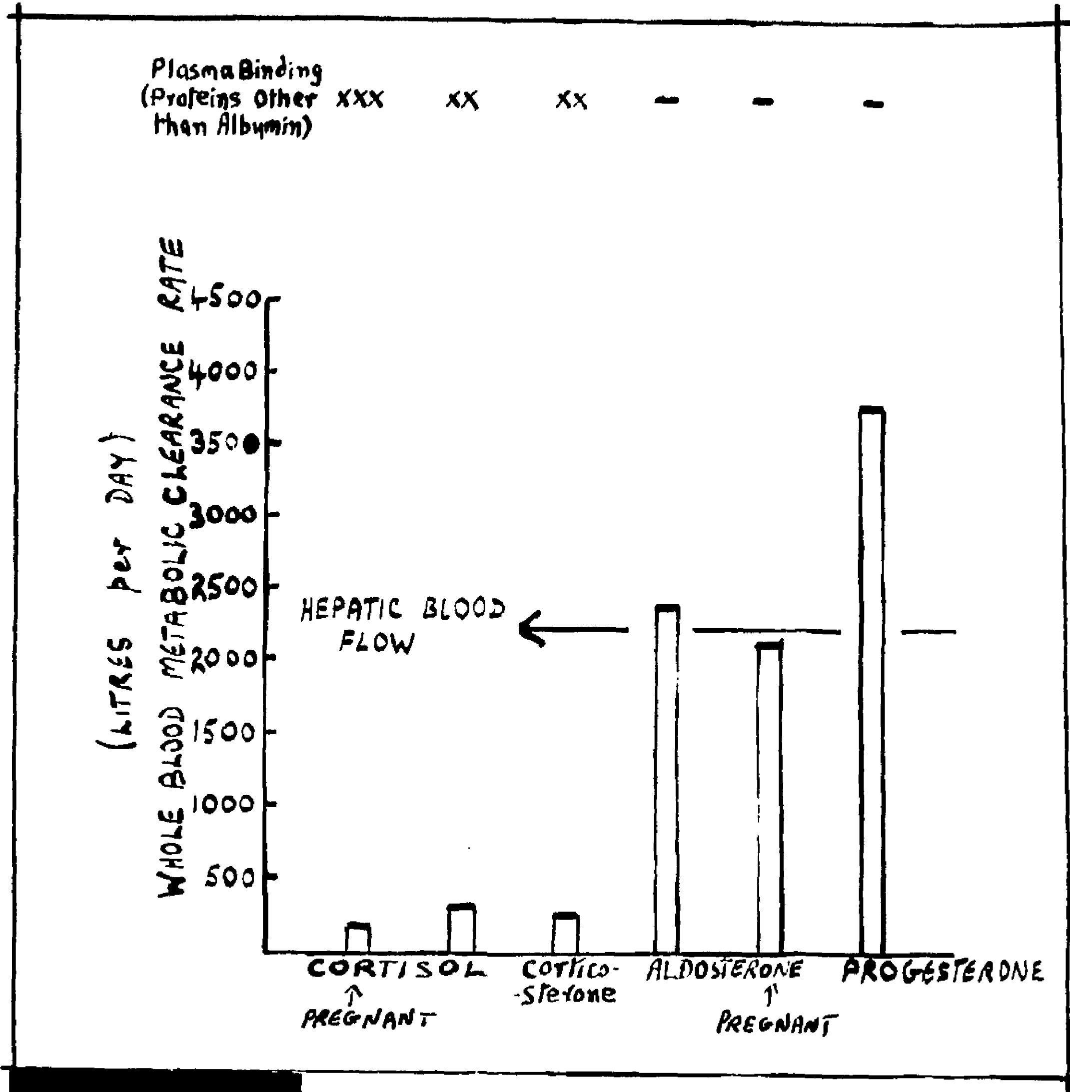


FIG.21.

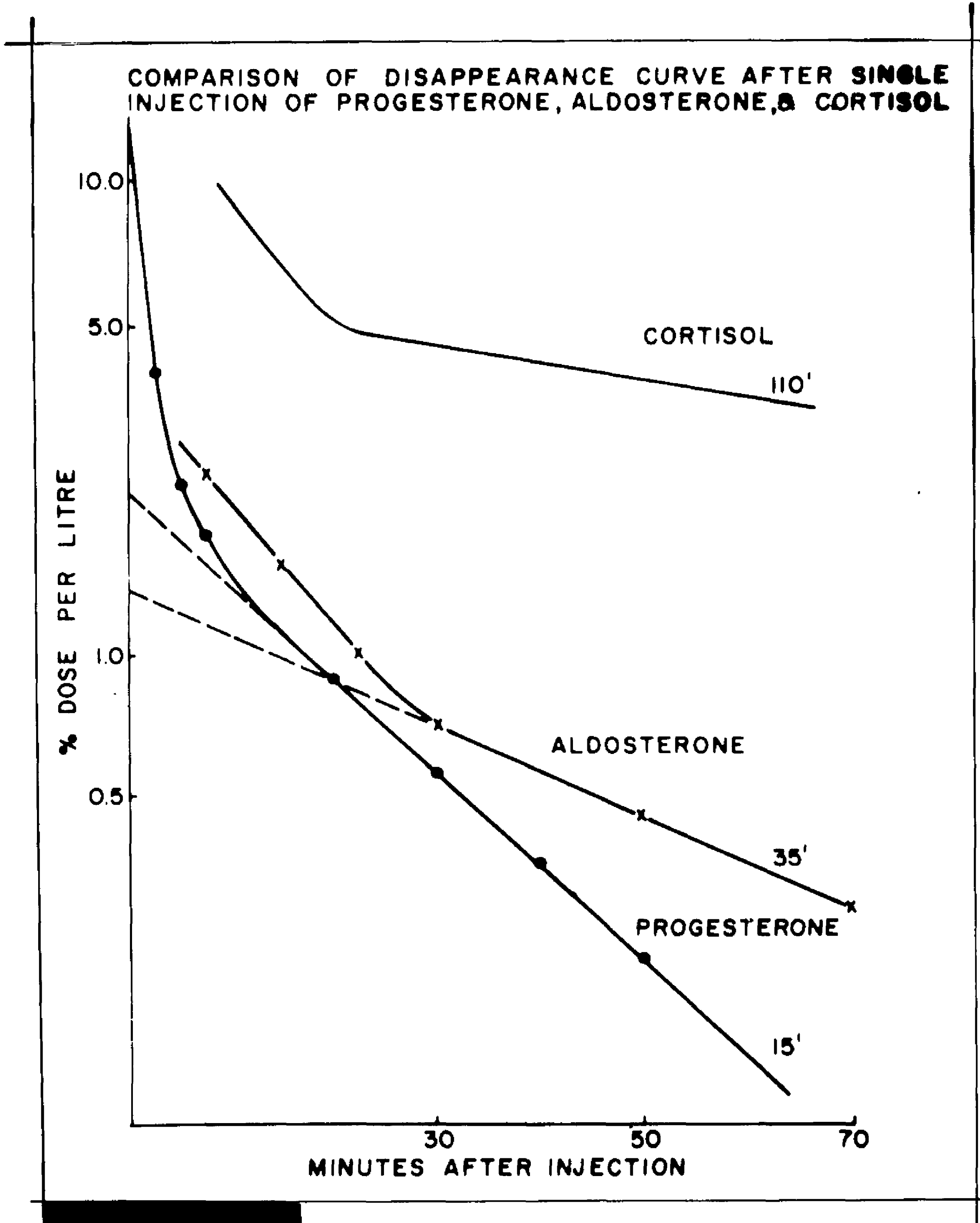


FIG. 22.

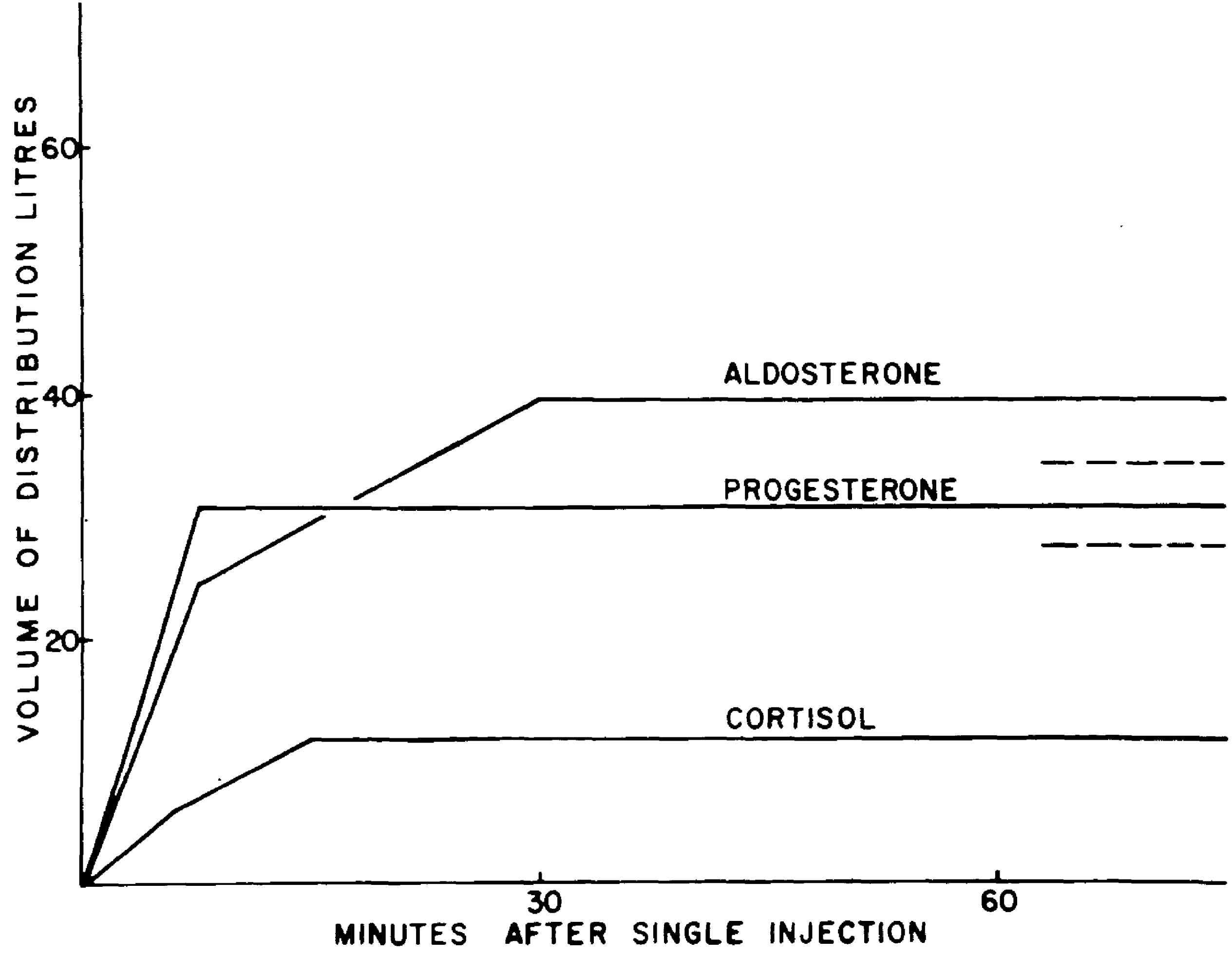


FIG. 23.

P = progesterone secretion rate, i = $\frac{P}{M}$.
The plasma progesterone levels so calculated take no account of possible diurnal variation and the values are, for normal males – 0.053 to 0.143 microgrammes per 100 ml. (Mean 0.091) and for castrate females – 0.032 to 0.034 micrograms per 100 ml. (Mean 0.033). These values just fail to differ significantly (see Results page 118; t test = $0.05 < P < 0.1$) but the M values do not differ significantly so that "i" may show a difference of significance when a larger number of subjects are studied.

Such low levels of plasma progesterone cannot be measured by the chemical methods noted previously. However labelled reagent methods of progesterone estimation have been recently developed which can measure such minute quantities of progesterone. Woolever & Goldfine (1962) estimated the plasma progesterone level in males to be 0.66 micrograms per 100 ml. and Riondel, Tait, Tait & Little (1962) estimated it to be

0.036 to 0.077 micrograms per 100 ml. There is a tenfold difference in these findings but the latter one is in keeping with the results for males in this thesis calculated by the indirect method. The progesterone secretion rate for males in this thesis is in keeping with the results of other workers. If the plasma progesterone level, as calculated by the indirect method were to have a tenfold error the metabolic clearance rate estimation would require to be ten times too great (i.e. 300 litres per day and not 3000 litres per day). The metabolic clearance rate of progesterone has not so far been studied by other workers but subsequent experiments although giving somewhat lower figures, continue to confirm the results in this collaborative work. (Tait, Little, Tait, Black, Riondel & Gut 1962, Little, Tait, Black & Tait, 1962).

b) Turnover time (TT). The only factor that is akin to the metabolic clearance rate (M) is

the Turnover Time (see Material and Methods, page 46) studied by Pearlman (1957a).

$$TT = \frac{i \times V_1}{P} = \frac{V_1}{M} = \frac{1}{K_2}$$ and the

turnover time so calculated in this thesis (see Results page 118) are for normal males 3.72 to 10.6 minutes (Mean 6.7) and for castrate females 5.85 to 6.27 minutes (Mean 6.06). Pearlman's figure for turnover time in pregnant women is some 3.3 minutes. Taking 300 mgm. per day as a reasonable mean figure for progesterone secretion rate (P) in late pregnancy, and 15 micrograms per 100 ml. of plasma as a reasonable value for plasma progesterone (i) in pregnancy, $M = \frac{P}{i}$ = 2000 litres per day. As these figures are reasonably in keeping with the results in this thesis a ten fold error in the metabolic clearance rate value is excluded.

c) Mean Total body content of Progesterone.

The total body content of progesterone at any instant (see Results page 118) is in

normal males - 17.62 to 36.57 micrograms (Mean 28.08) and in castrate females - 6.43 to 10.11 micrograms (Mean 8.27) and the difference is significant (t test = $0.02 < P < 0.05$). These values are remarkably small and this was the first time they have been estimated. Ejarque & Bengtsson (1962) have recently studied this aspect of progesterone metabolism and they calculated that the amount of progesterone stored in tissues and participating in dilution during radioisotope studies of pregnant women was 27.4 micrograms (Subjects 19 weeks pregnant: Progesterone secretion rate 75.5 mgm. per day). These low values reflect the rapid transport and metabolism of the steroid as shown by the disappearance curve of radioactive progesterone, and the low tissue levels of progesterone in the body as indicated by the minute value for plasma progesterone concentration.

Conclusions:

1) A method for estimating progesterone secretion rates utilising a radioisotope tracer technique is described and proof of specificity, accuracy and reproduceability established. The theory of calculation of secretion rates is discussed and the validity of this aspect of the method confirmed.

2) Normal male subjects and surgically castrate female subjects have the method applied for study of their progesterone metabolism and secretion rate. The initial investigations on male subjects contribute to the development of the method. The results of the study of the normal male subject and surgically castrate female subjects are compared.

3) The normal male subjects excrete significantly less total radioactivity in urine following injection of 2 microcuries of $7H^3$-progesterone (50.2 per cent of the injected dose) than the surgically castrate females (72.5 per cent of the injected dose) and this

aspect of progesterone metabolism is discussed.

4) The normal males produce significantly less radioactivity as urinary pregnanediol following injection of $7H^3$-progesterone (13 per cent of the injected dose) than the surgically castrate female subjects (18.9 per cent of the injected dose). It is noted that the ratio of urinary pregnanediol radioactivity to total radioactivity in urine is similar in these two groups.

5) Peak X - the other major peak of radioactivity recovered from the chromatographic separation of the urine extracts following injection of $7H^3$ progesterone-is significantly greater in the normal male subjects (2.66 per cent of the injected dose) than in the castrate females (1.25 per cent of the injected dose). The possible metabolic content of Peak X and the significance of this aspect of progesterone metabolism is discussed.

6) The normal males excrete significantly more urinary pregnanediol per day (458.7 micrograms) than the surgically castrate female

subjects (208.1 micrograms).

7) The normal male subjects have a significantly greater progesterone secretion rate (3.23 mgms. per day) than the surgically castrate females (1.071 mgm. per day). The secretion rate for the surgically castrate females is an estimation of the secretion rate of progesterone from the intact female adrenal glands. These results are discussed and it is suggested that the normal male testis secretes progesterone and that the normal female ovary secretes progesterone in the proliferative phase of the menstrual cycle.

8) A collaborative study (by Dr. Brian Little) of the disappearance rate of plasma radioactive progesterone following injection of $7H^3$-progesterone enables calculation of the volumes of distribution, the metabolism rate constants, and the metabolic clearance rate of progesterone. This method, its application, and the mathematical treatment applied to the calculation of the results are noted and points relevant to this thesis discussed.

9) The mean plasma progesterone level is calculated from the knowledge of the progesterone secretion rate and metabolic clearance rate. The normal male subjects have a higher mean plasma progesterone level (0.091 micrograms per 100 ml. plasma) than the surgically castrate females (0.033 micrograms per 100 ml. plasma) but the difference is not statistically significant for the numbers so far studied.

10) The total body content of progesterone is calculated from the knowledge of the mean plasma progesterone level and the final volume of distribution ($V_1 + V_2$) of the injected tracer amount of radioactive progesterone. The normal males have a significantly larger total body content of progesterone (28.08 micrograms) than the surgically castrate female subjects (8.27 micrograms).

11) Further study of progesterone metabolism in various groups of subjects in various clinical conditions by the application of this

method of investigation will allow a clearer understanding of the function of progesterone in physiological and pathological states, which hitherto has not been possible.

ACKNOWLEDGMENTS.

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APPENDIX I.

a) Equipment.

b) Chemicals.

c) Radioactive compounds.

d) Experimental Subjects.

e) Management of Subjects.

f) Radiation hazard.

a) Equipment.

Packard Tri-Carb Liquid Scintillation Spectrometer.

Beckman DU Spectrophotometer: 4 ml. quarts cuvettes.

Beckman Zeromatic pH Meter.

Tracerlab Precision Ratemeter.

International Centrifuge.

Scintillation Vials: 22 ml. glass; with plastic screw caps, silver foil lined: "Packard".

b) Chemicals.

Acetic Acid; Glacial: Reagent quality: "Du Pont".

Benzene; Redistilled: "Curtis".

Celite; "Johns-Manville" Celite 545.

Chloroform; Redistilled: "Curtis".

Ether; Anhydrous A.R.: "Mallinckrodt".

Ethyl acetate; Redistilled: "Curtis".

Ethyl alcohol; absolute; pure: "U.S. Industrial Chemical Co.".

Hydrochloric Acid; Reagent quality: - "Du Pont".

Ketodase; Brand of beef liver β glucuronidase (5000 Fishman units per ml.): "Warner-Chillcott".

Methanol; Redistilled: "Curtis".

Phosphomolybdic Acid: "Fisher".

P.O.P.O.P.: Scintillation grade "Packard".

P.P.O.: Scintillation grade: "Packard".

Sodium hydroxide; Reagent quality: "Merck".

Sodium bisulphite; Pure: "Merck".

Sulphuric acid: Conc; Reagent quality: "Du Pont".

Toluene; Sulphur free; Redistilled: "Curtis".

2.2.4 Trimethylpentane; Redistilled: "Curtis".

c) Radioactive Compounds.

H^3-7-progesterone; specific activity 30,000 microcuries per milligramme: "New England Nuclear Co.".

C^{14}-4- progesterone; specific activity 1.8 microcuries per milligramme "Picker Nuclear Co.".

C^{14}-1- acetic anhydride; specific activity 0.22 microcuries per milligramme. "Picker Nuclear Co.".

C^{14}- toluene; specific activity 0.18 microcuries/ per milligramme. "Picker Nuclear Co.".

H^3- Pregnanediol; randomly labelled; specific activity 15.32 microcuries per milligramme.

1 Curie = 3.7×10^{10} disintegrations per second.

1 Millicurie = 3.7×10^{7} d.p.s.

1 Microcurie = 3.7×10^{4} d.p.s.

= 2.22×10^{6} disintegration per minute.

= 0.416×10^{6} counts per minute (efficiency 1/5.4)

= 0.370×10^{6} c.p.m. (efficiency 1/6).

1 rontgen: corresponds to that quantity of radiation which distributes 83.8 ergs. of energy in 1 gram of air.

1 rontgen-equivalent physical (r.e.p.) is that quantity of radiation which will release an energy of 93 ergs. per gram of tissue.

The absorbed dose of any ionising radiation is the amount of energy imparted to matter by ionising particles per unit mass of irradiated material. The r.a.d. is the unit of absorbed dose and is 100 ergs. per gram.

(see Documenta Geigy Scientific Tables, 5th. ed. 1956).

d) Experimental Subjects.

Subject.

Normal Males.		Height.	Weight lbs.
*I	A.B.	6'1"	195
*II	L.S.	6'0"	170
*III	R.W.	6'0"	180
*IV	M.S.	5'7"	185
*V	R.S.	5'9½"	190
*VI	M.I.	5'8"	159
*VII	F.S.	5'8"	160
*IX	S.G.	5'11"	136
XII	R.O'R.	5'10"	172

Castrate Females.		Height.	Weight. lbs.	Age. years.	Post-operative days.
*VIII	P.S.	5'4"	136	37	6
*X	S.T.	5'2"	125	40	26
*XI	H.F.	5'5"	250	46	10
*XIII	H.T.	5'6"	125	48	34

e) Management of Experimental Subjects.

The $7H^3$ progesterone used in the experiments was obtained sterile in solution in toluene and in a concentration of 11 microcuries per millilitre from Dr. J.F. Tait, Worcester Foundation of Experimental Biology, Shrewsbury, Mass. U.S.A. Thus 0.2 ml. of this progesterone solution contained 2.2 microcuries of radioactivity. When 0.2 ml. was taken up

in 11 ml. of normal saline, 10 ml. of the saline solution contained 2.0 microcuries of radioactive progesterone.

All the single injection experiments were performed in the following manner.

The experimental subject was fasted overnight. The following morning two 0.2 ml. aliquots of $7H^3$ progesterone (11 microcuries per ml.) were taken using a sterile 0.2 ml. blow out pipette. The first 0.2 ml. was assayed for radioactivity in the Scintillation Counter. The second was added to 11 ml. sterile normal saline. 10 ml. of this saline solution was taken in a disposable 10 ml. syringe for injection into the experimental subject.

Prior to the injection, the subject emptied the bladder. The injection was made into the antecubital vein. After 5 ml. had been injected the time was noted (= zero time).

The syringe and pipette were destroyed and care was taken that the hands were washed

after each handling of equipment containing this relatively high radioactivity.

Blood samples were withdrawn from the experimental subject, 2½, 5, 7½, 10, 15, 20, 30, 50 or 70 minutes following the injection (taken from zero time) depending on the experiment. The first withdrawal of blood was taken from the arm not used for the radioactive injection. Blood was withdrawn into heparinised syringes in sufficient quantity to obtain 5, 5, 10, 10, 20, 20, 20, 40 and 100 ml. plasma respectively which were then extracted as noted in Appendix VIII.

Subsequent to the injection, the experimental subject collected all urine for the next four days. The collections were made in 2 litre winchester bottles containing 1.25 ml. toluene as preservative. The bottles were kept in a refrigerator during the day of collection and at the end of each day, the bottle containing the day's collection was transferred to the deep-freeze to await

extraction. When the urine was required for extraction (Appendix II) the bottles were thawed out at room temperature, each day's output measured, and the appropriate quantity taken for analysis. Thereafter the urine was not again frozen but retained in a refrigerator (0-4° centigrade) for further experiments as required.

f) Radiation Hazard.

The maximum permissible amount of H^3 (as water) in the body is 10 millicuries (Recommendations of the International Commission on Radiological Protection, 1950). Any data which suggests a lower figure uses an arbitrary safety factor. The soft tissue dose from natural radioactivity would appear to be about 2 m.r.a.d. per week (Spiers and Burch 1952). 10 microcuries of tritiated water in a single injection would give a total radiation dose of 2 m.r.a.d. (assuming 19 day half life and 40 litres total body water space). This dose would therefore appear to be safe for serial

injection and 1 millicurie reasonable for a single injection per year (total dose 200 m.r.a.d., maximum dose rate 45 m.r.a.d. per week; tolerance dose rate = 300 m.r.a.d. per week).

The safe dose of a tritiated steroid depends on knowledge of concentration in particular tissues and so far such knowledge is scanty. From the work of Davis, Plotz, Le Roy, Gould & Werber (1956), Davis & Plotz (1958), Zander (1961), Plotz (1961) and from the results contained in this thesis, some 70 to 90 percent of the radioactivity injected as progesterone can be accounted for at the end of four to six days. Assuming that 10 per cent of the radioactivity injected and unaccounted for by uranalysis (a reasonable figure for progesterone) is concentrated permanently by the yellow marrow (weight 1.5 Kg.) than from a dose of 100 microcuries the radiation to this tissue will be 12 m.r.a.d. per week, about six times the natural

background. In this research 2 microcuries have been injected.

The above considerations apply to normal adults. For a pregnant woman, if it is assumed that 10 per cent of the injected dose is concentrated permanently in the sensitive organs of the foetus (total volume 100 ml.), than a 2 microcurie injection would deliver a radiation dose rate to those tissues of 4 m.r.a.d. per week or about twice the natural background. Thus the technique is applicable to pregnant patients and the safety would be further assured by restricting the injection to the last trimester when the foetus is least radio sensitive.

Comparison of C^{14} and H^3 steroids confirms the superiority of H^3 steroids for this experimental programme. Although the detection efficiency for C^{14} is approximately three times that of H^3 for both flow counter and liquid scintillator methods, the radiation dose from C^{14} is about ten times that of H^3

so that the safety factor for tritium over C^{14} is about threefold when the quantities injected are adjusted to give the same amount of information. Furthermore the specific activity of tritiated steroids now available is about 300 times that of C^{14} labelled steroids. Allowing for the lowered detection efficiency the advantage of H^3 from the specific activity point of view over C^{14} is one hundred.

Tritiated steroids are therefore safer and more effective tracers than C^{14} labelled steroids. Contamination, from a safety angle, is thus less dangerous. However contamination of equipment which could vitiate results is more of a problem because of lack of sensitive monitors for H^3. However paper swabs can be taken (detection efficiency about 1 per cent on a flow counter) and this should not be a serious problem unless synthetic and analytical procedures are being carried out in the same laboratory.

Those who work with radioactive substances have to be aware of the dangers involved and the quantities of radioactivity being processed. Radiation detection badges must be worn, changed regularly, and readings charted for each period. The laboratory requires to be tested regularly for contamination. Equipment used for isotope research has to be kept free from contamination not only for personal safety but to ensure reliable scientific results.

Glassware was washed thoroughly after use as follows -

Rinsed with hot water.

Washed with detergent.

Steeped in cleaning solution overnight.

Rinsed with hot water.

Dried in a drying oven.

The cleaning solution was -

100 G Dichromate of potassium.

750 ml. Water.

250 ml. Sulphuric acid (Conc.).

APPENDIX II.

URINE EXTRACTION:

A) METHOD I.

B) METHOD II.

C) METHOD III.

D) METHOD IIIb.

E) METHOD IV.

Method I utilised 25000 units β glucuronidase for incubation, followed by toluene extraction. This proved to be an insufficient amount of ketodase. The toluene extract was slow to take to dryness and did not suit the facilities available in the laboratory.

Method II utilised acid hydrolysis, ether extraction, and a more refined benzene-alkali partition. Applying this method to urine already extracted after ketodase incubation resulted in unexpected pregnanediol spectrophotometer readings not related to radioactivity as detected by the scintillation spectrometer. This confirmed the detrimental effect of acid hydrolysis on pregnanediol assay reported by Bongiovanni and Clayton (1954) and Ronan, Parsons, Namiot & Wotiz (1960). The ether extract could be taken to dryness with greater facility.

Method III utilised 250,000 units of β glucuronidase for incubation, followed by

ether extraction, and benzene-alkali partition (Figure 24). This method proved satisfactory, was slightly modified (-Method IIIb), and thereafter used for the research work.

Method IV was evolved for the extraction of pregnancy urine which could be expected to contain some 60 mgm. of pregnanediol per day.

A) METHOD I.

1) Collect urine output daily in a Winchester bottle for four days following the single intravenous injection of 2 microcuries of $7H^3$ progesterone. Keep urine in refrigerator while daily output is being collected. When total output for a day is complete, transfer urine container to the "deep freeze" so that the urine is frozen solid till required for extraction.

2) Remove urine from "deep freeze" and allow to thaw out at room temperature. Measure urine volume for each day.

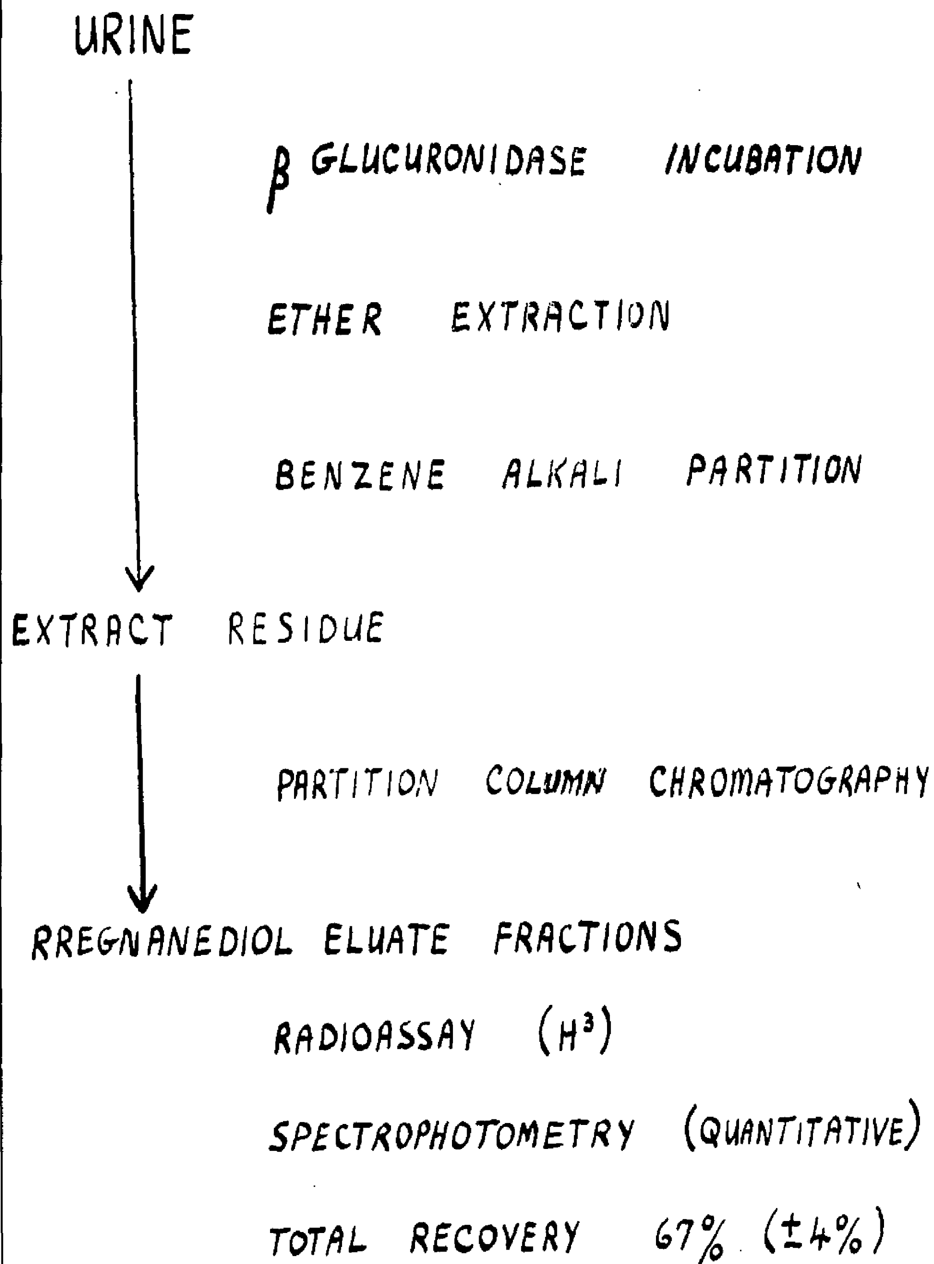


FIG. 24.

3) Ketodase Incubation. Take 500 ml. urine and transfer to 2 litre erlenmeyer flask. Adjust pH to 4.5 with 50 per cent acetic acid (about 0.5 to 1.5 ml. required). The pH was measured by a Beckman Zeromatic pH Meter or by appropriate pH paper. Add 50 ml. acetate buffer.

Buffer - Solution A = 0.2M. solution of acetic acid (11.55 ml. glacial acetic acid in 1000 ml. deionised water).

Solution B = 0.2M. solution of sodium acetate (16.4 G of $C_2H_3O_2$ Na. or 27.2 G $C_2H_3O_2$ Na $3H_2O$ in 1000 ml. deionised water).

28 ml. Solution A + 22 ml. Solution B = pH 4.5.

4) Add 5 ml. ketodase (Brand of β glucuronidase, 5000 Fishman units per ml.).

5) Incubate at $37^{o}C$. overnight with shaking in a temperature controlled water bath shaker.

6) Toluene Extraction. Extract with toluene in 2 litre separatory funnel

500 ml. once

250 ml. twice.

If an emulsion forms in the toluene extract and proves troublesome the toluene phase will require centrifugation - in the International Centrifuge at 2,500 r.p.m. for 3 to 5 minutes.

7) Wash toluene extract with 1/10 volume of N. sodium hydroxide twice.

8) Wash toluene extract with 1/10 volume of deioniser water twice.

9) Take toluene extract to dryness in a two litre flask with vacuum pump and at a temperature of not more than 50°C.

10) Dissolve residue in 10 ml. Chloroform - Methanol (equal parts), transfer to conical test tube and take to dryness with stream of nitrogen.

11) Add 100 gamma pregnanediol standard as carrier and again take to dryness.

12) Put on chromatographic column. (Appendix III).

B) METHOD II.

1) Measure 500 ml. urine into a 2 litre round bottom flask fitted with a condenser and bring to the boil.

2) Add 50 ml. 10N Hydrochloric acid cautiously through the condenser to the boiling urine and continue refluxing for 10 minutes. Thereafter cool the hydrolysed urine rapidly. (833 ml. Conc. hydrochloric acid to 1000 ml. with deionised water = 10N hydrochloric acid).

3) Extract the hydrolysed urine with freshly opened peroxide free ether in a 2 litre separatory funnel using 500 ml. and 250 ml. twice. Any emulsion in the ether phase was removed by centrifugation as previously described.

4) Wash the ether extract with deionised water - 25 ml. four times, and thereafter take to dryness in a 2 litre round bottom flask in a fume cupboard with water bath at not more than 50°C.

5) Further purify the extract by partitioning it between benzene (50 ml.) and N. sodium hydroxide (25 ml. four times) in a 125 separatory funnel.

6) Free the benzene phase from alkali by washing it with deionised water to neutrality (25 ml. five or six times). The pH was checked by pH meter or pH paper.

7) Take the benzene extract to dryness in a 100 ml. round bottom flask with vacuum.

8) Dissolve the residue in 10 mls. chloroform - methanol (equal parts), transfer to a conical test tube and dry down with a stream of nitrogen.

9) Add 100 gamma of standard pregnanediol as carrier and put extract on chromatographic column.

C) METHOD III.

1) Ketodase incubation of 500 mls. urine with 50 mls. ketodase as described.

2) Ether extraction and benzene-alkali partition as previously detailed.

3) Carrier pregnanediol was added to the

dried down benzene extract which was then taken up in mobile phase directly from the 100 ml. round bottom flask and put on the Column.

D) METHOD IIIb.

As Method III but no carrier pregnanediol was added as this had been shown to be unnecessary.

E) METHOD IV.

1) Urine daily volume made up to 2 litres and 500 mls. incubated with 50 mls. ketodase as before.

2) Ether extraction and water wash as described.

3) Benzene-alkali partition.

80 ml. benzene

40 ml. N. sodium hydroxide four times.

4) Benzene phase water wash to neutrality

40 ml. five times.

5) Benzene extract divided into three portions, dried down in 100 ml. round bottom flasks and put on a column directly from the flask as before. This method gave disappointing

recovery rates for pregnanediol and further modification would be required before a satisfactory precedure could be recommended for extraction of pregnancy urine. Probably not enough ketodase was utilised for the incubation and the chromatographic columns were overloaded with pregnanediol in the extracts.

The result of these extraction experiments suggest that 1) When this method is applied 250,000 units of β glucuronidase are required to free not more than 2 mgm. of pregnanediol from conjugation with glucuronic acid and 11) not more than 1.5 mgm. of pregnanediol (preferably 1.0 mgm.) should be in the extract put on the Columns.

It may be argued that since the results of such experiments are based on the calculation of the specific activity of the recovered pregnanediol, the recovery efficiency of the method is not so significant. However it is considered that the recovery efficiency

of any extraction method in such research work as this, should have a recovery rate of at least 50 per cent. This should be achieved for pregnancy urine by suitable modification of the present technique.

APPENDIX III.

Partition Chromatography.

Column Partition Chromatography.

Paper Partition Chromatography.

Counter Current Distribution.

Partition Chromatography.

Chromatography is a physical process which exploits differences in a physical property among a series of chemically similar substances. This property can be termed "relative solubility in two immiscible solvents".

Solubility can be thought of as reflecting the balance between the polar and non-polar properties of a molecule. Polar properties depend on the number of reactive, or hydrophylic groups, such as hydroxyl, ketone, halogen and amino groups. Non-polar properties depend on the non-reactive, hydrophobic groups or structures. These are typically long hydrocarbon chains or ring systems.

This distinction between polar and non-polar properties applies to both solvents and solutes. In the present instance the solutes are steroids. Table 1 lists some non-polar solvents in groups of increasing

Table 1. Some Non-Polar Solvents.

Least Polar	Medium	Non-Polar
Ligroin	CCl_4	$CHCl_3$
*Petroleum Ether	*Benzene	CH_2Cl_3
Hexane	*Toluene	*Ethyl Acetate
*Iso octane (224 T.M.P.)	*Ether	

* Solvents used in this research.

Table 2. Some Polar Solvents.

Miscible with Non-Polar.	Immiscible with Non-Polar
Ethanol	H_2O
Methanol	* H_2O: Alcohol
Acetone	Propylene Glycol
	Formamide

* Solvents used in this research.

polarity. The least polar have only hydrocarbon chains while the polarity increases as other groups appear. Table 2 lists some polar solvents. Those on the left are miscible both with water and with less polar solvents. Those on the right are immiscible with the less polar solvents. Solvent systems are composed of a mixture of non-polar and immiscible polar solvents, i.e. there are two layers each saturated with the other.

The non-polar component common to all steroids is the 4-ring system. As the number of oxygen groups, i.e. Ketone and hydroxyl groups, increases, their polarity increases. Double bonds have the same effect.

Steroids with only one or two oxygen groups, viz. gonadol hormones and their metabolites, are of low polarity and are soluble in non-polar solvents such as ether and benzene. Steroids with 5 or more oxygen

groups are of high polarity and are soluble only in more polar solvents such as ethyl acetate and chloroform. Thoy may even be slightly soluble in water.

The fact that certain polar and non-polar solvents are immiscible has provided a powerful analytical tool, i.e. distribution, or partition of a solute between two solvents which are contained in the same vessel.

The simplest application of this tool is the familiar extraction process. An aqueous solution, such as urine, containing both organic salts and steroids may be shaken with ether in a separatory funnel. After the layers have separated, about 98 per cent of the salts remain in the aqueous layer and about 95% of the steroids are in the ether layer. The separation is made possible by the tremendous difference, perhaps over 1000-fold, in the "partition coefficient" between ether and water.

The simplified distribution law states

that when a solute is dissolved in one phase which is in equilibrium with another immiscible phase, the solute will distribute itself so that the ratio of the concentration in the two phases is constant for a varying amount of solute. It is assumed that the temperature is fixed and the volume of the two phases are equal. The partition coefficient, P, is then defined as -

$$P = \frac{\text{Concentration of solute in non-polar phase}}{\text{Concentration of solute in polar phase}}$$

The constancy of P regardless of the amount of solute holds only under "ideal" conditions, usually a limited range of concentration and under circumstances where there is no association or dissociation of molecules.

Inorganic salts can be readily separated from other soluble steroids because their partition coefficients are very different. But a group of several steroids may have rather similar partition coefficients. In such a situation it requires many successive

partitions to separate them from each other. These successive partitions are affected by procedures or devices which move one phase of the solvent system past the other.

This may be visualised by considering that -

1) A substance with a high P will be mostly in the non-polar (upper, solvent) mobile phase, while -

2) A substance with a low P will be mostly in the polar (lower, aqueous) stationary phase.

Given a series of separatory funnels, each filled with equal volumes of two immiscible solvents, the solutes may be placed in the first funnel and shaken to attain distribution (Figure 25 a,b,c) equilibrium. After the two phases have had time to separate, each non-polar, or upper layer, may now be moved over into the adjacent funnel and the higher the P value of the solute, the more it will be moved. (Figure 25 d,e,f,g). As this process is repeated, the solute with the high

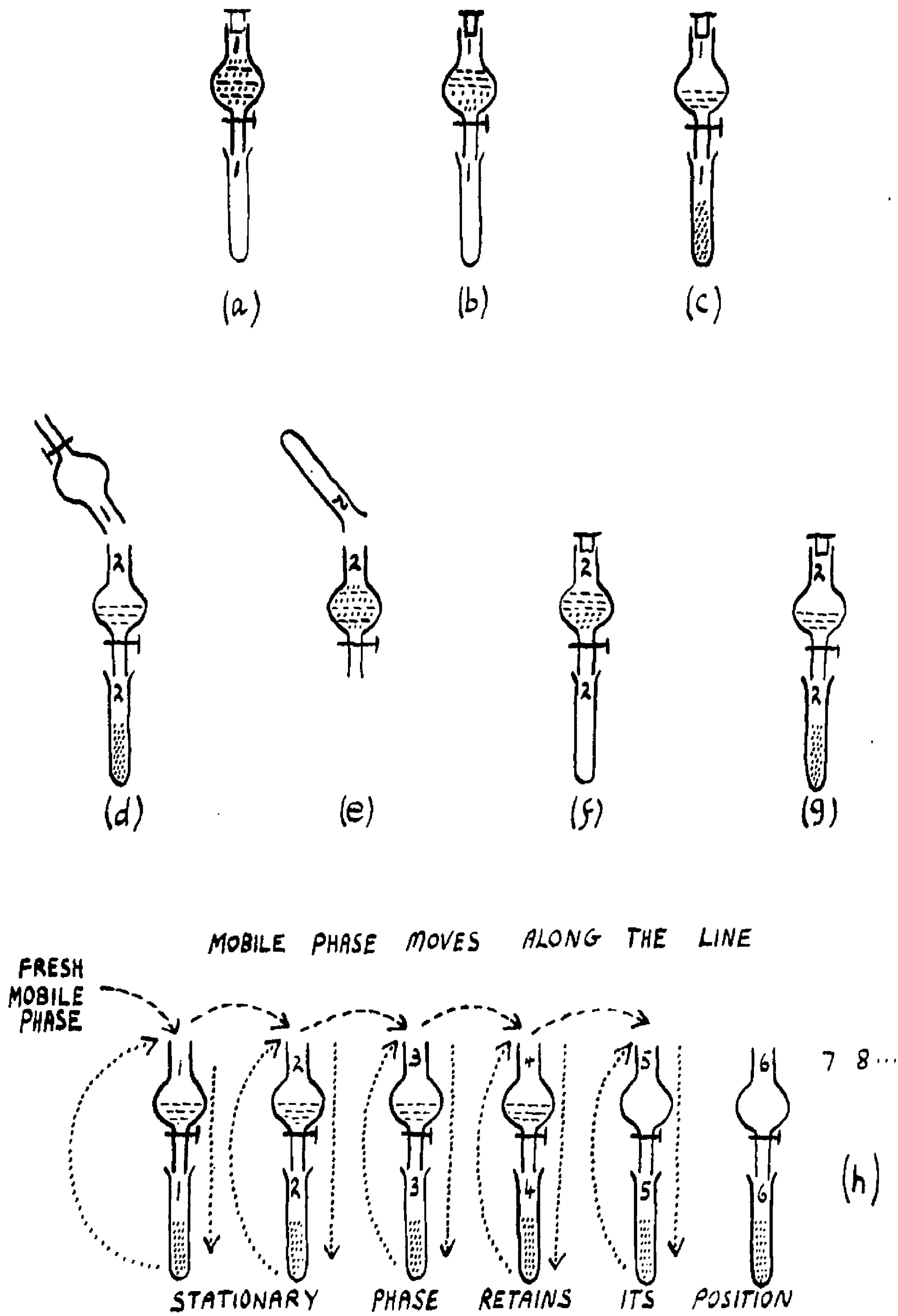


FIG.25.

P value will move down the line of funnels after fewer distributions, i.e. faster, than the one with a low P value.

The simplest example of this type of procedure is "counter current distribution". Here the solute "moves" along a row of tubes or separatory funnels as the upper layers are moved over one at a time and the speed of its progress depends on its partition coefficient. (Figure 25 h). After a number of equilibrations or transfers, the solute concentrations are distributed symmetrically over several tubes with the central or peak tube containing the greatest amount and lesser amounts trailing off in tubes to either side. This localisation of the solute is called a "band". Two solutes with sufficiently different P values will eventually be located in different groups of tubes or bands and thus be separated.

A partition column is simply a modified counter current distribution

apparatus which works by itself using the force of gravity. Instead of many tubes, each with stationary or "lower" phase held within it, there is only one tube filled with tiny globules of polar (stationary) phase. Then the non-polar (mobile) phase is allowed to flow down over these globules.

In order to achieve this immobilisation of the polar phase, a finely divided solid polar material or adsorbent is required. Adsorbents are selected which have a high surface area per unit weight i.e. each particle is porous and has many interstices. Various silicates are commonly used for this purpose as well as silica gel, celite and cellulose powder.

Such a polar adsorbent will attract and tightly hold the polar phase around itself: in effect a globule. The non-polar solvent or mobile phase can now flow over these globules without displacing the polar solvent. Yet the solute (steroid) will

distribute between these two phases very much as it does in a separating funnel. All these components packed into a tube constitute a partition column. (Figure 26).

In a partition column, the solute (or crude steroid extract) is applied in a thin band at the top. As the non-polar solvent flows down the column from a reservoir, partition occurs at each microscopic layer of globules so that a very large number of partitions are obtained as compared with those possible with a series of tubes.

Here a new term "column volume" should be defined. This term indicates the volume of mobile phase filling the interstices between the globules of stationary phase in a column. As is shown in Table 3 a substance which is essentially completely insoluble in the stationary phase and 100 per cent soluble in the mobile phase has an infinite partition coefficient and obviously will move with the

CHROMATOGRAPHY COLUMN

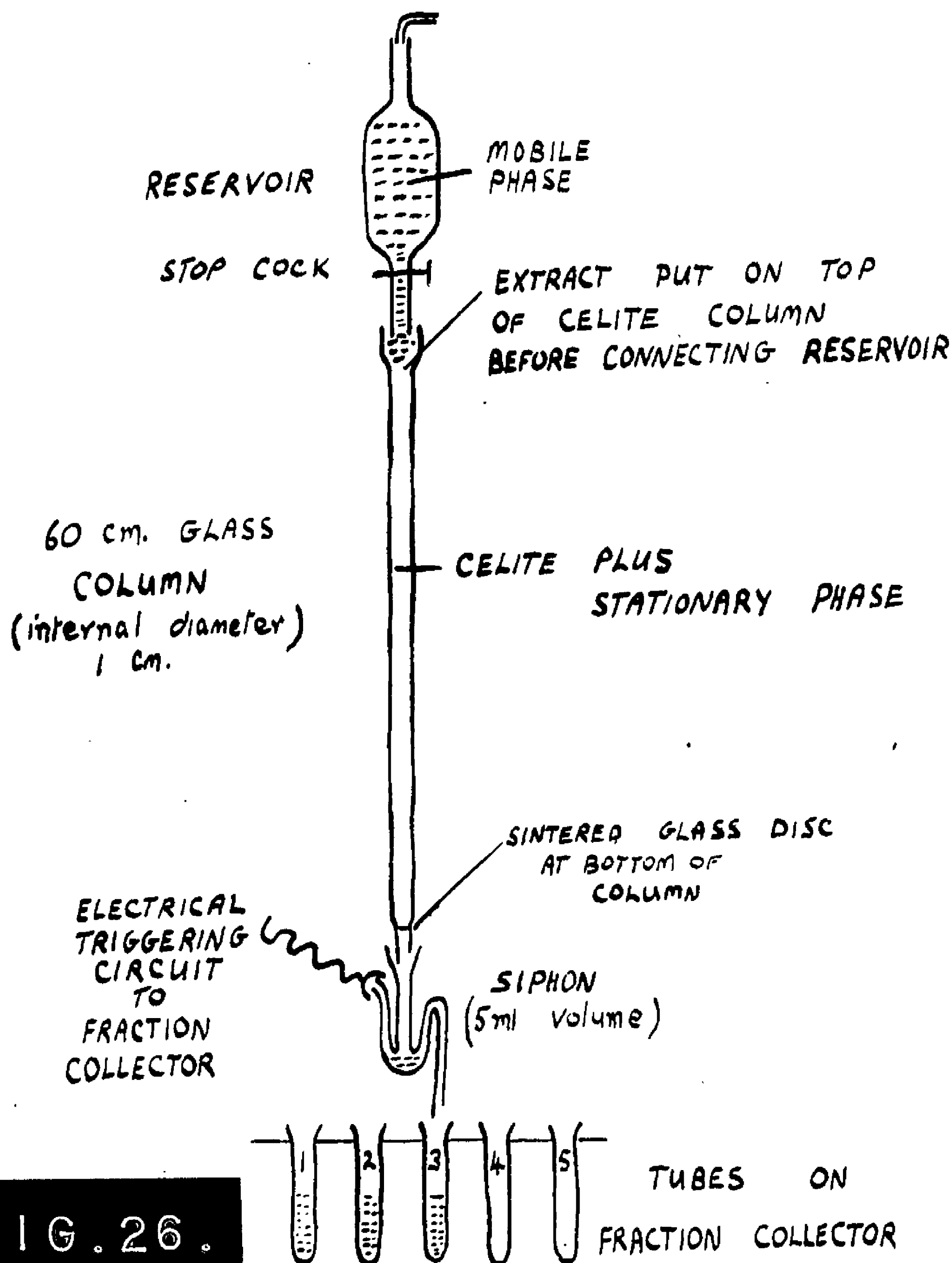


FIG. 26.

Table 3.

P	Distribution.	Mobility.	Column Volumes to Elute.	
	All in mobile phase	With solvent front	1	figures refer to peak band.
1.0	½ in stationary phase	½ of front	2	
0.5	2/3 in stationary phase	1/3 of front	3	
0.1	9/10 in stationary phase	1/10 of front	10	

solvent front. With another substance equally soluble in both phases, half of it will be motionless at any one time so that it will move only half as fast and require twice as much solvent to elute it. As the P values go down, mobility decreases and larger volumes are needed to elute the solute.

Thus mobility on a column is measured by the volume of solvent required to elute any given compound. It is clear that if the band width is not excessive two solutes requiring very different volumes can be separated physically simply by changing the receiving vessels at appropriate intervals.

Theoretically one should be able to calculate the elution volume of a solute (steroid) whose P value for the solvent system is known. In some instances, particularly for fast moving steroids, these calculations hold but in general the P values on a column are not the same as those in a separatory funnel and the steroids will move more slowly

than expected. This is probably due in part to some adsorption of the steroid directly to the solid adsorbent and partly to the fact that the dissolving powers of the bound polar phase have been altered. Consequently the operation of a partition column can only be approximated from a knowledge of P values and standardisation experiments must be performed on an impirical basis to establish mobilities and separations.

In paper partition chromatography the same general principles apply. The filter paper sheet constitutes the "supporting" polar adsorbent and holds the polar phase stationary in its interstices. In some systems the paper is dipped into the polar liquid and then blotted and in others (e.g. Bush System) the vapours of the polar phase are allowed to enter the paper while it hangs in a closed tank. Paper chromatography is usually performed for discrete separation of a single compound

as the bands can be located accurately. The solutes of interest are not allowed to run off the paper and are separated by cutting out the region where they are found.

Column Partition Chromatography.

A column was constructed to obtain separation of pure pregnanediol from the crude urine extracts. To obtain good separation and maximum purity of the pregnanediol the column was made 60 cm. long and the solvent system made more retentive towards the less polar steroids.

A) Construction of the Column.

1) The pure celite, as obtained from the manufacturers, was washed

a) overlay with conc. HCl and leave overnight (x 2).

b) Wash with distilled water till free from chloride ions.

c) Wash with methanol (x 3).

d) Wash with 2.2.4. trimethylpentane once.

e) Dry in an oven.

2) The mobile and stationary phases were equilabrated

Stationary phase - Methanol		480 ml.
	Deionised Water	120 ml.
Mobile phase - Toluene		550 ml.
2.2.4. Trimethylpentane		450 ml.

These volumes were placed in a two litre separatory funnel, shaken up, and allowed to stand overnight to separate out and equilibrate. Thereafter, the lower, stationary phase, was run off and kept in a stoppered bottle as was the upper mobile phase.

3) Thirty grammes of celite were weighed out and placed in a 600 ml. beaker. Fifteen millilitres of stationary phase were added and mixed in with a glass stirring rod for 20 minutes. Mobile phase was then added with mixing to make a slurry.

4) Glass chromatographic columns, 60 cms. long, with internal diameters of 1.1 - 1.3 cms.

were available. A column was packed firmly with the celite using a metal packing rod. The packing had to be even and during the procedure which took some 60 to 90 minutes, care had to be taken that the celite was covered with mobile phase and not allowed to dry out. When the column was packed, the top of the celite packing was made smooth and firm. The glass reservoir, with stop-cock was fitted to the top of the column, the column fixed upright in the fraction collector, and its delivery end directed into a 5 ml. siphon collector. The column was allowed to run overnight to equilibrate it and note its rate of flow. Thereafter the column was ready for use.

B) Management of the Column.

All materials submitted to partition column chromatography were put on the column in a similar manner.

The material for chromatography was taken to dryness in a suitable container, and

thereafter dissolved in 1 ml. of mobile phase.

The reservoir was detatched from the equilibrated column and the mobile phase pipetted from the top of the column to expose the celite. The millilitre of mobile phase with the experimental material in solution was transferred to the top of the column by Pasteur pipette. Half a millilitre of mobile phase was added to wash out the experimental substance container and this was transferred by pipette to the top of the column when the first millilitre had run into the column, leaving the celite exposed. A further 0.5 ml. of mobile phase was used to rinse the container and applied to the column as before. Thus the experimental material was applied to the column in a total of 2 ml. mobile phase, in such a way that a minimal amount was left behind in the sample container.

When the second 0.5 ml. of experimental

material in solution in mobile phase had run into the column, the reservoir was reconnected to the column, the air bubble at the top of the column eliminated and the column left running. At the same time, the 5 ml. siphon collector was emptied so that measured collections into the tubes contained in the fraction collector commenced from this time.

When sufficient tubes of eluate had been collected for any given experiment, the tubes were accurately serially labelled and numbered on removal from the fraction collector.

The column was then disconnected and the top one inch of celite removed from the top of the column. Previous to this, 4 grammes of celite had been weighed out in a 100 ml. beaker and 2 ml. mobile phase added and mixed in with a glass rod for 15 minutes. Mobile phase had been added to make a slurry and this celite mixture (similar to that used to make the column) was

packed on to the top of the column to replace the layer of celite removed. The column was then reconnected and allowed to run at least overnight to re-equilibrate and clean any remaining slow running components of the experimental material from the column. Thereafter the column was ready for another experiment. With the research programme progressing smoothly, the columns were running continuously, with experimental material being added approximately every three days, the column being treated as described above, between each experimental procedure.

The fraction collector was a simple mechanical device. It consisted of a metal wheel with four concentric circular rings of holes at the periphery into which were placed the tubes for collecting the eluate. Thus four columns could be run simultaneously. Each column delivered into a 5 ml. siphon collector which delivered 5 ml. aliquots of

eluate into the tubes in the fraction collector wheel. One siphon was attached to an electrical device which triggered the wheel mechanism to advance round the tubes one at a time after the siphon filled a tube with its 5 ml. of contents. The siphon connected to the triggering device was that of the fastest column if more than one column was in use and in practice this method of collection was efficient. During the equilibration of a column, there was no need to use the fraction collector, the eluate being collected from the siphon into a beaker. The siphons themselves were washed in mobile phase after each experiment, and thorough cleaning was completed by the passage of eluate during the equilibration of the column.

If the column was not in use, the stop-cock of the reservoir was turned off and the delivery end of the column immersed in a conical flask containing mobile phase. Thus the column was never allowed to dry out

in any of its length at any time.

Paper Chromatography:

Paper chromatography was utilised
1) to confirm the efficiency of the column solvent system to separate pregnanediol from various other steroid metabolites of progesterone when they were included in a Bush paper chromatography system. The results of this would reflect the relationship of these steroids in the column eluate should they be included in a column extract.
2) to relate the radioactivity in the eluate tubes from the columns during the initial tests of column efficiency, to known metabolites of progesterone when compared by paper chromatography.
3) to separate pregnanediol diacetate from any unacetylated residue in the acetylation experiment.

Paper chromatography was performed by the Bush System (Bush 1952) as follows.

The solvent system was the one used for column chromatography. A cylindrical glass tank was utilised (Diameter 14" Height 30"). The top was closed by a sheet of plate glass with a central circular hole closed by a rubber stopper. The top of the tank was smeared with vaseline to achieve a satisfactory seal when the plate glass lid was in position.

The tank was lined by two sheets of chromatographic paper, one with a square window cut in it. Two metal shelves were fixed opposite each other inside the tank some six inches from the top. The mobile phase saturated the lining sheets and lay on the bottom of the tank. The stationary phase saturated two chromatographic paper cones which sat into glass beakers on the bottom of the tank, the beakers containing stationary phase. Thus the two phases were able to saturate the air in the tank without the fluid phases coming into contact with each other. The tank was left closed overnight

to equilibrate before use.

A length of chromatographic paper was cut from an intact sheet. The width varied with the number of samples to be run simultaneously. No more than six samples were run at one time so that the maximum width used was 7 inches. To run 5 samples, for example, a 6 inch strip of chromatographic paper was cut. A line (a) 10 cms. from one end was drawn across it by pencil and another line (b) 2.5 cms. further on from the first line. The lines were marked off in inches and lines drawn in to form 6 squares. The intersections on line (a) marked the points for application of the samples.

The prepared paper was contained in a folded full sheet of chromatographic paper to keep it clean, the lined end of the strip protruding to allow application of the samples. This end of the paper was suspended between 4 pieces of plate glass so that there would be no contamination during application

of the samples. The experimental samples were pipetted drop by drop on to the appropriately labelled marks and dried quickly with a stream of pure nitrogen so that each sample was applied on a small circular area of paper about 0.5 cm. in diameter.

The paper was then fixed in a metal trough, the end with the samples being in the trough, and suspended in the equilibrated tank so that the paper did not come into contact with the lining papers or cones. The tank was closed and left overnight for the paper to equilibrate. Next morning mobile phase was added to the trough through the hole in the plate glass cover. The chromatogram was allowed to run for 3 to 5 hours, i.e. until the front was some 7 to 10 cms. from the end of the paper. The tank was protected by a cardboard sheath to protect it from air currents but otherwise the papers were run at room temperature.

As a rule, the samples were "walked up" from line (a) to line (b) with "walking up" solution before being placed in the tank to equilibrate.

"Walking up" solution - equal parts Chloroform
Ethyl acetate
Methanol

This was achieved by inserting the marked end of the paper in a tank of "walking up" solution to a depth of some 2 cms. from line (a). The tank was situated in a glass container so that the paper was in an atmosphere saturated with "walking up" solution. The solution was allowed to rise by absorption till the line of wetting was level with line (b). Thereafter the paper was removed and dried at room temperature with an electrical fan hair drier. The papers were walked up twice. When walking up was used, line (b) was the start line for the chromatogram, when walking up was not used, the start line was line (a).

When the chromatogram had run for a sufficient length of time in the tank, i.e. until the front was some 7 to 10 cms. from the end, the paper was removed and dried at room temperature with the hair drier. The front was then accurately determined by viewing the paper in ultraviolet light.

Thereafter the paper was cut into 1 inch strips, the centre of each strip being in line with the point of application of the samples.

The strips were then treated to show up the steroid spots.

Colour reagent - Phosphomolybdic acid
10 G in 100 ml. ethanol.

This solution was sprayed evenly into each strip which was immediately suspended in an oven at 80°C and developed for 4 minutes. This resulted in the paper turning a lime green and the steroid spots standing out as purple areas. Comparison of the separation of steroids by paper chromatography is

measured by the rate of movement of the steroid in relation to the front. The starting line for the running of the chromatogram is known. The solvent front is marked on the paper at the end of the run. The steroid spots are stained with phosphomolybdic acid and the centre of the steroid spot marked.

Rf = rate of movement of steroid in relation to the solvent (mobile phase) front

$$= \frac{\text{starting line to mid-point of steroid spot in cms.}}{\text{starting line to front in cms.}}$$

Recovery of a steroid from a paper chromatogram is achieved by elution of the steroid from the segment of paper where it is separated. This area can be determined by cutting the individual steroid running strip in half, staining one half and eluting the corresponding area of the other half. A known standard of the steroid may be run simultaneously, stained, and the corresponding experimental strip steroid area taken for

elution. If the steroid is labelled with C^{14}, the paper can be scanned for radioactivity in an automatic radio chromatogram scanner (Tracerlab Precision Ratemeter) and the radioactive area of the strip taken for elution.

Elution: The segment of strip containing the required steroid is cut from the chromatogram strip. This segment is then cut into small strips (3 mm x 20 mm approx.) and transferred to a test tube. Absolute methanol is then added to the test tube to cover the paper strips at the bottom. The strips are left to elute for 4 hours.

The strips are then removed, squeezing out the methanol as much as possible with a glass rod. The steroid thus remains in the methanol in the test tube and is available for further analysis.

<u>Counter Current Distribution.</u>

A simple 8 tube counter current distribution was used to compare the C^{14} and H^3 radioactivity of an experimental sample, and the C^{14} radioactivity of a control sample of pregnanediol, each of which had been acetylated with 1-C^{14}-acetic anhydride.

The solvent system used was basically that described by Pearlman (1957b).

Mobile phase : Petroleum ether - 250 ml.

Stationary phase : 90 per cent methanol - 250 ml.

These volumes were measured into a 1 litre separatory funnel, shaken up and left to equilibrate and separate overnight.

Eight 25 ml. separatory funnels were set up, numbered from 1 to 8. Ten ml. of mobile phase were added to the first funnel. Eight 30 ml. test tubes were numbered 1 to 8. An acetylated sample was transferred to test tube 1 and taken to dryness. Ten ml. of stationary phase was added to each test tube and the tubes placed below their corresponding

separatory funnels. (Figure 25)., (page 193).

The contents of tube 1 were transferred to funnel 1 and shaken up. After the phases settled the lower (stationary) phase was run off into tube 1. The remaining upper mobile phase was transferred from funnel 1 to funnel 2. Ten ml. of fresh mobile phase were added to funnel 1. The two phases of tube and funnel 1 and tube and funnel 2 were mixed in the respective separatory funnels, the lower phase separated into the respective tubes, and the contents of funnel 2 transferred to funnel 3 and the contents of funnel 1 transferred to funnel 2. Ten ml. of fresh mobile phase were again added to funnel 1. The process was repeated till all the funnels contained mobile phase which had been passed along the line, the first aliquot of mobile phase having had eight transfers.

The separatory funnels were emptied into their corresponding test tubes each of which now contained 20 ml. of equal volumes

of mobile and stationary phase. The contents were taken to dryness preparatory to estimation of the radioactivity of their contents. (Appendix VI).

APPENDIX IV

A) SCINTILLATION SPECTROMETRY.

B) PACKARD TRI-CARB SCINTILLATION SPECTROMETER.

C) STATISTICAL ERROR OF COUNTING.

D) EXPERIMENTAL APPLICATION.

1) Sample Composition.

2) Machine Setting.

3) Standard Samples.

4) Efficiency of Machine Settings.

5) Efficiency of Counting Experimental Samples.

6) Results of the trials upon which the above efficiencies were based.

A. Scintillation Spectrometry.

Reynolds (1950), Reynolds, Harrison & Salvini (1950), and Kallman & Furst (1950 & 1951) independantly announced that dilute solutions of fluorescent substances in aromatic solvents are useful as radiation detectors. At first these liquid scintillators were used in place of solid crystals in detecting penetrating radiation; however the discovery was soon made that another real usefulness of liquid scintillators is in counting low level β emitting isotopes.

Since the maximum energy of most β particles is low the particles are absorbed readily in passing through matter. This absorption phenomenon hampers the counting of low level β emitters in the solid phase because the sample itself absorbs its own radiation. "Self absorption" is eliminated, however, when the sample is dissolved with the scintillator or fluorescent substance

in some suitable solvent. In addition "4 π geometry (100 per cent geometry) is obtained when the sample is placed in solution since the disintegrating atom finds itself completely surrounded by molecules of scintillator.

Aside from considerations of high efficiency and sensitivity achieved by counting the sample in solution, there are further benefits to be realised from liquid scintillation counting. The time and effort involved in preparing sample planchets for solid phase counting or in conversion of the solid matter to gas may be compared with the ease of placing the sample in solution. Another advantage of liquid scintillation counting is that relatively large samples may be counted easily and efficiently.

There are, of course, some disadvantages connected with liquid scintillation counting. In many cases the sample itself is insoluble in a desirable solvent and must be finely

ground or precipitated and suspended in the scintillator solution. This may introduce self absorption problems, particularly in tritium counting and nullify some of the advantages of liquid scintillation counting. Also the equipment involved is complicated and consequently expensive. In general, however, the advantages associated with liquid scintillation counting - efficiency, sensitivity and ease of sample preparation - greatly outweigh the disadvantages.

The idea of liquid scintillation counting is to dissolve the sample with a fluorescent material in a suitable solvent giving essentially 100% geometry and no self absorption. Then if it were not for three important facts the vial containing this solution could simply replace the sodium iodide crystal in the gamma spectrometer and β counting could be done with the same electronic equipment used for gamma counting. The facts which make the method impracticable

for β counting are -

1) The β energies involved are much lower than the γ energies usually encountered.

2) Liquid scintillators do not emit light as efficiently as solid crystals.

3) Photomultipliers produce a large and variable number of thermal noise pulses even at very low temperatures. Unfortunately the amplitude of the thermal pulses is essentially the same as the amplitude of the pulses due to β decay events so they cannot be eliminated by pulse height selection as they are with gamma counting. To eliminate these thermal pulses the use of cooling and a coincident arrangement of two photomultipliers is necessary.

Cooling in a freezer greatly reduces the rate of thermal pulses and by having two photomultipliers view the sample vial containing the solution of radioactive sample and liquid scintillator, legitimate light pulses are seen simultaneously by both.

An electronic coincidence circuit is provided to pass such pulses and to reject thermal noise pulses which occur at random in the two photomultipliers and rarely chance to coincide in time.

The sample used in liquid scintillation counting is best considered as four separate topics -

The solvent.

The Scintillator.

The radioactive material.

The Vial.

The Solvent.

Perhaps the primary requisite of the solvent is that it permits an efficient transfer of energy from the β particle to the scintillator and allows the light emitted from the scintillator to be transmitted with very little absorption. If the solvent tends to absorb the β particle energy, the process could be called "thermal" or "chemical" quenching since the β particle energy is

dissipated as heat. Should the solvent tend to absorb the light emitted by the scintillator this would be termed "colour" quenching.

Another important attribute of the solvent is that the radio-active matter being assayed should be soluble in it. While solubility is desirable in terms of efficiency and reproduceability there are instances where no suitable solvent can be found. In many of these cases the material in question can be ground very finely or precipitated and suspended in the solvent. While suspension counting invariably yields a lower efficiency due to self absorption and colour effects, fine grinding of the radio-active matter will yield reproduceable results with good efficiency. This method generally is applied only to C^{14} or isotopes having higher maximum energies. With tritium, the self absorption problem may be so great that the counting efficiency is a function of the particle size.

A third quality desirable in the solvent is that it should not freeze at counting temperatures. Freezing of the solvent will produce erratic results; however, in many cases the freezer temperature may be raised to allow counting with the desired solvent. In general the freezing point of the solvent is the factor which determines the temperature of the Tri-Carb Spectrometer freezer.

For liquid scintillation counting, the solvents in general use tend to be either aromatic hydrocarbons or aromatic ethers but other compounds are used where solubility is a factor - e.g. in tritium water counting, an ethanol toluene mixture is often used to hold the water in solution. Since the amount of water which can be counted in this way is low and the efficiency is decreased by ethanol, 1-4 dioxene is often used as a solvent. Here many problems appear. Dioxene freezes at +12°C and although the

water tends to depress the freezing temperature, the addition of another substance to depress the freezing point may be desirable. Naphthalene often is added to help restore the fluorescence of the dioxene-water mixture. Also purity of the dioxene is quite critical.

The Scintillator.

The Scintillator or fluor is used in liquid scintillation counting to convert the β particle energy to light quanta. There are several fluors from which to chose all of which are organic. The main difference between one fluor and another is solubility in the particular solvent being used and the "self quenching" exhibited. Self quenching is the process whereby a fluor tends to absorb the light emitted.

In general one finds that for a given solvent the counting efficiency increases with increasing scintillator concentration. Some fluors tend to reach a plateau prior to the solubility limit where the efficiency is, to

a good approximation, independent of concentration. Clearly this is a desirable region in which to operate since efficiency is not affected by inaccuracies in scintillator concentration. For other fluors the solubility limit may be reached before the plateau occurs. Occasionally one finds that the counting efficiency first rises and becomes level and then falls with increasing scintillator concentration. This decrease in efficiency is due to self quenching becoming evident at high concentrations.

The addition of a "secondary fluor" is advantageous. The "secondary fluor" acts to shift the wavelength of the light emitted by the "primary fluor" to a region where the photomultipliers are more sensitive. One finds that minute quantities of secondary fluor may give quite impressive increases in counting efficiency particularly in tritium counting where the energies involved are low;

however with carbon counting some increase in efficiency is also noted.

The Radio-active Matter.

Dissolved or suspended in the solvent is the radio-active matter being assayed. There are many isotopes being counted with Tri-Carb Liquid Scintillation Spectrometers.

	Half Life	Maximum β Energy.
H_3	12.3 years	0.018 M.V.
C_{14}	5700 years	0.15 M.V.

The Vial.

A part of the sample which frequently is not given sufficient consideration is the sample vial. Normally the vial is made of glass. All glasses contain K^{40} in varying amounts and this has been found to constitute a significant part of the background. Since for a given concentration of K^{40} the background will be lower where the vial is smaller, the investigator should try to use as small a vial as practicable. For many

purposes a 22 ml. vial will suffice. The difference in background due to K^{40} from the glass of one manufacturer to that of another is quite striking.

Some work has been done on sample vials made of materials other than glass. Some of these are quartz, vicor and polyethylene. These materials tend to produce a lower background than does glass but their cost is generally higher. This is a matter which must be resolved by the individual investigator since some experiments require very low background while others may have much higher tolerable background. Another source of background is activation of the glass by ultraviolet light. After exposure to strong sunlight or fluorescent light a high background may be observed. This phosphorescence decays with a half life which may be in the order of a few hours. In some cases the effect has been seen to persist for several days.

Another important point in chosing sample vials is the optics which the vial provides. The sides should not be extremely thick and should possess uniform optical transmission. The optical absorption properties of the glass should be such that the counting efficiency is not impaired. In general the investigator will find the best results are obtained when he reproduces his optics as nearly as possible from one sample to another and in line with this marking vials on the bottom or sides is discouraged. Indeed the vials themselves should be handled as little as possible. They can be carried about in boxes or lifted by the screw cap. The glass vial should be polished with tissue when the sample is ready for cooling in the freezer prior to counting.

The use of polyethylene or cork-tinfoil screw caps also is recommended although some users prefer polyethylene snap caps. The

contamination which can result from an unlined or paper lined screw cap has been known to yield unreproduceable results - particularly in tritium counting.

For a standard, a sealed vial is to be preferred since oxygen quenching is reduced and deterioration of the scintillator is virtually eliminated. Sealed standards usually are prepared by bubbling nitrogen or argon gas through the solvent immediately prior to sealing.

B. Packard Tri-Carb Scintillation Spectrometer.

The block diagram of the instrument is shown in Figure 27. When there is a decay event, the emitted β particle excites the scintillator and light is produced. These light quanta are reflected about the polished aluminium sample chamber and eventually some of the quanta reach the "analyser" (A) photomultiplier and some reach the "monitor" (B) photomultiplier. This results in a pulse at the anode of each photomultiplier,

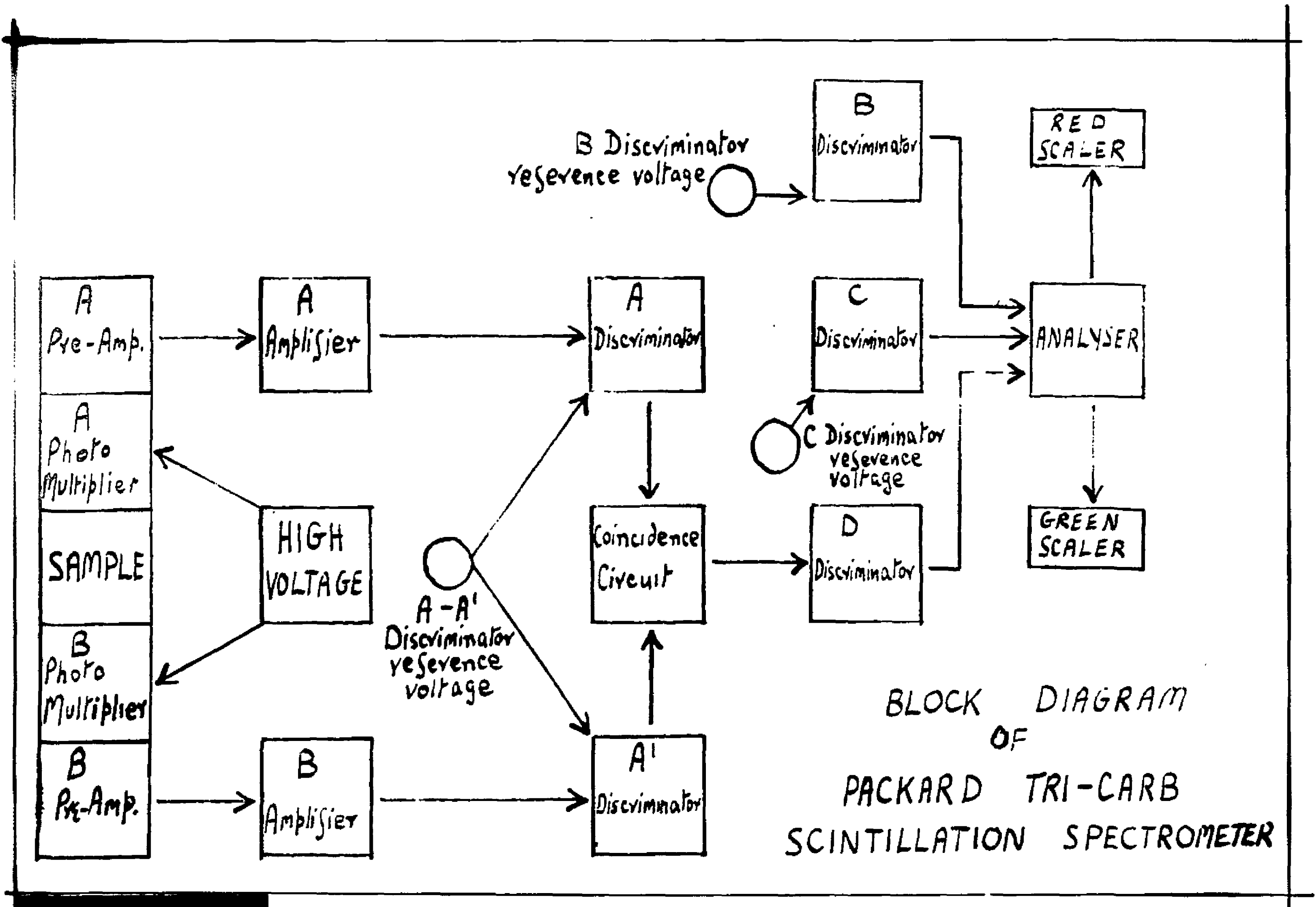


FIG. 27.

a pulse at the output of each pre-amplifier and a pulse at the output of each amplifier. The two amplifier output pulses will, in general, not be the same size; however, if both are larger than the A-A' reference voltage, there will be pulses at the outputs of the A and A' discriminators. These pulses enter the coincidence circuit which acts to give a large output pulse when there are two input pulses within the resolution time of the coincidence circuit. If there is only one input pulse the coincidence circuit produces a very small output pulse. The large output pulses of the coincidence circuit triggers the D discriminator and the output of the D discriminator is the signal which may be counted.

Whether or not the output of the D discriminator is counted depends upon whether or not the A amplifier output pulse was larger than the B and C discriminator reference voltages and upon the setting of

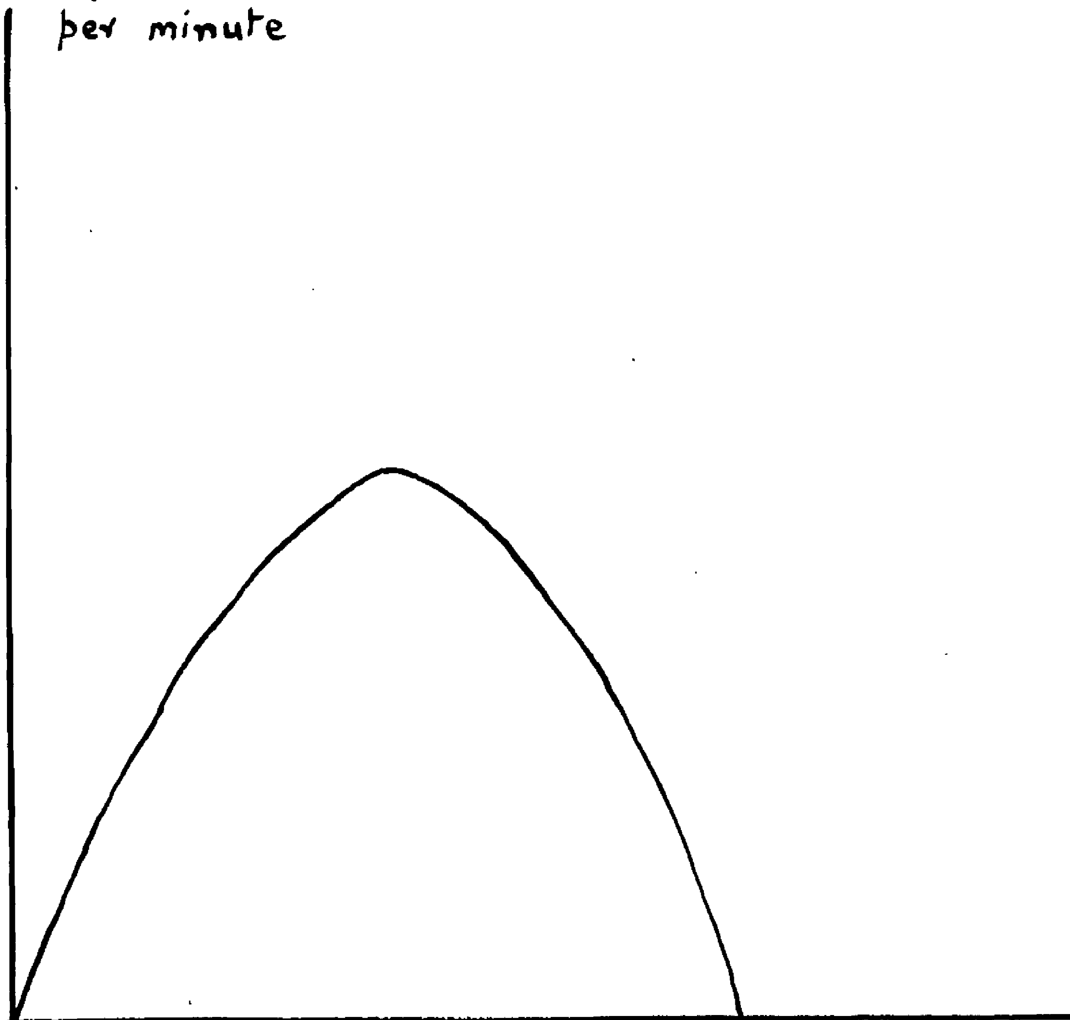
the analyser mode switch. This switch, which controls the manner in which the analyser functions, has three positions for normal counting operation. Depending upon the switch position, the counting information is displayed on the "red" and "green" scalers as follows -

Switch Position.	Red Scaler.	Green Scaler.
2	AA' - B	B-C
3	AA' - B	AA'-C
4	AA' - C	B-C

For example when the analyzer mode switch is in position 2 and a pulse at the output of the A amplifier is larger than the AA' reference and smaller than the B reference, the output (if any) of the D discriminator is counted on the red scaler. If the pulse at the output of the A amplifier is larger than the B reference and smaller than the C reference, the pulse, if any, at the output of the D discriminator will be counted on the green scaler.

An important point to note is that the D discriminator output pulse results from a coincidence of two pulses down the two channels but all analysis is done on the output of the A amplifier (i.e. on the output of one photomultiplier - the analyser). The other photomultiplier (the monitor) acts only to establish coincidence events.

Figure 28 shows the spectrum of pulses due to β decay events at the A and A' discriminator inputs of Figure 27. Since the β particle energy and pulse height due to the β decay event are proportional, the abscissa could be plotted in MeV or volts. For the present discussion it is convenient to consider the abscissa scaled in volts of pulse height and the ordinate scaled in particles or pulses per minute. Figure 28 is the pulse spectrum resulting solely from β decays. There are other pulses present



IDEALISED PULSE SPECTRUM AT THE AA' DISCRIMINATOR INPUT DUE TO β DECAY

FIG. 28.

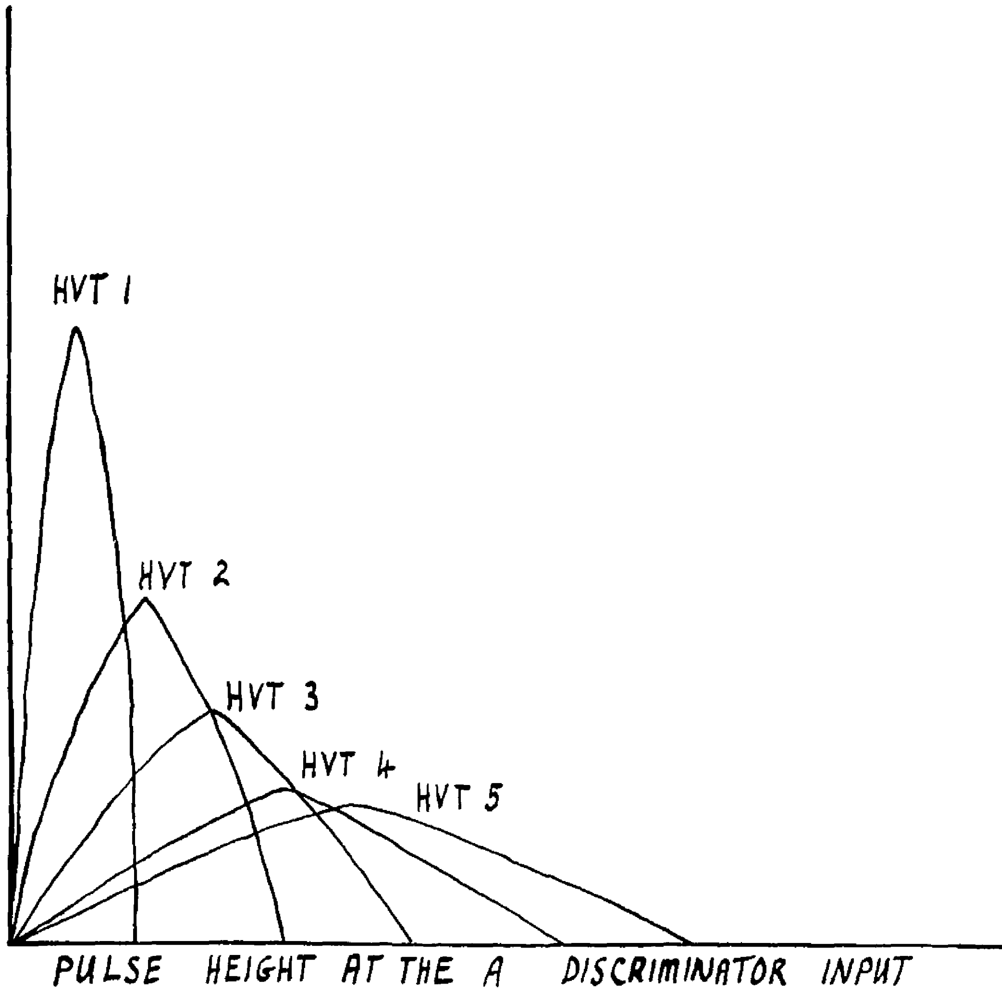
due to thermal noise pulses and 'shot noise' in the pre-amplifier but these will be considered later.

Figure 28 deserves some further comment before proceeding. The β spectrum is "continuous", as opposed to a typical gamma spectrum which has discrete "peaks". The β spectrum has pulses of near zero energy and pulses of near "cut off" or maximum energy. Between these extremes the spectrum rises smoothly then falls. This sort of spectrum is a characteristic of β emitters.

The constant of proportionality between the height of a pulse and the energy of the β particle which created the pulse is a function of the gain or amplification of the linear channels. This gain is a strong function of the high voltage applied to the photomultipliers. Therefore one parameter which is omitted from Figure 28 is the setting of the Tri-Carb Spectrometer high

voltage supply.

Figure 29 shows a family of curves plotted with high voltage tap switch setting as a parameter. This tap switch is the primary gain control of the Tri-Carb Spectrometer. The voltage applied to the photomultiplier is approximately 680 volts on tap 1 and rises approximately 85 volts per tap. In Figure 29 the important points to observe are that the shape of the spectrum changes as the high voltage is increased in such a way that the cut off point extends to higher voltages (pulse heights), and the peak of the curve assumes lower valves. The explanation is as follows - Since an increase in high voltage results in an increase in gain, those pulses which were near the cut off voltage before the high voltage was raised must be larger afterwards. Therefore the cut off point will occur at a larger voltage. Since the cut off has moved to higher values, the peak must be reduced in magnitude,



IDEALISED PULSE SPECTRA AT THE A DISCRIMINATOR INPUT DUE TO β DECAY FOR SEVERAL HIGH VOLTAGE TAPS

FIG. 29.

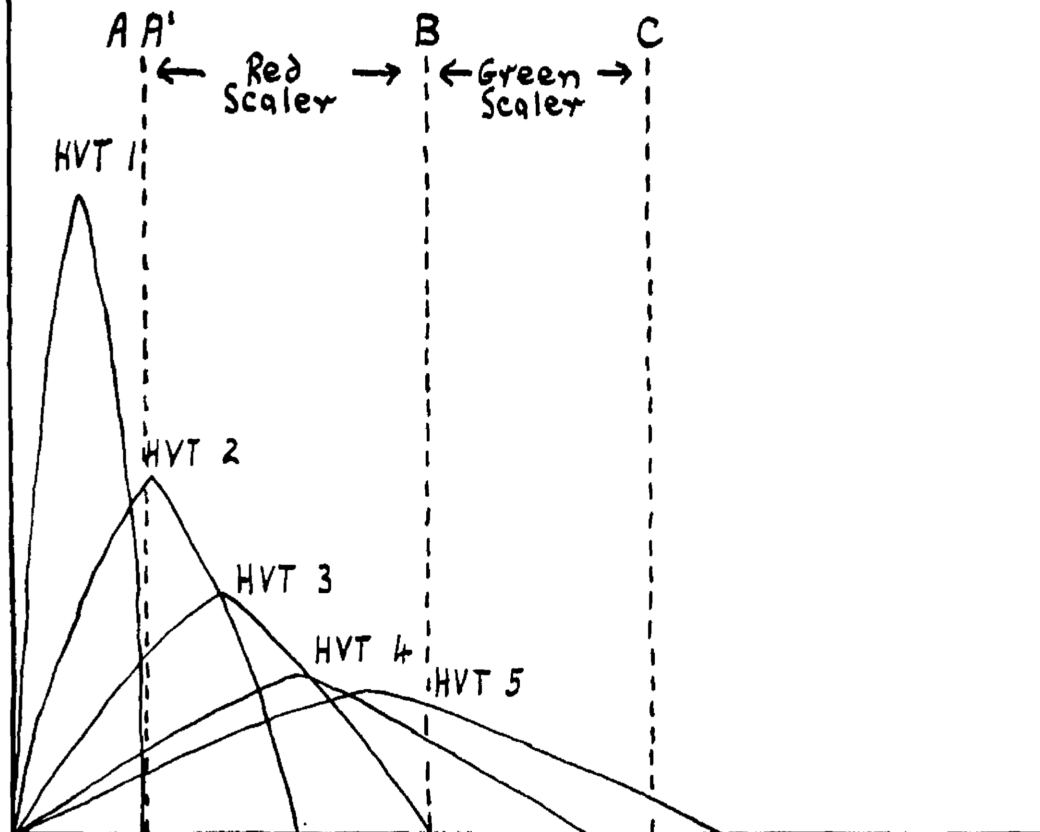
because the area under the curve must be constant (i.e. there is a given amount of activity).

Figure 30 shows Figure 29 with the discriminator reference levels added. Typical settings for the discriminator levels might be A-A' - 10 volts.

B - 50 volts.

C - 100 volts.

In Figure 30 the count that is recorded on a scaler is equal to the area of a spectrum included between the limits of discrimination. If the high voltage is set at tap 1, there is no count on either scaler because the spectrum lies entirely below the A-A' reference. On high voltage tap 2, the red scaler counts perhaps 50% of the pulses and the green scaler counts nothing on high voltage tap 3, the red scaler counts perhaps 70% of the pulses and the green scaler still counts nothing. When the high voltage is changed to tap 4, the

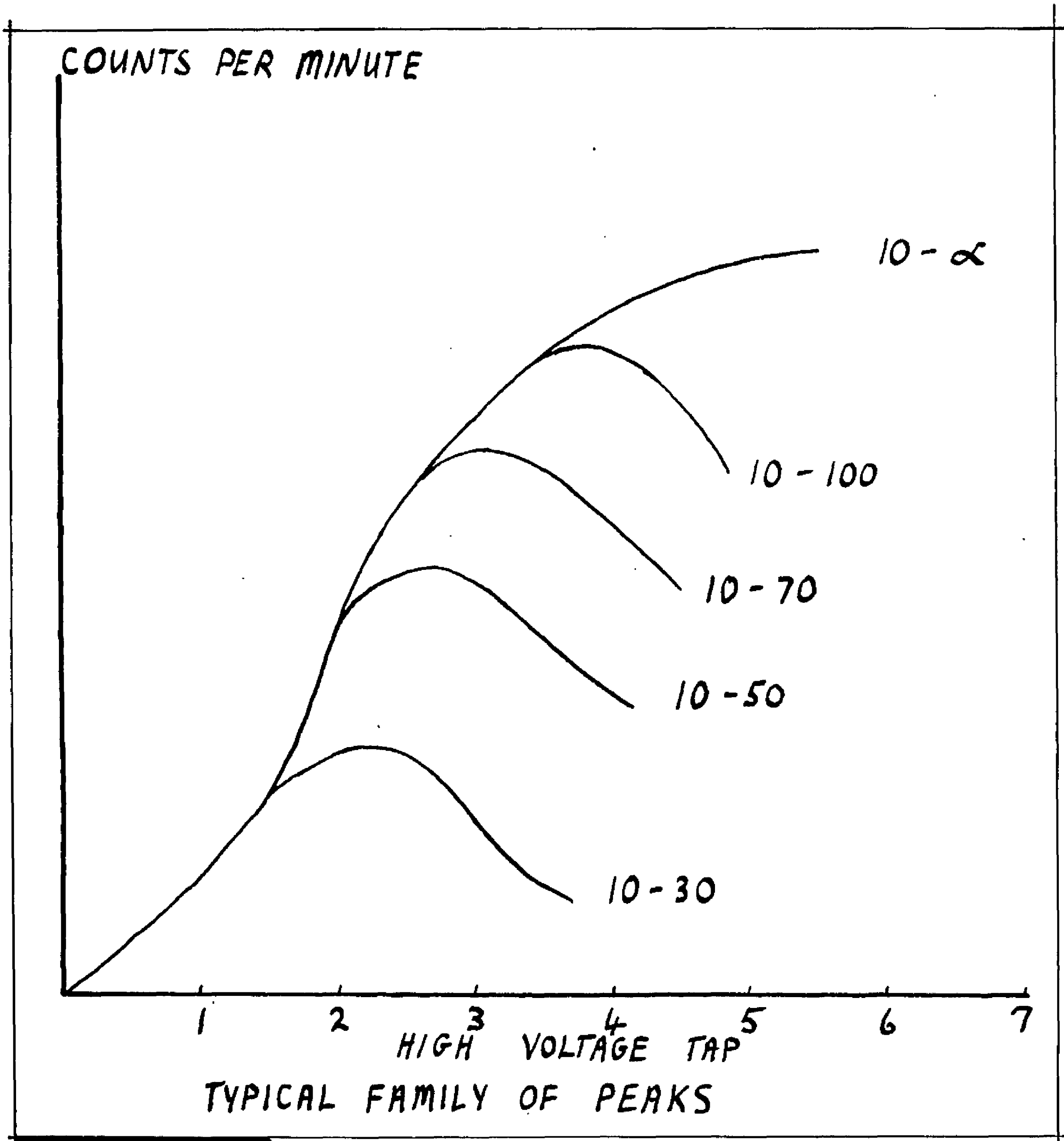


IDEALISED PULSE SPECTRA AT THE A DISCRIMINATOR INPUT DUE TO β DECAY FOR SEVERAL HIGH VOLTAGE TAPS

FIG. 30.

green scaler begins to count and the red scaler remains roughly constant. On tap 5 the count on the red scaler has decreased while the count on the green scaler still is increasing. If the high voltage tap switch setting is further increased, the
switch setting is further increased, the
count on the red scaler continues to decrease and the count on the green scaler reaches a peak and then begins to drop.

The important point to notice is that as the spectrum is shifted through the "window" the count rises, reaches a peak, and falls. The reason the count falls is that there is an upper limit to the window. If there had been no upper limit, the count would have risen and tended to become flat. If the window had been narrower (10-30 volts, eg.) the peak would have occurred at a lower high voltage tap and the counting efficiency at the peak would have been less. Figure 31 shows a typical family of "peaks" taken with a Tri-Carb liquid scintillation



TYPICAL FAMILY OF PEAKS

FIG. 31.

spectrometer.

In normal counting operations with the Tri-Carb Liquid Scintillation Spectrometer, a window is set on the discriminator reference controls, and the high voltage tap switch is adjusted so that the counting rate in this window is a maximum. The tap position required to achieve this condition will vary with the amount of quenching present in the sample. Where the isotope has a low maximum β energy, or is badly quenched, a higher voltage will be required to obtain a peak for a given window than where the isotope has high maximum β energy or is relatively unquenched. There are two reasons for counting with a high voltage tap setting that gives a maximum number of counts in a window. The first is that this gives the maximum counting efficiency possible for the particular window setting. The second is that operation in this condition is more stable. The solid curve in

Figure 32 shows a β spectrum where the high voltage has been adjusted so that there is maximum counting rate in the window. The dashed curve shows what happens when a decrease in gain occurs. Such a shift might arise where the line voltage changes, tubes age, etc. Note that the two curves cross roughly in the centre of the window and that, while counts are added to the left of the cross-over, they are lost on the right. The same argument holds where the gain increases by a slight amount. The consequence of this is that small drifts tend to be rejected and for this reason operation where the high voltage setting is adjusted so that there is a maximum counting rate in the window is termed "balance point" operation.

The selection of the window is another point deserving of attention. It is true that as one widens the window the background increases. Strictly speaking, the

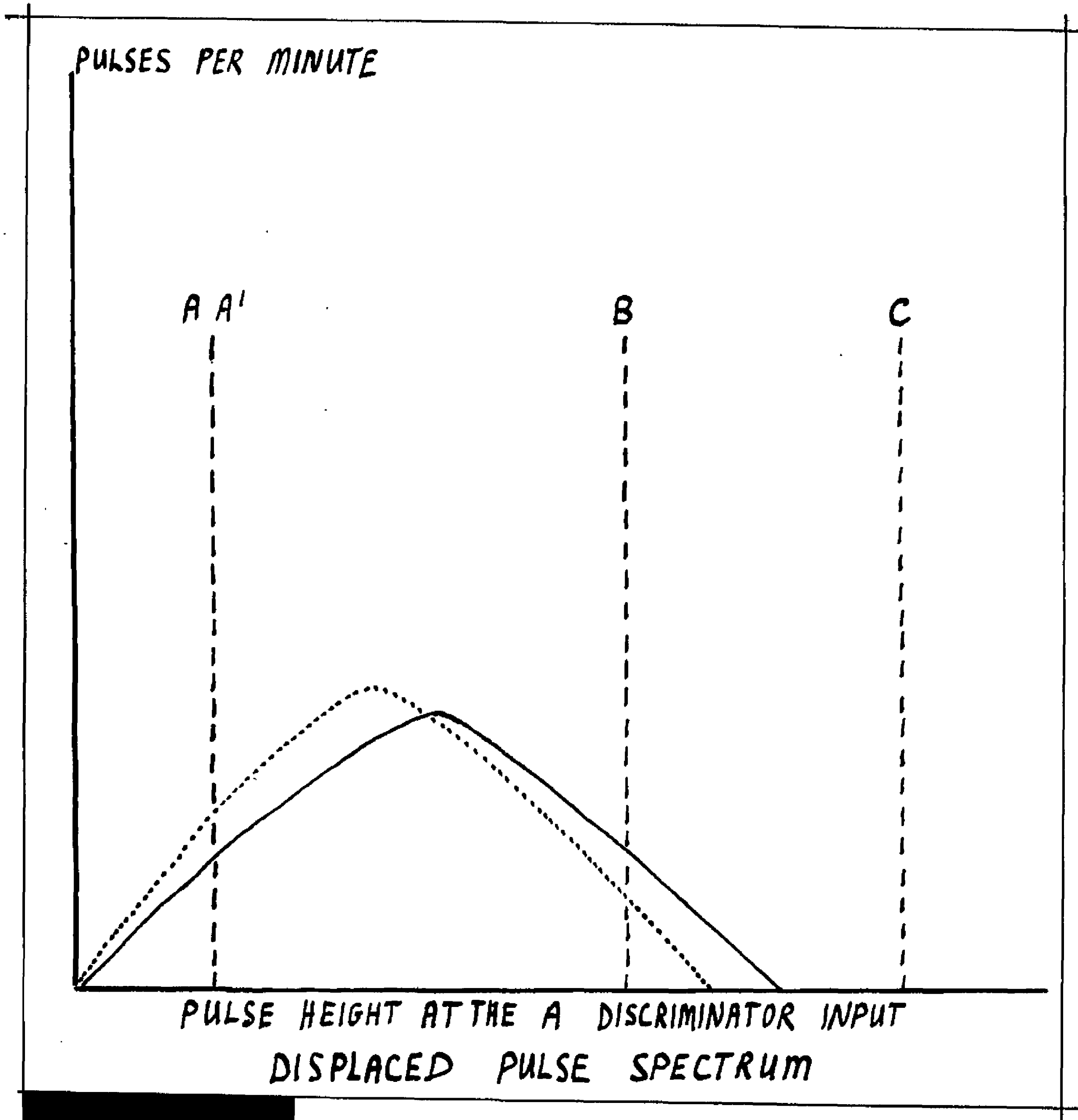


FIG. 32.

statistical uncertainty introduced by the background is least where the window is adjusted so that the sample rate squared divided by the background is a maximum. This condition usually is achieved on a Tri-Carb Liquid Scintillation Spectrometer with a window of approximately 10 volts to 50 volts. These limits vary somewhat from one instrument and environment to another. Where an experiment such as carbon dating requires that the least possible statistical error be introduced by the background, the operator normally would go through a process of determining the optimum window. For routine counting, the time and effort involved would not be justified.

Figure 32 shows two spectra. If the solid curve were considered to be a pure unquenched spectrum then the dashed curve might be the result of quenching. If the machine were being operated so that the

red scaler counted in the AA' - B window and the green scaler counted in the B-C window, then there would normally be a more or less constant ratio of count rates in the two windows for a pure unquenched sample. When quenching occurs, as indicated by the dashed curve the ratio will change radically. This provides a simple monitor for quenching. The ratio normally will vary somewhat due to the statistical nature of the radiation and the fact that the green scaler is not counting in balance point operation. However the ratio is a quick and convenient method of keeping a check on quenching without making repeat counts.

One basic reason that the Tri-Carb Liquid Scintillation Spectrometer is such a valuable instrument is that it can perform pulse height analysis. Pulse height analysis allows the user to assay samples labelled with two isotopes, discriminate against the majority of the background while

still counting a particular isotope with good efficiency, achieve a very stable operating point and monitor quenching continuously.

The fundamental assumption underlying the pulse height analysis system in the Tri-Carb Liquid Scintillation Spectrometer is that the pulses at the amplifier outputs are proportional in height (voltage) to the energy of the beta particles which created them. This is a good assumption; since the amplifiers and pre-amplifiers are linear devices, the light energy emitted by the fluor is proportional on an average basis to the β particle energy and the photomultipliers produce electrical pulses the voltage of which tends to be proportional to the light energy incident on the photocathode. Thus there are four devices cascaded; each linear, to produce a linear channel.

Where the ratio of the maximum

energies of the two isotopes is four or five to one or greater, the two isotopes may be counted at one operation by adjusting the high voltage tap switch so that one isotope is counted efficiently in one window and inefficiently in the other and the other isotope vice versa.

Figure 33 shows the idealised pulse spectra of two isotopes. With the analyser mode switch set to position 2, where the efficiency for each isotope in each window is known, the activity of each isotope can be calculated by simultaneous equations.

(a) <u>Simultaneous Equations.</u> <u>Method:</u>

$Rr = A1 \times E1r + A2 \times E2r$ where

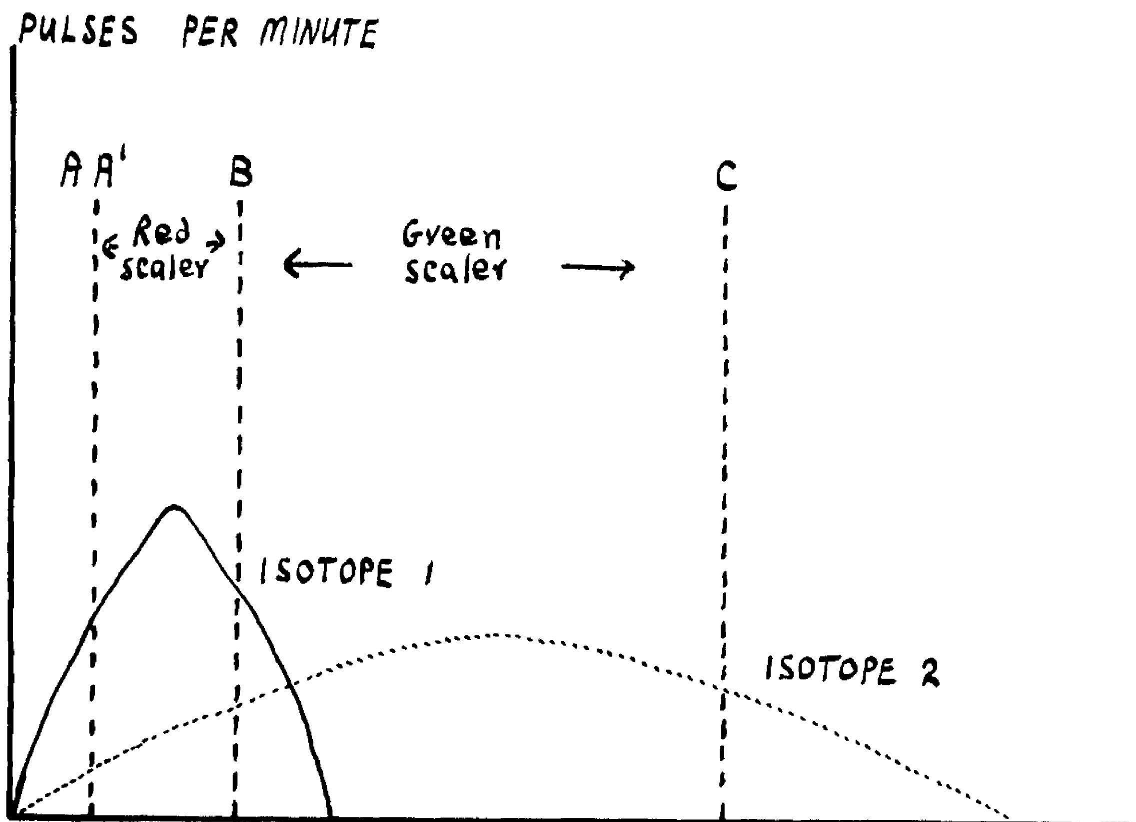
Rr = count rate on the red scaler.

A1 = the activity of isotope 1.

E1r = the counting efficiency of isotope 1 in the AA' - B range (i.e. in the red scaler).

A2 = the activity of isotope 2.

E2r = the counting efficiency of isotope 2 in the AA' - B range.



IDEALISED PULSE SPECTRA AT THE A DISCRIMINATOR INPUT DUE TO β DECAY FOR TWO ISOTOPES

FIG. 33.

$Rg = A1 \times E1g + A2 \times E2g$ where

Rg = count rate on the green scaler.

$E1g$ = counting efficiency of isotope 1 in B-C range (i.e. on the green scaler).

$E2g$ = counting efficiency of isotope 2 in the B-C range.

Therefore A1

$$\text{Therefore } A1 = \frac{Rr - Rg\left(\frac{E2r}{E2g}\right)}{E1r - E1g\left(\frac{E2r}{E2g}\right)}$$

$$A2 = \frac{Rr - Rg\left(\frac{E1r}{E1g}\right)}{E2r - E2g\left(\frac{E1r}{E1g}\right)}$$

These equations take the form of ratios of differences. Where the numbers involved are large and the differences are small, errors may develop which are quite sizeable and for this reason it is not feasible to count double-labelled samples if the energies of the isotopes involved are relatively close to one another. In practical applications, most operators have found that significant results cannot be obtained where the ratio of the energies of

the two isotopes is less than four.

(b) Discriminator Ratio Method:

In the discriminator-ratio method, the photo-multiplier voltage and discriminator control settings are similar to those used in the simultaneous equation method. This method uses the ratio of window AA' - B counts per minute to window B-C counts per minute. Equations are then derived that utilise the discriminator ratio for each isotope instead of the counting efficiency used in the previous method.

Where N1 = net c.p.m. window AA' - B

N2 = net c.p.m. window B-C

H1 = net c.p.m. of H^3 in window AA' - B

H2 = net c.p.m. of H^3 in window B-C

C1 = net c.p.m. of C^{14} in window AA' - B

C2 = net c.p.m. of C^{14} in window B-C

$$a = \frac{H2}{H1}$$

$$b = \frac{C2}{C1}$$

Since $N1 = H1 + C1$

and $N2 = H2 + C2$

then $H1 = \frac{bN1 - N2}{b-a}$

and $C2 = \frac{b(N2 - aN1)}{b-a}$

(c) Screening Method:

This method involves the use of the high voltage tap switch to compress or expand the pulse spectra. Figure 34 shows the spectra of the double labelled sample at a low high voltage tap and shows the same spectra after the high voltage has been increased. By the use of standards, the counting efficiencies can be determined for the two conditions.

Where C = c.p.m. C^{14}

H = c.p.m. H_3

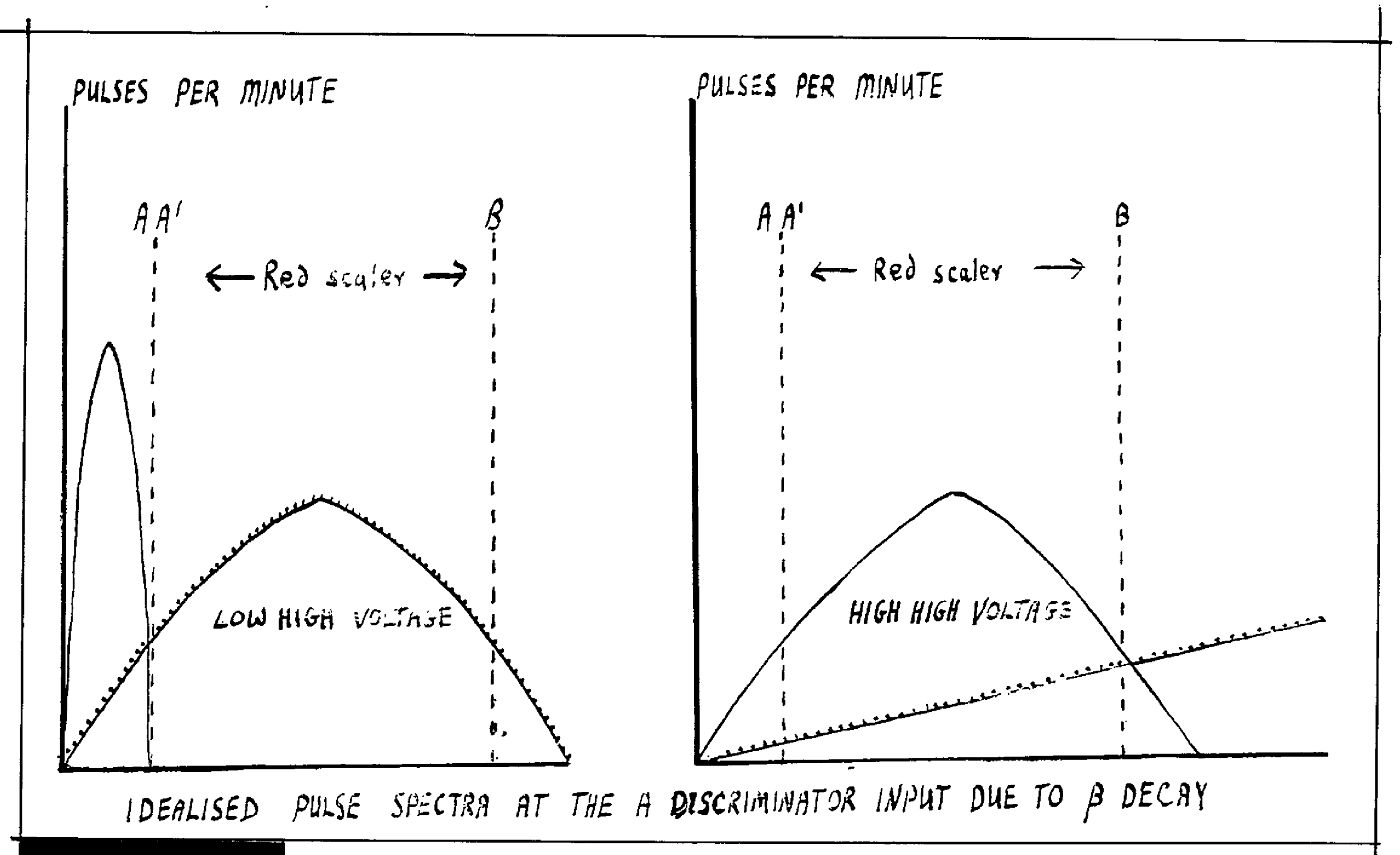
$N1$ = net c.p.m. at low voltage.

$N2$ = net c.p.m. at higher voltage.

$c1$ = C^{14} efficiency at low voltage.

$c2$ = C^{14} efficiency at higher voltage.

$h1$ = H_3 efficiency at low voltage.



IDEALISED PULSE SPECTRA AT THE A DISCRIMINATOR INPUT DUE TO β DECAY

FIG. 34.

$h2 = H_3$ efficiency at higher voltage.

Then $N1 = Hh1 + Cc1$

and $N2 = Hh2 + Cc2$

For example, in the application of the screening method in the acetylation and counter current distribution experiments

$c1 = 100\%$ (1) (Arbitrarily)

$c2 = 30\%$ (1/3)

$h1 = 1\%$ (1/125)

$h2 = 100\%$ (1) (Arbitrarily)

$$\text{Thus } C = \frac{N1 - Hh1}{c1} = N1 - Hh1 = N1 - \frac{H}{125}$$

$$\text{and } H = \frac{N2 - Cc2}{h2} = N2 - Cc2 = N2 - \frac{C}{3}$$

$$\text{Therefore } C = \frac{475}{474} N1 - \frac{N2}{158}$$

$$\text{and } H = \frac{475}{474} N2 - \frac{125}{474} N1$$

The same remarks with regard to error apply here since, if the two isotopes do not give widely separated pulse spectra, the analyses of data will require the subtraction of large numbers to obtain small differences.

This method was selected for use in the calculation of this programme because it was easily applied and was within the required degree of accuracy when simplified to -

$$C = N1 - \frac{N2}{150}$$

$$H = N2 - \frac{N1}{3}$$

(see Okita, Kabara, Richardson & LeRoy, 1957).

In liquid scintillation counting, the phenomenon of quenching is of great interest. Quenching as previously mentioned is a process occurring in the sample which results in attenuated pulse heights. Substances which materially affect pulse heights (and counting efficiency) are called "quenchers". Other materials which tend to decrease pulse heights (and counting efficiency) more or less in inverse proportion to their concentration are termed "diluents".

Figure 35 shows the effect of a quencher, pyridine, on a sample. The initial total volume of the sample is 16 ml. and the total activity is 115200 cpm. The important factors to note are that as the volume of quencher is increased, the overall integral efficiency is decreased and the peaks shift to the right with decreased peak counting efficiency. These effects are observed for C^{14} and isotopes more energetic than C^{14}. With tritium the principal effect is a decrease in counting efficiency; the shifting phenomenon is not as prominent because the photomultipliers are working at essentially single photo electron levels.

In normal counting operations, quenching is encountered in varying degrees in all samples. The consequence of this is that often some doubt may exist with regard to the counting efficiency for a particular sample. The usual procedure is

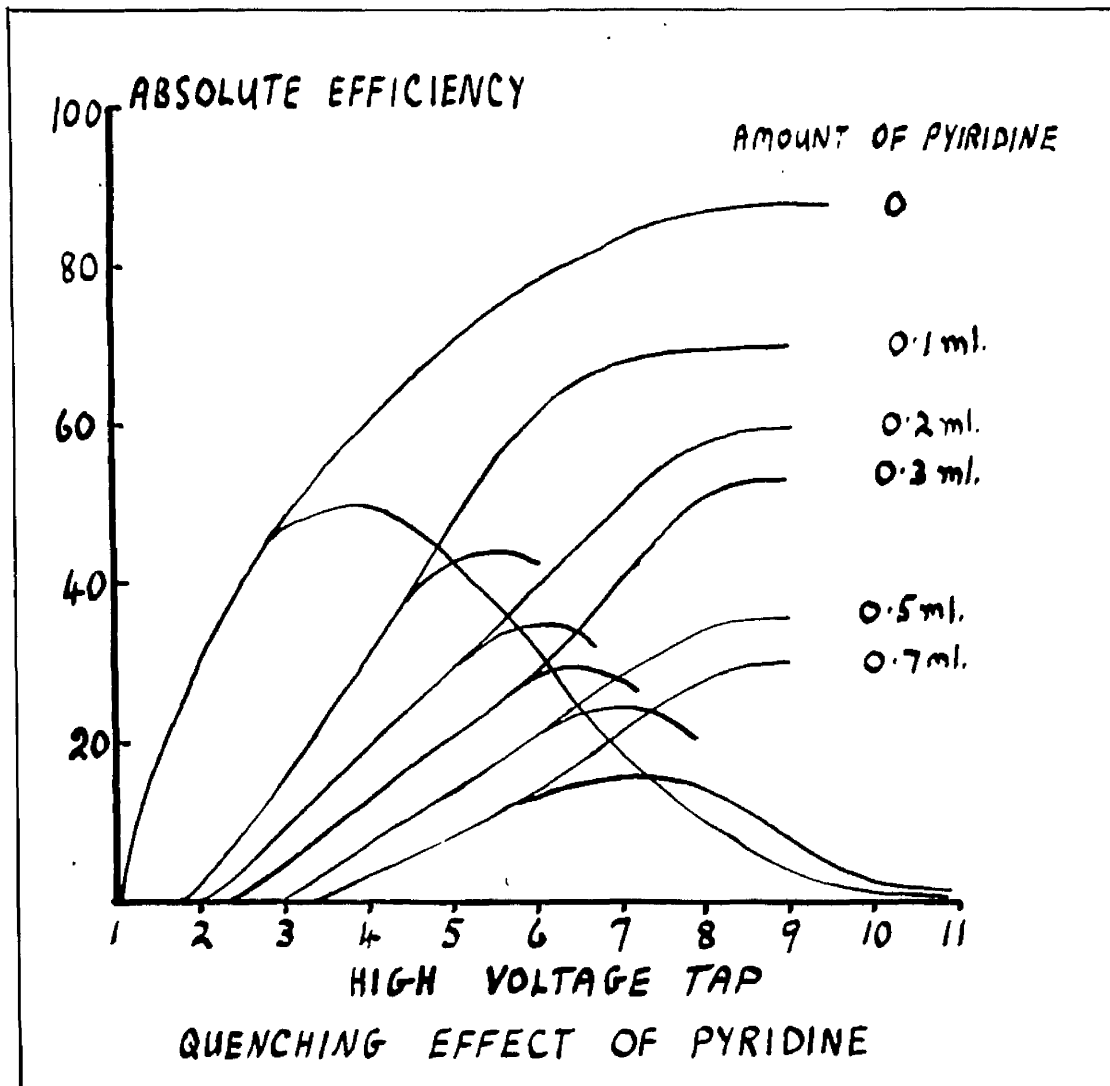


FIG. 35.

to employ an internal standard to determine the counting efficiency. By this method a known amount of a standard of the same isotope is added to the sample after it has been counted. The sample is then recounted and the increment in count, together with the amount of radioactivity which was added, may be used to compute the counting efficiency for the sample. This method yields good results where the sample is quenched to a moderate degree. When the sample is severely quenched, the method of internal standards may tend to give erroneous results and it may be necessary to dilute the sample to reduce the degree of quenching.

Since the degree to which a sample is quenched is a function of the ratio of the volume of quencher to the volume of solvent, a qualitative measure of quenching may be obtained by adding a volume of solvent and recounting. Where a sample is moderately quenched there will be only a slight increase

in counting efficiency; however if the sample is markedly quenched there will be a large increase.

The background which is encountered with the Tri-Carb Spectrometer arises from the following -

1) K^{40} in phototubes and vial
2) Cosmic and local and radiation
3) Accidentals.

K^{40} in the glass of the phototubes and the vial (where glass vials are used) is present in an amount in the order of 0.2%. When K^{40} decays it emits a rather strong β particle (1.33 Me V) which can create a Cerenkov radiation. The Cerenkov radiation can be observed by both photo-multipliers simultaneously and can result in a count being registered. Attempts to avoid this source of background usually have centred about the use of quartz vials and quartz faced photo-multipliers. The objection to the use of quartz is that the vials cost approximately 50 times as much as low K^{40}

glass vials and the photomultipliers cost approximately 10 times as much as glass faced photomultipliers. For most investigators these costs are prohibitive. The usual practice, then, is simply to use low K^{40} (or polyethylene) vials. For routine tracer work, this is an adequate precaution.

Cosmic and local and radiation contribute a portion to the background which varies considerably with location. In general the pulses due to cosmic radiation are so large that they are not registered in the normal counting window. An exception to this is encountered where very energetic isotopes are being counted and the amplifier attenuator is decreased to allow a peak in the normal window. The sources of local radiation are so varied that it is difficult to make a general statement in this regard. In practice, however, it is advisable to locate the instrument as far away as possible from radiation sources.

The accidentals (counts that result from coincident thermal noise) contribute a very small fraction of the background. This is true principally because the photomultipliers are selected and cooled, and because the coincidence resolution time is so short. The criterion applied is that the accidental rate must be less than 2 c.p.m. in a 10-50 volt window at the peak for an unquenched H_3 standard. In carbon counting, the accidental rate is well below 1 c.p.m. and is negligible for more energetic isotopes.

The only background that is meaningful in liquid scintillation counting is that encountered with the particular solvent system, quenching conditions etc. that apply to the sample. Just as quenching will decrease the counting efficiency for a sample, it will decrease the background counting efficiency. It is necessary therefore to prepare a background sample identical to the sample under assay with the exception of

the radioactivity to establish accurately the background.

For purposes of verifying the proper operating condition of the instrument it is necessary to have a background standard. The background standard should consist of a volume of scintillator solution in a low K^{40} ampoule which is preflushed and sealed. It is desirable to count the background prior to, and at the completion of the day's counting activity.

C. Statistical Error of Counting.

Radioactive decay being a random process, the closeness with which the rate observed during a finite counting time approaches the "true" rate increases with the total number of disintegrations observed. Since every determination involves a background correction, calculation of the uncertainty in the corrected count rate must take into consideration the uncertainty in this background rate.

The standard deviation, S.D., of any measurement in which N random events are recorded is simply $\pm\sqrt{N}$. If a total of 400 counts is recorded, for example, S.D. = ± 20 counts, and the chances are about 2 in 3 that the true count lies between 380 and 420, and 19 in 20 that it lies between 360 and 440. Standard deviation expressed in counts per minute is $\pm\frac{\sqrt{N}}{t}$ where t is the time in which N counts are recorded. Whatever the total time over which N counts are recorded, the percentage S.D. both for total counts and count rate can be simply calculated from the same expression $\pm\frac{\sqrt{N}}{N} \times 100 = \frac{100}{\sqrt{N}}$

These formulae apply equally to sample counts and background counts and the S.D. for the final corrected count must take account of the error in each.

Standard deviation for corrected count rate is given by

$$S.D._{obs-bg} = \pm \sqrt{S.D.^2_{obs} + SD^2_{bg}} = \pm \sqrt{\frac{N_{obs}}{t^2_{obs}} + \frac{N_{bg}}{t^2_{bg}}} \quad \text{---(1)}$$

$$S.D._{obs-bg} = \pm \sqrt{\frac{(cpm)_{obs}}{t_{obs}} + \frac{(cpm)_{bg}}{t_{bg}}} \quad \text{----equation (2)}$$

Where -

S.D. obs = Standard deviation for the gross sample count rate (background plus sample).

S.D. bg = Standard deviation for the background rate.

S.D. obs-bg = Standard deviation for the corrected or net sample count rate.

N obs = Total number of gross sample counts observed in time t obs. (background plus sample).

N bg = Total number of background counts observed in time t bg.

(cpm) obs = Gross sample count rate (background plus sample).

(cpm) bg = Background count rate.

(cpm) obs-bg: corrected or net sample rate.

For samples with activity many times background, the standard deviation can, with very little error, be calculated from the net sample count rate alone. For example, if (cpm) bg = 30, t bg = 30 minutes, (cpm) obs = 200, and t obs = 5 minutes, S.D. obs-bg calculated by equation (2) is ± 6.5 cpm; calculated from the net

sample rate alone it is ± 5.8 cpm. In other words, when the background rate is much lower than the sample rate, the error in the background, if the background has been counted for a reasonable length of time, will contribute only a negligible portion of the error in the overall determination. For this reason, when all samples to be assayed are more than 5 times background it is generally not necessary to count the background for more than 5 or 10 minutes. On the other hand if (cpm) bg = 30, t bg = 2 minutes, (cpm) obs = 40 and t obs = 10 minutes, then the true S.D. obs-bg would be ± 4.4 cpm and that calculated from the net sample rate alone would be ± 1 cpm.

It is common practice in many laboratories to count sample and background long enough, when practicable, to bring the standard deviation for the corrected count down to ± 5% which suffices for most biological studies. Most experimental

samples in this research work were counted to obtain a standard deviation within ± 5 per cent.

D. Experimental Application.

1) Composition of Sample.

The sample used in liquid scintillation counting was -

a) Solvent - Toluene and ethanol

b) Fluor - Primary : 2.5 - diphenyloxazole (P.P.O)

Secondary : 1.4-bis-2 (5 phenyloxazolyl)-benzene (P.O.P.O.P.)

Scintillation Fluid : P.P.O. : 4.0 G.

P.O.P.O.P.: 0.1 G.

Toluene (containing 2 per cent ethanol) to one litre.

10 ml. Scintillation fluid was used for each sample. When urine was to be assayed, the Scintillation fluid would not hold it in solution. Ethanol had to be added to keep the water content of the urine in solution.

For 0.3, 0.4 or 0.6 ml. urine add

6 ml. absolute ethanol to

10 ml. Scintillation fluid.

c) Vial - 22 ml. glass vials with silver foil lined plastic screw cap (glass of low K^{40} content; supplied by Packard). Vials were used once and then destroyed.

d) Radioactive material.

$7H^3$ progesterone

H^3 pregnanediol - standard and experimental

Tritiated urine

Pregnanediol C^{14} diacetate - experimental

H^3 Pregnanediol C^{14} diacetate - experimental

C^{14} Toluene - standard (two strengths of approximately 200 c.p.m. per 0.01 ml. and 20 c.p.m. per 0.01 ml.

2) <u>Packard Tri-Carb Scintillation Spectrometer Setting.</u>

a) Analyser Mode Switch.

Position 2

i.e. Red Scaler Counting AA' - B

Green Scaler Counting B - C

All readings recorded were taken from the Red Scaler.

b) Discriminator Reference Levels.

AA' - 10 volts

B - 50 volts

C - 100 volts

c) High Voltage Tap (HVT)

Tap 3 for C^{14} counting

Tap 7 for H^3 counting

d) Freezer Temperature : minus 3 to 4 degrees Centrigrade. All samples were cooled in the freezer overnight before statistical counting.

e) Preset Timing Switch.

The first five experiments were performed before the Scintillator was converted to the Automatic Model. For these, the timing switch was set to the appropriate time for statistical counting for each sample. When the automatic sample changer was connected the switch was set for 30 minutes.

f) Preset Count Switch.

This was usually set at 1000,000 (10^6). If the samples contained enough radioactivity to obtain statistical results in short counting

periods, the switch was set at 10,000 (10^4).

3) Standard samples.

The following standard samples were included in each series of experimental samples counted.

a) Beckman Tri-Carb Spectrometer Standard (Series C. No. C-241)

Isotope.	Radioactive Chemical Form.	Activity.
C^{14} ($MC^{14}S$)	Benzoic Acid	27.900 d.p.m.
H^3 (MH^3S)	Toluene	83.200 d.p.m.

Only the standard appropriate to the isotope being counted was included. This is a check on the normal functioning of the machine.

b) Beckman Tri-Carb Spectrometer Standard Blank. (MB).

This is a further check that the machine is running normally with a satisfactory background level.

c) Scintillation Fluid Blank. (SB).

10 ml. Scintillation fluid in a vial. The background for a standard is the solution

in which the standard is counted. This also confirmed that the scintillation fluid had no radioactive contamination and that the vials were not contaminated.

d) Experimental Blank. (EB)

e.g. 10 ml. scintillation fluid plus 0.5 ml. column eluate from equilibrated column prior to elution of experimental extract.

The background where the experimental sample is concerned, must always be a sample blank or a blank equivalent to the sample.

e) Experimental Standard.

10 ml. Scintillation fluid containing some 5000, 13000 or 26000 c.p.m. of tritiated pregnanediol to monitor the efficiency of the machine for counting the experimental H^3 pregnanediol. The standard H^3 pregnanediol (H^3IS) was made up in solution in ethanol in two concentrations - approximately 210 c.p.m. per 0.01 ml. and 20 c.p.m. per 0.01 ml.

f) Experimental Injection Solution.

0.2 ml. of the experimental $7H^3$ progesterone (2.2 microcuries per 0.2 ml.) was taken at the time of making up the experimental injection solution and placed into a vial to which 10 ml. scintillation fluid was added. This enabled the c.p.m. injected to be calculated under similar conditions to the c.p.m. recovered.

4) <u>Efficiency of Packard Tri-Carb Spectrometer Settings.</u>

a) Counting efficiency C^{14} at H.V. Tap 3 = 1/2

b) Counting efficiency H^3 at H.V. Tap 7 = 1/6

c) Relative counting efficiency C^{14} at H.V. Tap 7 = 1/3

where counting at H.V. Tap 3 is taken as 1.

d) Relative counting efficiency H^3 at H.V. Tap 3 = 1/125

where counting at H.V. Tap 7 is taken as 1.

e) H.V. Tap 7 gave the maximum efficiency for counting H^3 and H.V. Tap 4 gave maximum efficiency for counting C^{14}. H.V. Tap 3 was selected for counting C^{14} because when

counting combination of C^{14} and H^3 the drop in H^3 counting efficiency from H.V. Tap 4 to H.V. Tap 3 was much greater than the fall of C^{14} counting efficiency so that H.V. Tap 3 was the most efficient setting for differential counting of C^{14} in this combination. Similarly H.V. Tap 8 was preferable to H.V. Tap 7 for H^3 differential counting. H.V. Tap 8 was used for the plasma experiments where the low levels of radioactivity required maximum differentiation. H.V. Tap 7 however was used throughout the urine experiments to obtain peak counting efficiency for the tritiated pregnanediol. The differentiation at H.V. Tap 7 was adequate for the acetylation and counter current distribution experiments.

5) Experimental Sample Counting Efficiency.

The experimental samples were

a) 0.5 ml. column eluate from each tube collected from the column plus 10.0 ml. scintillation fluid.

This system reduced the counting efficiency

of H^3 pregnanediol by a factor of (0.98 to 0.97) so that experimental tritiated pregnanediol results needed correction by a factor of (1.02 to 1.03.). This quenching factor (QF) was a constant for the experimental work and could be neglected as it was well within the error of the method. However, it is included in the calculations with the correction factor for the ratio of the molecular weights of progesterone and pregnanediol (0.98) and the secretion rate equation is simplified to

$$\text{Secretion Rate} = \frac{\text{c.p.m. injected}}{\text{c.p.m. recovered}} \times \begin{array}{c}\text{pregnanediol}\\ \text{mgm. per day}\end{array} \times 0.96$$

b) Urine 0.3, 0.4 or 0.6 ml. plus Scintillation fluid 10 ml. plus absolute ethanol 6 ml. The daily urine collections had to be thoroughly shaken up before these samples are taken. The addition of the 6 ml. ethanol caused considerable quenching in these samples so that the counting efficiency had to be calculated for each sample by the use of an internal

standard. The internal standard was a solution of H^3 pregnanediol in ethanol (H^3IS).

The samples were counted statistically. Thereafter a known amount of internal standard was added to each sample and the samples again counted statistically. The experimental blank for this procedure was urine from a subject who had not been injected with radioactivity.

Efficiency = (c.p.m. sample + Internal Standard) - sample background

minus

(c.p.m. sample - sample background)

divided by

c.p.m. Internal Standard - standard background.

The reciprocal of the efficiency will give the factor by which each sample count has to be corrected for the loss due to quenching. (Q.F.). This method of assaying the tritium content of urine was a modification of the method described by Flood, Layne, Ramacharan,

Rossipal, Tait & Tait (1961).

6) <u>Results.</u>

The results upon which the above conclusions have based are noted below -

a) High Voltage Tap efficiency for C^{14} and H^3 (Tables 4, 5 and 6).

b) Counting efficiency of experimental standard H^3 pregnanediol sample. (Table 7).

Table 4.

Sample: Radioactive standard plus 10 ml. scintillation fluid.

Experimental blank: 10 ml. scintillation fluid plus 0.2 ml. ethanol.

Counting: Five minutes.

Results: All corrected for time; Background correction only at HVT 3 and 7.

Sample.	HVT 2 cpm	HVT 3 cpm	HVT 4 cpm	HVT 5 cpm	HVT 6 cpm	HVT 7 cpm	HVT 8 cpm
C^{14} Standard 0.2 ml.	2111	3911	4480	3485	2287	1288	851
H^3 Standard 0.2 ml.	21	39	686	2006	3700	4827	4121
C^{14} and H^3 Standard of each 0.2 ml.	1780	3837	4928	5497	5850	5773	4806
EB		11				28	

Table 5.

Samples and Standards as before.
Counting: For five minutes.
Results: All corrected for time and background.

Sample.	HVT 2 cpm	HVT 3 cpm	HVT 4 cpm	HVT 5 cpm	HVT 6 cpm	HVT 7 cpm	HVT 8 cpm
$MC^{14}S$	6308	14245	15766	12427	8176	4852	2932
$MH^{3}S$	12	110	3758	6357	11371	13810	12163
MB	10	13	17	23	28	30	28
SB	8	11	16	22	29	28	25
EB	12	11	12	18	25	29	23
C^{14} Std. .05 ml.	31	73	89	76	43	28	16
H^{3} Std. .05 ml.	7	1	5	42	70	98	92
C^{14} & H^{3} Std. (.05 ml. each)	36	77	104	121	132	136	115
Column Blank + H^{3} & C^{14} Std. of each 0.5 ml.	31	77	116	137	143	138	117
	37	76	116	126	145	134	124

Table 6.

Sample: Radioactive standard plus 10 ml. scintillation fluid.
Counting: One minute.
Results: Not corrected for background.

Sample.	HVT 3 cpm	HVT 4 cpm	HVT 5 cpm	HVT 6 cpm	HVT 7 cpm	HVT 8 cpm
0.1 ml. C^{14} Progesterone.	38817	51928	45860	31581	19871	11913
	39136				19807	11340
	38847				19964	11432
0.2 ml. H^3 Progesterone.	3446	63146	238011	483762	591750	549150
	3138				600548	549369
	3183				598496	549664
					591142	550703
					587936	548962
0.1 ml. H^3 Progesterone.	2231	35346	140470	276692	344526	319867

Table 7.

Experimental samples a) H^3 standard plus 10 ml. Scintillation Fluid.
b) recounted plus 0.5 ml. Column Eluent.

Experimental blank : 0.5 ml. Column Eluent plus 10 ml. Scintillation fluid [for correction of b) results]

Scintillation blank : 10 ml. Scintillation fluid [for correction of a] results).

Sample.	Counts.	Time.	cpm.	B.C.	SD±	QF
MH^3S	26660	2	13330			
SB	864	30	29			
EB	944	30	31			
a) 0.2 ml. H^3IS	43977	10	4398	4369)	21	
0.2 ml. H^3IS	43629	10	4334	4334)		
0.5 ml. H^3IS	52341	5	10468	10439)	45	
0.5 ml. H^3IS	52549	5	10510	10481)		
0.5 ml. H^3IS	52400	5	10480	10451		
b) + .5 ml. Eluent	43234	10	4323	4292		1.02
+ .5 ml. Eluent	42932	10	4293	4262		1.02
+ .5 ml. Eluent	50849	5	10170	10139)	45	1.03
+ .5 ml. Eluent	50944	5	10189	10158)		1.03
+ .5 ml. Eluent	51924	5	10385	10354		1.02

APPENDIX V.

A) Absorption Spectrophotometry.

B) Preparation of Sample.

C) Colour Reaction.

D) Reading in Spectrophotometer.

E) Calculation of Results.

F) Trial Readings of Pregnanediol and Pregnanetriol.

A. Absorption Spectrophotometry.

Absorption spectrophotometry is based on the observation and comparison of absorption spectra. The absorption spectrum - a curve showing the amount of radiant energy absorbed at each wavelength - is a characteristic property of chemical compounds: i.e. each such chemical compound has its own absorption spectrum. Basically the spectrophotometer provides absorption data to assist the chemist in quantitative and qualitative analysis.

Transmittance (T) is the ratio of the radiant energy transmitted by the sample (P) to the energy incident upon the sample (Po). Both radiant energies must be obtained at the same wavelength with the same spectral slit width. $T = \frac{P}{Po}$.

Transmittance is usually given in percent. Since it is seldom possible to measure these radiant energies directly because of the presence of a sample cell,

it is customary to consider the transmittance of the sample as the ratio of the light transmitted by the cell and the sample to the light transmitted by some arbitrary standard. In other words, the sample is compared to a standard. This standard is often the cell filled with the liquid in which the sample is to be dissolved. The transmittance of this standard, of course, is defined as 100%. Therefore when the transmittance of a sample is given, it is necessary to specify the standard with which the sample was compared.

Absorbance (A) is the negative logarithm to the base 10 of the transmittance $A = -\log T = \log \frac{1}{T}$ (T is expressed as a decrease fraction, not in %). As with transmittance measurements the standard with which the sample was compared should always be included with the absorbance data. The absorbance of the standard is defined as zero.

All substances transmit some portions of the electromagnetic spectrum and absorb

others. The colour of red glass, for example, results from the fact that it absorbs light of short wavelength, the green and the blue, and transmits light of long wavelengths, the red and some yellow light. The concentration of the solution and its thickness will further affect the absorbance. If the absorbance is plotted against wavelength, the spectral absorbance curve of the sample is obtained. Just as each component has its own absorption spectrum, a mixture of two or more components will yield an absorption spectrum containing characteristics of all the components. The absorbance at each wavelength is the sum of the absorbances of the components of the mixture at that wavelength; the contribution of each component is proportional to the concentration and its absorptivity at that wavelength.

Problems involving the identification of organic components can be solved by

spectrophotometric techniques through use of the "matching fingerprint" method. If the spectrocopist has an extensive library of absorbance curves of known compounds, positive identification of the unknown is established in a very short time if the unknown spectrum matches any of the known spectra.

Usually in quantitative work the constituents of the sample are known and it is desired to determine the concentration of one or more of them. If, however, it is necessary to decide what components are present this information may be obtained from the history of the sample, by ordinary qualitative chemical analysis or by comparing the spectrum of the mixture with the spectra of various pure samples as discussed above.

Quantitative spectrophotometry is based upon the fact that the absorbance of an absorbing material is dependent on its concentration. If the absorbance is directly proportional to the concentration the system

is said to obey Beer's Law (In much practical spectrophotometric work the cell length is a constant and therefore it cancels out in Beer's Law Calculations).

Mathematically A = abc where A = absorbance

a = absorptivity (unit area per unit mass)

b = length of light path

c = concentration (mass per unit volume).

Assuming that Beer's Law is obeyed strictly it would be very simple to obtain the concentration of an unknown by simply comparing the absorbance of the unknown with the absorbance of a known sample. Concentration of the unknown could then be readily obtained by simple arithmetic, i.e. by proportion.

In practice, however, slight deviations from Beer's Law are noted. In making quantitative measurements it is necessary, therefore, to plot the absorbances of a series of standards (Solutions containing known concentrations of the absorbing material) against their concentrations. In these graphs, concentration

is given linearly on one axis and absorbance is given linearly on the other axis. Usually a slightly curved line passing through the axis is obtained. When the absorbance of the unknown has then been obtained its concentration can be easily read from the graph.

The applicability of Beer's Law depends on the adherence to certain requirements. The samples must be compared with a 100 per cent reference in which the concentration of the absorbing substance is zero; the absorbing substance must not participate in an equilibrium in the solution which would be shifted by change in concentration; essentially monochromatic light must be used for absorbance measurements; and absorbance measurements must be made at wavelengths where the absorbance does not change appreciably with wavelength -- in a horizontal portion of the absorbance curve.

The absorbance of a mixture at a

particular wavelength is the sum of the absorbance of all the components of the mixture.

Transmittance or absorbance measurements are always made by comparing a sample to a standard (or reference). As the standard would obviously have a transmittance of 100% compared with itself, it may be called the 100% reference. It is possible to define various standards but usually the standard is a "blank" - i.e. a solution identical in composition with the sample except the absorbing material being measured is absent. In making the spectrophotometric measurement, the operator sets the instrument at 100% by using the 100 per cent reference. The sample is then placed in the light path and its transmittance or absorbance read.

The 100% reference contains none of the absorbing substance. In the Beer's Law graph discussed above, therefore, the 100%

reference will be at zero on the concentration axis. In this instance the 100% reference may be considered to be one of the series of standards used in preparing the graph. Often quantitative techniques are developed where the standard is a known concentration of the sample. Use of such a standard allows a greater measuring accuracy and precision of measurement particularly where samples are stable and where strong absorption bands must be used for measurement.

B. Preparation of Sample for Spectrophotometry.

1) Standard solutions of

5 β pregnane - 3α, 20α-diol

pregnane - 3, 17, 20 - triol

pregnane - 3α, 17α, 20α-triol

pregnane - 3α, 20α-diol diacetate

in ethanol, 12.5 mgm. in 25 ml. were made up and kept in the refrigerator. "Bisulphite Sulphuric" acid was prepared as described previously (Materials and Methods, page 39), placed in a dark brown glass bottle with cap

and kept out of direct light. A Beckman D.U. spectrophotometer was used for the spectrophotometry with the samples contained in 4 ml. Quartz Cuvettes.

2) <u>Samples:</u> They were made up in small boiling tubes

a) An acid blank was contained in each series. This was 4 ml. of "bisulphite-sulphuric" acid, which was processed with the other samples and used to check the purity of the "acid", the reading of the experimental blank and the functioning of the spectrophotometer.

b) The experimental blank was made up in identical fashion to the experimental samples except that the steroid content was absent. For experimental samples from the column, the experimental blank consisted of a similar volume of eluate from the column which was collected prior to the extract front.

c) Standard Samples: of known amounts of the steroid to be measured were obtained by

taking appropriate aliquots from the standard solutions. These were also made up in a similar way to the experimental samples. When column samples were to be read, equivalent volumes of column eluate were added to the standard samples from the column eluate used for the experimental blanks. Thus each series of experimental samples from any given column were accompanied by an acid blank, experimental (or column) blank, and standard samples.

d) Experimental Samples. The serially labelled tubes of column eluate were available. Two ml. (in some instances 3 ml. and 0.2 ml.) were taken from the tubes with significant pregnanediol radioactivity as estimated by Scintillation Counting and transferred to small boiling test tubes. The radioactivity was contained in two peak tubes as a rule. One tube on each side of the peak tubes were also included so that 4 tubes from each column were usually assayed

for pregnanediol by spectrophotometry.

C. Colour Reaction.

All the samples, appropriately labelled, were taken to dryness and then transferred to a vacuum dessicator. One empty labelled tube was included for the "acid" blank. A vacuum was produced by vacuum pump and the tubes left overnight.

They were then removed from the dessicator, 4 ml. "bisulphite-sulphuric" acid added to each and gently rolled to ensure that all the contents were taken up. Thereafter they were transferred to a boiling water bath for 4 minutes, care being taken that during the incubation no moisture entered the test tubes.

At the end of 4 minutes the samples were removed from the water bath and allowed to cool at room temperature for 20 minutes, by which time they were ready for reading.

D. Reading in the Beckman D.U. Spectrophotometer.

D. Reading in the Beckman D.U. Spectrophotometer.

The silica cuvettes were washed in absolute methanol, wiped dry on the outside with tissue and when dry inside, the contents of selected tubes were decanted into them. As a rule three cuvettes were read in a series - the experimental blank (100 per cent reference or zero absorbance), and the two peak experimental tubes, for example, being read as one series so that identical conditions would be present during their measurement. The cuvette carrier however could take four samples if required. The Silica Cuvettes were washed thoroughly in absolute methanol after each sample had been read. Thereafter they were allowed to dry out before the next sample was transferred for reading.

The absorbance of the experimental samples were compared with the experimental blank at various wavelengths. When a spectrum was being measured, readings were taken within the range of 320μ to 500 μ.

When quantitative measurements were being taken the readings for pregnanediol were at 390 μ, 425 μ and 460 μ and for pregnanetriol 410 μ, 440 μ and 470 μ.

E. Calculation of Results.

Quantitative calculations were based on a correction factor (Allen 1950)

$$\text{Correction O.D.}425 = \text{O.D.}425 - \frac{(\text{O.D.}390 + \text{O.D.}460)}{2}$$

for pregnanediol

and

$$\text{O.D.}440 = \text{O.D.}440 - \frac{(\text{O.D.}410 + \text{O.D.}470)}{2}$$

for pregnanetriol

where O.D. = optical density or absorbance.

The absorbances of the standard samples were read in a similar manner, and the mean reading for the absorbance of 25 gamma of the standard calculated. Comparison of the absorbance of the experimental samples with the standard mean for 25 gamma enabled the experimental sample absorbance to be converted to weight.

When a spectrum was being measured, the absorbances at the various wavelengths were used to compare the experimental sample spectrum with that of a standard sample, the experimental blank, the experimental sample, and the standard sample being put through the machine in series.

F. Trial Readings.

Absorption spectra for 25, 50 and 100 gamma aliquots of pregnanediol and pregnanetriol were obtained using the above technique (Tables 8 and 9). Corrected absorbance readings for 25, 50 and 100 gamma aliquots of pregnanediol and pregnanetriol were obtained. (Tables 10 and 11).

The results indicated that the spectrophotometer technique functioned satisfactorily and was reproduceable.

Table 8. Absorption spectra of standard aliquots of pregnanediol.

Wavelength Microns.			Gamma				
	25	25	50	50	100	100	Acid Blank.
375	.095	.095	.207	.204	.405	.455	0
380	.088	.090	.194	.192	.389	.430	
385	.085	.085	.190	.190	.390	.418	
390	.089	.091	.197	.198	.405	.440	
395	.093	.091	.211	.215	.437	.480	
400	.108	.106	.239	.241	.500	.545	
405	.120	.120	.267	.269	.570	.608	
410	.139	.134	.303	.305	.650	.676	
415	.149	.146	.329	.331	.721	.729	
420	.157	.155	.345	.348	.745	.750	
425	.158	.155	.350	.355	.753	.752	
430	.157	.153	.349	.356	.752	.752	
435	.158	.152	.350	.357	.760	.750	
440	.155	.150	.341	.351	.741	.728	
445	.147	.143	.323	.331	.696	.689	
450	.129	.125	.290	.299	.620	.605	
455	.110	.107	.246	.253	.520	.504	
460	.093	.090	.203	.206	.422	.412	
465	.076	.074	.168	.168	.345	.337	
470	.066	.063	.143	.142	.292	.286	
475	.060	.057	.125	.126	.260	.255	
Allen Correction	.067	.065	.150	.153	.340	.326	

Table 9. Absorption spectra of standard aliquots of pregnanetriol.

Wavelength Microns.	Gamma 25	25	50	50	100	Acid Blank.
340	.180	.099	.169	.184	.357	0
350	.122	.102	.164	.182	.350	
360	.110	.091	.162	.175	.343	
370	.103	.090	.164	.173	.341	
375	.103	.089	.167	.172	.347	
380	.104	.093	.174	.184	.365	
385	.109	.100	.190	.196	.398	
390	.120	.110	.215	.218	.446	
395	.135	.128	.248	.254	.515	
400	.156	.150	.301	.300	.611	
405	.185	.177	.356	.352	.715	
410	.215	.209	.427	.412	.840	
415	.246	.242	.492	.480	.970	
420	.274	.272	.554	.540	1.10	
425	.290	.295	.591	.580	1.19	
430	.307	.313	.622	.612	1.26	
435	.315	.322	.640	.631	1.30	
440	.310	.320	.631	.628	1.29	
445	.290	.300	.591	.590	1.20	
450	.245	.258	.500	.503	1.04	
455	.191	.203	.393	.400	.810	
460	.142	.148	.292	.295	.600	
465	.103	.107	.209	.218	.434	
470	.073	.078	.153	.155	.315	
475	.058	.064	.125	.125	.248	
480	.052	.056	.110	.110	.217	
485	.048	.053	.104	.104	.201	
Allen Correction.	.166	.176	.341	.344	.712	

Table 10. Spectrophotometry Readings of standard aliquots of pregnanediol.

Gamma	Wavelength Microns 390	425	460	Allen Correction
25	.125	.211	.129	.084
25	.135	.215	.123	.086
50	.275	.454	.284	.176
50	.220	.405	.225	.182
100	.470	.817	.478	.343
100	.473	.825	.488	.345
Acid Blank.	0	0	0	0

Table 11. Spectrophotometry Readings of standard aliquots of Pregnanetriol.

Gamma	Wavelength Microns 410	440	470	Allen Correction
25	.159	.265	.041	.165
25	.184	.289	.072	.161
50	.390	.631	.136	.368
50	.378	.616	.145	.354
100	.796	1.25	.288	.708
100	.824	1.31	.335	.730
Acid Blank.	0	0	0	

APPENDIX VI.

PROOF OF PURITY OF PREGNANEDIOL SAMPLE.

A) Absorption Spectrum.

B) Acetylation.

C) Counter Current Distribution.

D) Infra-Red Analysis.

A) Absorption Spectrum.

Absorption spectra of 25, 50 and 100 gamma standard pregnanediol were obtained at the beginning of the research programme. (Appendix V, Table 8, page 288).

Absorption spectra of 50 gamma of standard pregnanediol and an experimental sample of pregnanediol (Experimental Subject VII, Day 3 urine, column tube 12 - usual 2 ml. aliquot) were read at the time of Experiment VII. (Figure 36).

Absorption spectra of 10 and 25 gamma standard pregnanediol were obtained at the end of the research project. (Volume II, Page 40).

These spectra confirmed that the colorimetric method of pregnanediol estimation continued to be efficient and reproduceable. The absorption spectrum of the experimental sample was identical to that of the standard pregnanediol, (Results, page 69), and confirms that the experimental sample is pregnanediol.

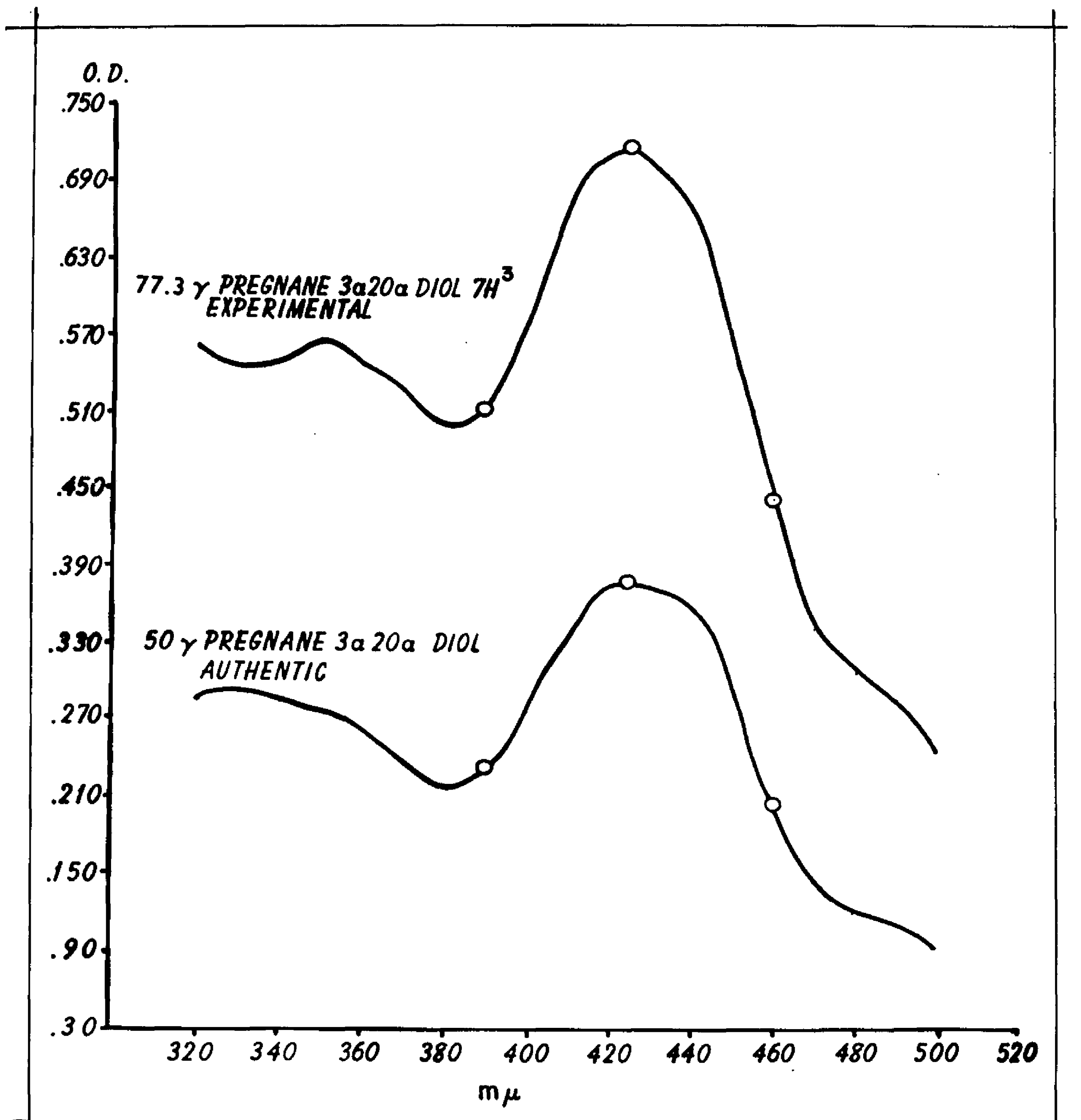


FIG. 36. Absorption spectra of experimental and standard samples of pregnanediol.

B) Acetylation.

The experimental sample for acetylation was Patient I, day 1, tubes 11 and 12 contents after 0.5 ml. had been removed from each for scintillation spectrometry and 2.0 ml. from each for absorption spectrophotometry. The 2.5 ml. of eluate remaining in each of these two tubes were pooled and dried down with a stream of nitrogen. Three hundred gamma standard pregnanediol were added and taken to dryness.

This composite sample was dissolved in 8 ml. methanol, 0.5 ml. was taken for scintillation counting and 0.5 ml. for absorption spectrophotometry so that the specific activity of this pre-acetylation sample could be calculated. Three and a half millilitres were taken and dried down with a stream of nitrogen in a test tube. Five hundred gamma of standard pregnanediol were placed in another test tube and similarly dried down. These two latter samples were

acetylated, one the experimental sample and the other the control sample.

Radioactive acetic anhydride was used for the acetylation (1 - C^{14} - acetic anhydride). To 0.6 ml. pyridine were added 1.8 ml. C^{14} acetic anhydride and 0.5 ml. of this mixture was added to each of the samples for acetylation which were then incubated in a water bath at 70°C for 30 minutes.

Thereafter 4.5 ml. distilled water was added to each tube and the contents transferred to 25 ml. separating funnels and extracted with 15 ml. ethyl acetate three times. The two ethyl acetate extracts were taken to dryness in test tubes with a steam of nitrogen.

The residue of the experimental sample was taken up in 0.5 ml. methanol-chloroform (equal parts). The control sample was taken up in 1 ml. methanol-chloroform and divided into two 0.5 ml. portions. The three samples were run on a Bush paper chromatography system with 50 gamma pregnanediol as a standard

control. The chromatography system was that described in Appendix III, three "walkings-up" being used. (Pages 207 to 214).

The following strips were stained with phosphomolybdic acid -

1) Pregnanediol standard control strip
2) One of the acetylation control strips

The following strips were scanned for radioactivity in the Tracerlab Precision Ratemeter. (Figure 37).

1) The unstained acetylation control strip
2) Experimental acetylation strip

The stained pregnanediol control strip showed the pregnanediol at the expected distance from the start line. The strip of the acetylation control showed steroid with the front.

The Precision Ratemeter tracing of the radioactivity (C^{14} only) showed the radioactivity peak with the front where the post acetylation steroid appeared on staining one acetylation control

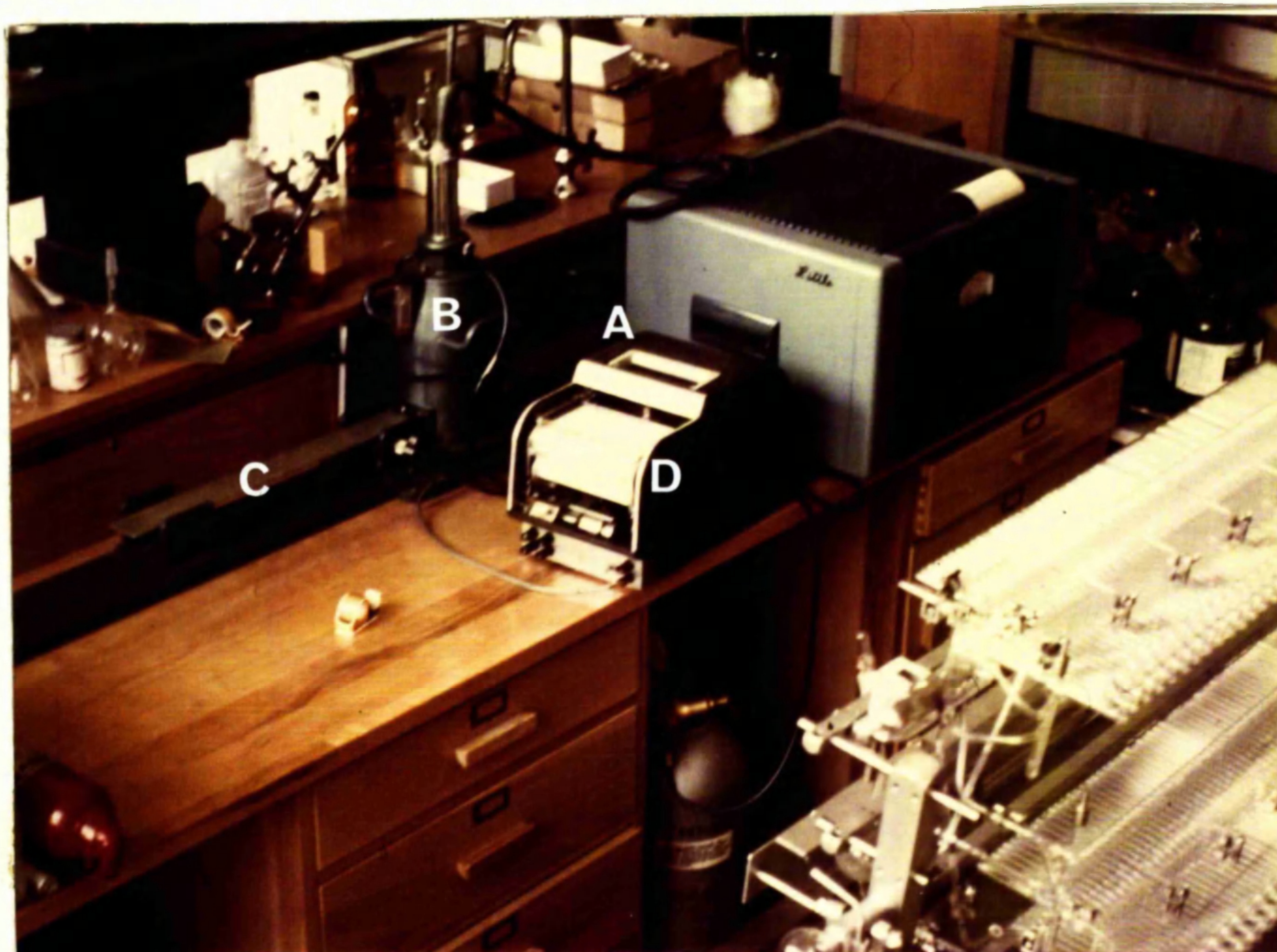


FIG.37. A.PRECISION RATE METER.
B.SCANNER FOR RADIOACTIVITY.
C.CHROMATOGRAM STRIP CARRIER.
D.TRACING RECORDER PEN AND PAPER.

strip. (Figure 38).

The 6 cm. of the strip of the acetylation control at the front which showed up the radioactivity and equated the steroid area on the stained control strip, and the corresponding 6 cm. of the experimental acetylation sample strip which also showed the radioactivity peak, were cut out and separately eluted in methanol.

The methanol eluates were dried down and redissolved in 2 ml. methanol.

From each were taken 0.2 ml. for scintillation counting, and 1.0 ml. for absorption spectrophotometry. This enabled the calculation of the specific activity of the derivative of the experimental pregnanediol which had been made (viz. pregnanediol diacetate). The remaining 0.8 ml. aliquots were submitted to Counter Current distribution. (See later).

The specific activities of tubes 11 and 12 were constant within the error of

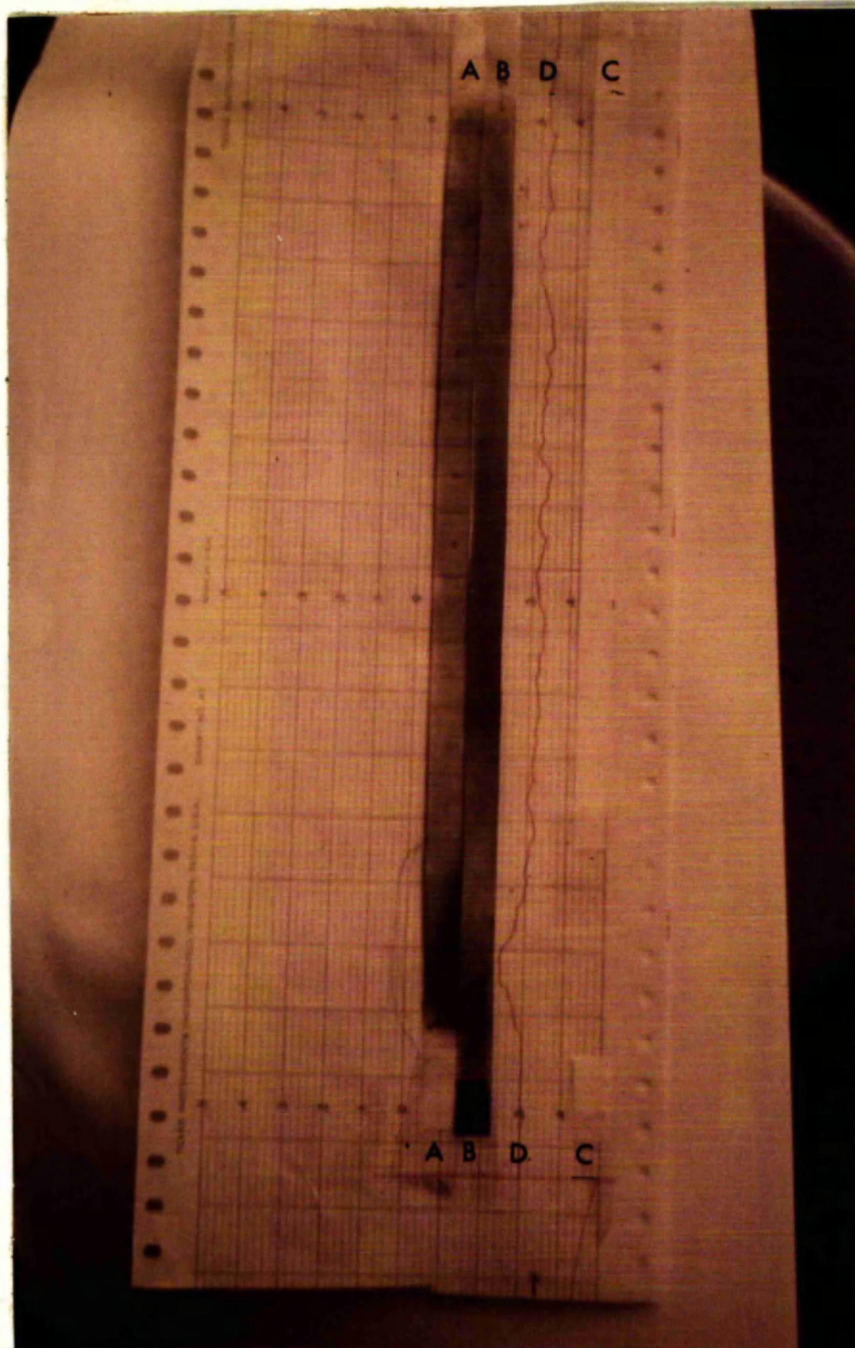


FIG.38. A.ACETYLATION CONTROL STRIP.
B.PREGNANEDIOL CONTROL STRIP.
C.ACETYLATION SAMPLE STRIP.
RADIOACTIVE AREA CUT FOR ELUTION.
D.RADIOACTIVITY TRACING OF C.

±5 per cent. The corrected specific activity of the diluted pre-acetylation experimental sample and the mean specific activity of tubes 11 and 12 were constant within the error of ± 10 per cent.

The specific activities of the pre-acetylation experimental sample and the post-acetylation experimental sample were constant within the error of ± 6.4 per cent.

The corrected specific activity of the acetylated experimental sample (correction for the dilution of the pre-acetylated sample with 300 gamma standard pregnanediol) and the mean specific activity of tubes 11 and 12 were constant within the error of ± 3.2 per cent.

Thus the specific activities of the experimental samples remained constant (i.e. within the error of the experimental method) after dilution with standard pregnanediol and after converting the experimental sample

to pregnanediol diacetate. This is further confirmation that the experimental sample is pure pregnanediol.

Details of the results of these experiments are contained in - Results: Proof of Purity of Experimental Pregnanediol. Volume II, page 41 to 44 and Results, page 70 to 71.

C) <u>Counter Current Distribution.</u>

The remaining 0.8 ml. aliquot of the acetylated experimental and control samples were dried down and submitted separately but simultaneously to the counter current distribution procedure detailed in Appendix III. (Pages 215 to 217).

The eight tubes from these two counter current distributions were taken to dryness. The contents of each tube were then taken up in 10 ml. Scintillation fluid (5 mls., 2.5 mls. and 2.5 ml. portions to ensure complete transfer of steroid from tube to vial) and transferred to appropriately marked

scintillation vials.

The vials were then assayed for radioactivity in the Scintillation Spectrometer at Tap 7 for H^3 and Tap 3 for C^{14} content. (See Appendix IV).

The distribution curves of C^{14} and H^3 radioactivity achieved by counter current distribution of the experimental sample were compared with the C^{14} distribution curve of the control sample which could only be pregnanediol diacetate. (For Detailed Results see Volume II, page 45-48).

These three curves were similar indicating that the experimental H^3 pregnanediol C^{14} diacetate was identical with standard control pregnanediol C^{14} diacetate and was a third confirmation of the purity of the experimental pregnanediol sample. (Results, page 72 to 73).

Infra-Red Analysis:

As a fourth and final proof of the specificity and degree of purity of the

method of extraction and separation of pregnanediol, a sample of experimental pregnanediol was submitted to an independent authority for infra-red analysis.

As a sample of some 5 mgm. of experimental pregnanediol was required, and since the pregnanediol output of the experimental groups was so small, the sample for I.R. analysis was obtained from urine from two pregnant women near term.

One day's output of urine was collected from each of the two pregnant women and one quarter of each daily total was extracted by Method IV after 80.000 c.p.m. H^3 pregnanediol was added to each sample as internal standard.

The pregnanediol tubes from the column, were confirmed by absorption spectrophotometry and radio active assay and the tubes with both significant radioactivity assay and quantitative pregnanediol content were pooled to give some 7 mgm. pregnanediol.

The total confirmed pregnanediol eluate from the pregnancy urine extractions, thus pooled in a 50 ml. conical graduated centrifuge tube, was taken to dryness. Ten millilitres of methanol was added to dissolve the residue and to ensure that all the residue went into solution, the tube was heated in water bath at 50°C. Distilled water (20 ml.) was also heated in the water bath.

Thereafter the sample tube was removed from the water bath and the distilled water gradually added to the methanol by pasteur pipette and with stirring by glass rod till crystallisation of the pregnanediol was achieved. (Some 10 ml. distilled water required).

The tube was then left to settle in a refrigerator overnight. Next morning, the tube was centrifuged in the International Centrifuge at 1500 r.p.m. for 2 minutes.

The mother liquor was decanted off,

and the procedure repeated three times as above. After the fourth mother liquor had been decanted, the pregnanediol crystals were taken to dryness under a stream of nitrogen and sent to Lewis L. Engel, Ph.D., Associate Professor of Biological Chemistry, Harvard Medical School, who had kindly consented to undertake Infra-red Analysis of the sample.

He reported that the experimental pregnanediol spectrum agreed in all respects with his reference sample. He converted a portion of the experimental pregnanediol sample to the acetate and this spectrum agreed in all respects with his reference sample of pregnane-3α, 20α - diol diacetate. (Results page 74 to 75). Full details of this independent confirmation of the purity and specificity of the experimental pregnanediol are contained in - Volume II, page 49-65).

APPENDIX VII.

Theory of Secretion Rate Calculation.

The calculation of the secretion rates of steroid hormones from the specific activity of a urinary metabolite after a single injection of the radioactive hormone (radioactivity injected divided by the specific activity of the metabolite) has proved to be a simple and powerful in vivo method (Pearlman 1957 a, and b; Pearlman, Pearlman & Rakoff 1954; Cope & Black 1958, Peterson 1959; Jones, Lloyd Jones, Riondel, Tait, Tait, Bulbrook and Greenwood (1959); Ayres, Garrod, Pearlman, Tait, Tait & Walker (1957); Ulick, Laragh & Lieberman (1958).

This expression, radioactivity injected divided by specific activity of a metabolite, ($P = \frac{R}{SA}$)- is correct assuming that a single compartment model with instantaneous mixing can adequately describe the transport and metabolism of steroids. This type of model has been applied with some success in interpreting the metabolism of corticosterone

and cortisol in man (Mignon, Sandberg, Decker, Smith, Paul & Samuels 1956; Mignon, Sandberg, Paul & Samuels 1956; Peterson 1959). The disappearance of radioactivity in plasma after the injection of labelled cortisol and corticosterone suggests that the use of a two compartmental model would be more exact (Robertson 1957) but analysis of the data indicated that a one compartmental model can be used as a first approximation for many purposes. However, when the volume of distribution is much greater than seems to be the case for these steroids, a more complex model must be used. This is probably necessary for all steroids where binding to plasma proteins is weak such as progesterone, oestrone, oestradiol and oestriol.

Laumas Tait & Tait (1961 a and b) have critically examined the validity of the calculation of secretion rates from the specific activity of a urinary metabolite

and the problem has been recently discussed by Gurpide, Mann and Vandewiele (1962). Before considering this in more detail the following definitions are necessary.

A Tracer is a labelled form of a substance. Ideally the label makes the labelled form detectable by the observer without affecting its behaviour in the system being studied.

Carrier is unlabelled material of the same substance added to a sample containing a tracer as an aid in chemical processing or estimation. A logical extension of this meaning is to include the unlabelled form of the substance being traced which is normally present in the system.

Specific Activity denotes the ratio of the amount or concentration of the tracer to that of the total (labelled and unlabelled) substance. Any units which define the ratio may be used - e.g. microcuries per milligramme or counts per minute per milligramme.

Pools or Compartments. For mathematical purposes the constituents of a living system can be represented as being located in distinguishable phases or volumes designated as pools or compartments. The boundaries of these compartments may but do not necessarily conform to anatomical boundaries. For example, the blood plasma is a relatively easily defined compartment. The location of the progesterone pool or bicarbonate pool is more difficult to define but perhaps no less clear in concept.

Transfer. Transport of a substance into and out of a compartment, and chemical synthesis and degradation, result in appearance and disappearance of the substance in the compartment. The term "transfer" denotes unidirectional processes of either kind when the mathematics are the same for both kinds.

Exchange implies a one for one substitution of atoms or molecules or simultaneous and

equal transfers into and out of a compartment.

Steady State. This term is applied to compartments where the rates of removal of the substances being studied are equalled by the rates of replacement so that the concentrations and amounts of the substances being studied are constant during the period of observation. Constant values of transfer are also usually specified or implied in mathematical treatments but are not required by the definition of steady state. Of course during an experiment the tracer itself is not in a steady state but it is assumed to be introduced in an amount sufficiently small not to disturbe the steady state of its unlabelled counterpart either by its quantity, by the affects of its radiation, or by its pharmacological effects.

Turnover. Exchange processes produce a "turnover" of the substance in a given compartment.

Turnover time is the time interval required

for the amount of a substance transferred into or out of a compartment in the steady state to be numerically equal to the amount present in the compartment.

Turnover Rate has been used in two senses.

1) as the reciprocal of the turnover time or fraction per unit time, giving turnover rate the dimension of Time $^{-1}$.

11) as the amount of substance that is turned over per unit time, giving turnover rate the dimension of Mass ÷ time.

In this thesis the word "rate" (as in turnover rate, exchange rate, transfer rate, secretion rate) will imply dimensions of Mass ÷ time and the term "rate constant" will be used when the dimensions Time $^{-1}$ are meant.

Secretion Rate is the amount of a substance produced in a pool or compartment system per unit time.

The following consideration of the validity of secretion rate calculation is

taken from Laumas, Tait & Tait 1961 a and b.

Figure 39 shows a general model which seems suitable for describing the transport and metabolism of steroids. In this model, it is assumed that there are five general pools. The plasma pool is connected to a general extracellular space (V_E^C) and a much smaller volume (V_E^L) representing the extracellular volume of the liver. It can be assumed that metabolism of steroid is negligible in these spaces and that the transfer from the plasma can be described by the rate constants K^L to the liver and K^C to the general extracellular volume. Steroid is also transferred (K_1^L) from the liver extracellular space (V_E^L) to the liver intracellular space (V_I^L), which has been shown to be the site of intense catabolism of steroids (metabolism rate constant K_2^L). Transfer also occurs (rate constant K_1^C) from the extracellular volume (V_E^C) to the general intracellular volume (V_I^C) where metabolism

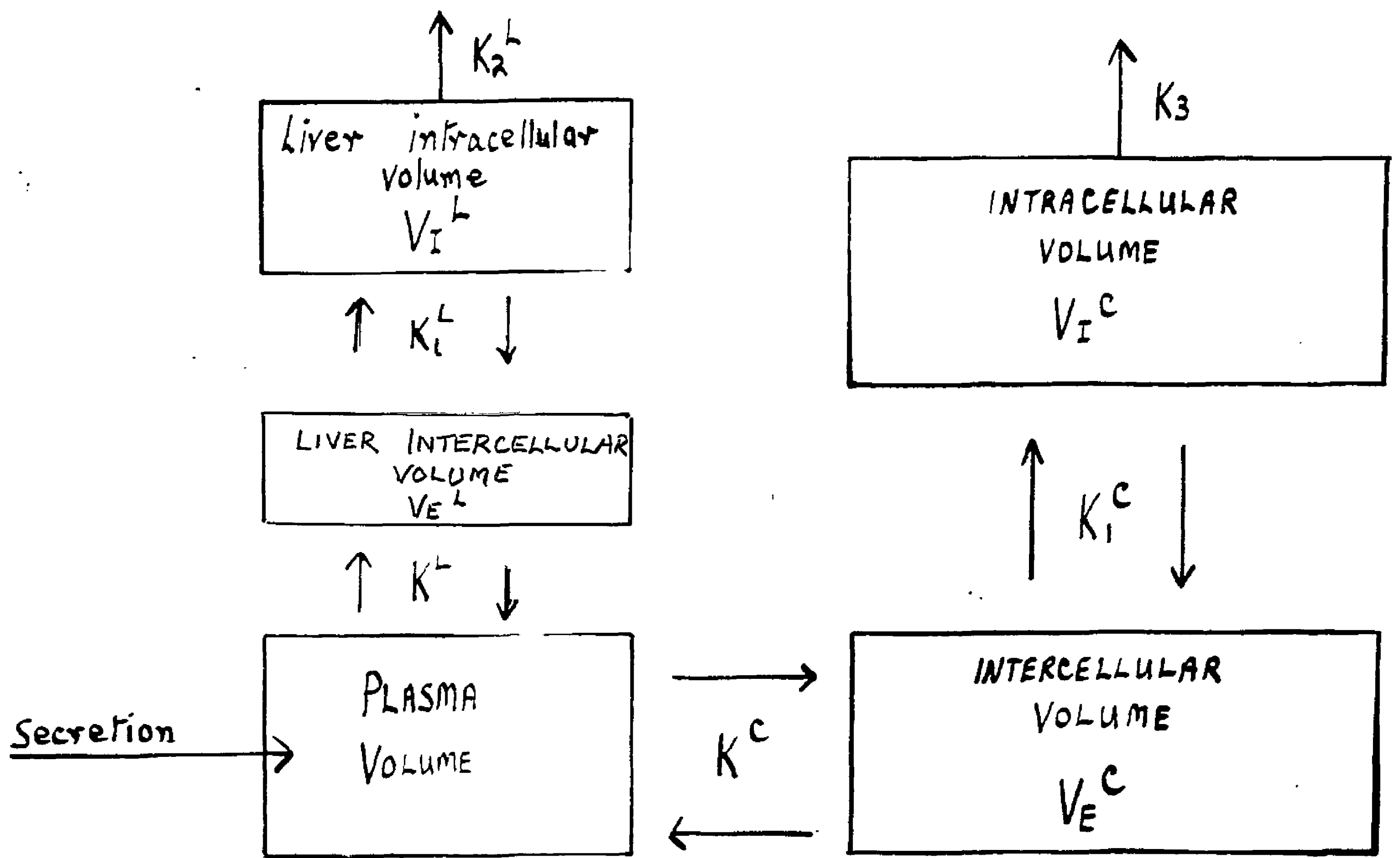


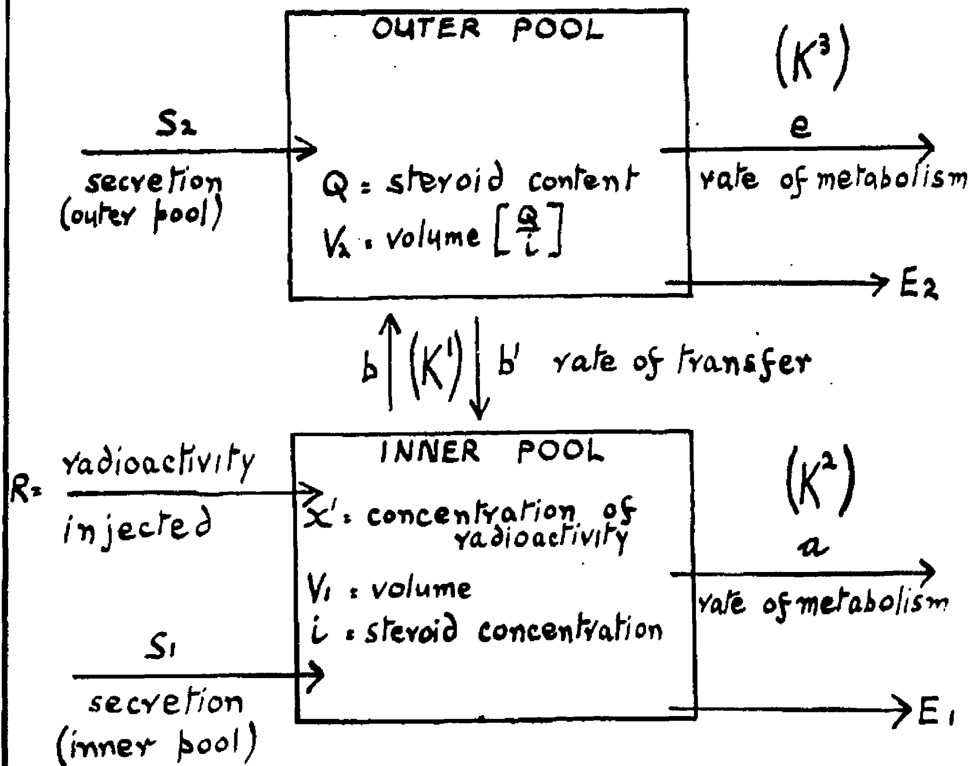
FIG. 39.

occurs at a rate given by the constant K_3.
It has been shown (Peterson 1959) that even
when the steroids are strongly bound to
plasma proteins, their rate of transfer from
plasma to the extracellular volumes is
fairly rapid. It seems, therefore, that
in the general case K^L and K^C are very
large and that the plasma, the extracellular
space V_E^C and liver extracellular volumes
can be regarded as one general extracellular
volume. Also, if the liver intracellular
volume is small compared with this general
extracellular space and the rate of transfer
between these pools is reasonably large,
then these two spaces can be regarded as
one general inner pool V_1 (Figure 40).
It should be emphasized that this does not
require that the rate of transfer from the
liver extracellular space into the liver
cells should be as great as the transfer from
the plasma to the extracellular volume. It
is sufficient that the volume of the liver

MODEL FOR STEROID METABOLISM

$$M = \frac{S_1 + S_2}{i}$$

$$= \frac{\text{total secretion rate}}{\text{plasma concentration of steroid}}$$



$$f = \frac{1}{1 - \frac{S_2 e}{S(S_2 + b)}} \qquad w = \frac{1 + \left(\frac{a}{a+e}\right)\frac{S_1}{b}}{1 + \left(\frac{E_1}{E_1+E_2}\right)\frac{S_2}{b}}$$

E_1 and E_2 = rate of excretion of a particular metabolite

FIG. 40.

should be much smaller than the total extracellular space (in man, the liver would be about 5% of the total extracellular volume) and that the rate of transfer from plasma to liver should be appreciable. This latter process may still be the rate limiting step under certain conditions for the metabolism of steroids by the liver. The lowering of the total rate of metabolism of cortisol when the hormone is more strongly bound to plasma proteins, as in pregnancy, may be explained by the operation of this rate limiting step (Mills 1959: Mills, Schedl, Chen & Bartter 1960). In this case, it may be necessary to use the more complex model (Figure 39) to explain the experimental results. However, as a preliminary step, towards a rigorous treatment of the secretion rate method, it would appear to be adequate to use a two compartmental model (Figure 40). One pool (the inner pool) would consist of the plasma volume, extracellular volume and

the liver volume, total space V_1 ml. total steroid content I and metabolic rate constant K_2. The other pool (outer pool) would consist of the intracellular volume, total volume V_2 ml., total steroid content Q and metabolic rate constant K_3. The transfer rate constant between the two pools would be K_1. Both the endogenous hormone secretion and the single injection of radioactive hormone are assumed to enter the inner pool where instantaneous mixing occurs. It is also assumed that when steroid enters the outer pool it is instantaneously mixed there. Then following this injection and in the non isotopic steady state let

I = total steroid content of inner pool

x = fraction of injected radioactivity in inner pool at time t

a = total steroid metabolized in inner pool in unit time

Q = total steroid content of outer pool

z = fraction of injected radioactivity in outer pool at time t

e = total steroid metabolized in outer pool in unit time

b = total steroid transferred from inner pool to outer pool in unit time

b^1 = total steroid transferred from outer pool to inner pool in unit time

then, if all radioactivity is measured specifically as the hormone itself and first order reactions apply for the individual steps

$$- \frac{dx}{dt} = \frac{b.x}{I} - \frac{b^1.z}{Q} + \frac{a.x}{I}$$

$$\frac{dz}{dt} = \frac{b.x}{I} - \frac{b^1.z}{Q} - \frac{e.z.}{Q}$$

then where $\Theta_1 = \frac{b}{I}$; $\Theta_2 = \frac{b^1}{Q}$; $\Theta_3 = \frac{a}{I}$ and $\Theta_4 = \frac{e}{Q}$

and $b - b^1 = e$ in the steady state so that

$$\Theta_2 = \frac{b^1}{Q} = \frac{b-e}{Q}$$

$$\frac{d^2z}{dt^2} + (\Theta_1 + \Theta_2 + \Theta_3 + \Theta_4)\frac{dz}{dt} + (\Theta_1\Theta_4 + \Theta_3\Theta_2 + \Theta_3\Theta_4)z = 0$$

$$\frac{d^2x}{dt^2} + (\Theta_1 + \Theta_2 + \Theta_3 + \Theta_4)\frac{dx}{dt} + (\Theta_1\Theta_4 + \Theta_3\Theta_2 + \Theta_3\Theta_4)x = 0$$

and $z = Ce^{-\alpha t} + De^{-\beta t}$ $\quad x = Ae^{-\alpha t} + Be^{-\beta t}$

where $\alpha + \beta = \theta_1 + \theta_2 + \theta_3 + \theta_4$ and

$$\alpha\beta = \theta_1\theta_4 + \theta_3\theta_2 + \theta_3\theta_4$$

with the boundary conditions

$x = 1$ when $t = 0$ and $z = 0$ when $t = 0$

$$z = \frac{\theta_1}{\beta - \alpha} \left(e^{-\alpha t} - e^{-\beta t}\right)$$

$$x = \frac{\theta_2 + \theta_4 - \alpha}{\beta - \alpha} \cdot e^{-\alpha t} + \frac{\beta - \theta_2 - \theta_4}{\beta - \alpha} \cdot e^{-\beta t}$$

This treatment and result are analogous to that obtained by Campbell, Cuthberton, Mathews & McFarlane (1956) using a simpler model for albumin metabolism. It assumes that no re-cycling of radioactivity into the hormone occurs following metabolism, e.g. incorporation of released isotope into acetate then into hormone by biosynthetic processes. This is highly unlikely for a stably labelled hormone.

Also, let E_1 = rate of excretion (combination of formation of metabolite and its renal clearance) of one particular E_2 = rate of excretion of one particular metabolite from the outer pool. Then rate of radioactivity excreted as this
rate of radioactivity excreted as this
metabolite from

$$\text{both pools} = \left\{ \frac{E_1 x}{I} + \frac{E_2 z}{Q} \right\} R$$

where R = radioactivity injected.

Therefore total radioactivity excreted as metabolite which is specifically measured and is not derived from any other hormone is given by

$$\frac{E_1 R}{I} \int_0^\infty x \, . \, dt + \frac{E_2 R}{Q} \int_0^\infty z \, . \, dt$$

$$\int_0^\infty x \, . \, dt = \frac{\theta_2 + \theta_4}{\alpha\beta} \; ; \qquad \int_0^\infty z \, . \, dt = \frac{\theta_1}{\alpha\beta}$$

then total radioactivity excreted as metabolite =

$$R \frac{\left\{ \frac{E_1 \; (\theta_2 + \theta_4)}{I} + \frac{E_2 \; \theta_1}{Q} \right\}}{\theta_1\theta_4 + \theta_3\theta_2 + \theta_3\theta_4} \text{ ——— Equation} \quad [9]$$

$$= R \left\{ \frac{E_1 + E_2}{a + e} \right\}$$

also total metabolite excreted is

$$(E_1 + E_2) \quad T$$

where T is time of urine collection during which all (98%) of the radioactivity in the form of the particular metabolite is excreted.

Then specific activity of the metabolite

$$S\,A = R \left(\frac{E_1 + E_2}{a + e} \right) \quad X \quad \frac{1}{(E_1 + E_2)\;T} \qquad [10]$$

and $\frac{R}{S\,A} = (a + e)\;T$

In the steady state, the rate of secretion of the hormone (P) will be equal to the total rate of metabolism. Then after a single injection secretion in time T = (P) = (a + e) T where T = time of urine collection and S A = specific activity of metabolite,

and steroid metabolised = steroid secreted in the non isotopic steady state.

This therefore proves that, if the body is represented by two pools with the hormone and radioactive steroid introduced into one and the same pool and metabolism occurring in one or both pools, the calculation is valid.

If the radioactive hormone is continuously infused so that the specific activity of the steroid is constant throughout the body, then providing there is no differential isotope effect $\frac{R}{SA}$ must be exactly equal to P. In practice this would be a difficult method to apply as after the continuous infusion, the amount of radioactive metabolite in the body would be unknown.

In the general case, if the hormone is secreted at rate S_1 into the inner pool and S_2 into the outer pool, then $S_1 + S_2 = P$ (total secretion rate). If no

accumulation occurs in either pool

$$S_1 - a - b + b^1 = 0$$

$$S_2 - e - b^1 + b = 0$$

and $b^1 = b + S_2 - e$, $\theta_2 = \frac{b + S_2 - e}{Q}$

Then substituting in equation [9]

$\frac{R}{SA} = (a + e)\ T \times w =$ Secretion rate (P)

$\times$

Single injection factor (w)

Where $$W = \frac{1 + \left(\frac{a}{a+e}\right)\frac{S_2}{b}}{1 + \left(\frac{E_1}{E_1+E_2}\right)\frac{S_2}{b}} \quad [11]$$

Generally the calculation $\frac{R}{SA} = P$ (i.e. $w = 1$) will be correct unless the hormone and radioactive steroid are not introduced into the same compartment and metabolism occurs in the inner and outer compartments.

Using a two compartmental model with metabolism occurring in both pools, the calculation is rigorously valid if

certain precautions are taken. However if the hormone is secreted into both compartments and the radioactive steroid is injected into only one, then the calculation may not be correct in certain circumstances as noted by Laumas, Tait and Tait (1961), although it is highly unlikely that these special circumstances would arise. The assumptions underlying this mathematical treatment must be critically examined. These are as follows:

(a) The radioactive and secreted steroid enter the same compartment and are rapidly mixed there. One criterion for this to be so is that the mean circulation time of the plasma should be shorter than the mean life of a radioactive steroid molecule following the first rapid mixing in the inner pool (mean life = $\frac{1}{K_1 + K_2}$). Studies with radiosodium have shown that the circulation time betweeen arm and foot is about fifty seconds (Low-Beer 1950). The mean life of

a radioactive progesterone molecule, as defined, is 2.13 minutes for the normal male ($\frac{1}{K_1 + K_2} = \frac{1}{676}$: mean values) and 2.87 minutes for the castrate female ($\frac{1}{K_1 + K_2} = \frac{1}{501}$: mean value) (see Results, page 114). Therefore, the requirement for rapid mixing is met for this steroid. However, this may not be the case for steroids with a much shorter metabolic half life.

(b) The amount of steroid injected must not be great enough to alter the normal transport and metabolism (i.e. to disturb the values of V_1, V_2, K_1, K_2, E_1, and E_2) of the hormone. It is therefore advisable when applying this method to use material of the highest possible specific activity for injection and to restrict the administered amount to about 1% total hormone content of the body pool.

In this research project 2 microcuries of $7H^3$ progesterone were injected. The specific activity of this radioactive steroid

was 30,000 microcuries per milligram so that the weight of steroid injected was 1/15 of a gamma. The mean total body content of progesterone is 28 micrograms for the normal males 8 micrograms for the castrate female (see Results, page 118).

(c) The injected radioactive steroid is in the same chemical form or is metabolized and transported in an identical manner to the secreted hormone. The tritiated progesterone used in this programme should comply with this.

(d) The isotope is stably attached to the hormone during the formation of the metabolite to be analyzed and the incorporation of the heavier atom into the hormone molecule does not alter its behaviour in the body.

It seems highly likely, due to the stable position of the isotope and the low mass ratio of the radioactive and natural isotope, that this requirement is satisfied for ($4-^{14}C$) steroids. Unfortunately, the

preparation of (4-^{14}C) aldosterone and certain other steroids of sufficiently high specific activity to satisfy the requirement of (b) does not seem possible at the present time. Pearlman (1957 a, b) has administered a mixture of (4-^{14}C) and (16-^{3}H) progesterone and found that the ratio of (^{14}C) to (^{3}H) was identical in the injected progesterone and excreted pregnanediol. Similarly Peterson (1959) found the $^{3}H/^{14}C$ ratio in an injected mixture of (4-^{14}C) and randomly labelled (^{3}H) corticosterone to be the same as in the excreted metabolites. Laumas, Tait & Tait (1961a) reported similar results with 16-^{3}H aldosterone and 7-^{3}H aldosterone. Thus it appears that within the limits of present experimental investigation the use of both (7-^{3}H), (16-^{3}H) and in certain conditions randomly labelled steroids, all in the form of the natural hormone, can be satisfactory for the application of the method. However, it is also clear that a judicious choice of the metabolite to be analyzed must

be made depending on the position of the isotope in the steroid molecule, e.g. it would be impossible to use the specific activity of the 17-oxosteroid metabolite after administration of (16-^{3}H) cortisol. Pregnanediol is the metabolite of progesterone isolated in this work and the $7H^{3}$ atom is not involved in the metabolism of progesterone.

(e) E_1, E_2, V_1, V_2, K_1 and K_2 should be constant (s) during the time of urine collection.

For this requirement to be met, particularly in the investigation of pathological conditions, the patient must be in a steady state, e.g. the amount of oedema or ascitic fluid should be constant. This appears to be particularly important for steroids such as progesterone, aldosterone which tend to distribute into a large body volume. If this is assured, then the major potential cause of error arising from this

assumption would appear to lie in diurnal variation. Usually it is necessary in applying the method to progesterone, for reasons to be discussed later, to collect urine for at least 96 hours. It is possible that the formation and renal clearance of a particular metabolite (E) may vary during the time of the urine collection. This could arise from the nature and specificity of the protein binding of steroids in plasma. However, tetrahydrocortisol, tetrahydrocortisone glucoronides and pregnanediol glucuronide are probably only weakly bound and hence, their renal clearances will be reasonably independent of concentration and E_1 will be constant with time as is required for the expression to be valid. It is highly unlikely that progesterone, or any metabolite, is strongly bound to plasma proteins and hence the E values are probably constant. However, there is little information on the

diurnal variation of volumes or other rate constants.

(f) The metabolite investigated is derived exclusively from the injected steroid.

It is considered that pregnanediol is, for all practical purposes exclusively derived from secreted progesterone.

(g) Finally, that all the radioactivity in the form of the metabolite analyzed is collected. The expression for radioactivity excreted as the metabolite is

$$\frac{RE_1}{I}\int_0^\infty x.dt + \frac{RE_2}{Q}\int_0^\infty z.dt$$ and this implies

the requirement stated. It is clearly sufficient, considering the other errors involved, for the time of urine collection to be long enough for about 97% of the total possible radioactivity to be excreted as the metabolite. That this has been achieved by 4 day collection of urine in this project is shown by the result of the experiments (see Results, page 94).

It has been noted that for all the assumptions examined except (a) the values for the specific activity of different metabolites will tend to disagree if the requirements are not satisfied. A comparison of these values therefore seems to be the most generally applicable method for testing the validity of the calculation of secretion rate. Pearlman (1957) and Pearlman and Contractor (1960) have produced from experiments on pregnant women utilising a single injection of $16H^3$ progesterone comparable secretion rates for progesterone calculated from pregnanediol and pregnaneolone.

The radioactive method examined here has considerable advantages in the estimation of secretion rates over the conventional methods which aim to measure as many excreted metabolites as possible to gather the same information. A knowledge of the secretion rate can be gained from a measurement on one metabolite and it is not necessary to

recover all the compound for the calculation to be valid. Generally, the measurement of the specific activity is no more difficult than the estimation of the excretion of the one particular metabolite by conventional methods; in practice it may be easier because of the presence of the radioactive indicator. The advantage of the radioactive method is most apparent when there is an alteration of the pattern of metabolism or in the rate of excretion of metabolites. In this case, for the conventional methods to give the same information, all metabolites by every route of excretion would have to be measured. It should be noted that if the radioactive hormone is given orally and the amount absorbed is estimated from total urinary excretion of radioactivity (Cope & Black 1958) the calculation of secretion rate will not be valid if the route of excretion of metabolites alter. The advantage of the radioactive method is not

particularly great in cases of renal dysfunction when the route of excretion is not disturbed. When a steady state is reached in such a condition, the renal clearance will remain unaltered and, provided accumulation of metabolites does not affect secretion, a measurement of the excretion of the major urinary metabolites will still reflect the rate of production. The radioactive method will give the same information but care must be taken to collect all the radioactivity as the metabolite analysed. The advantage of the use of the radioactive method in this condition will be to reveal the abnormality in renal clearance (see Discussion, page 131) rather than to give a better measure of the secretion rate. One potential disadvantage of the radioactive method might lie in the limitation on repeated measurements imposed by rules of radiation hygiene and also the necessity to allow for complete excretion of

radioactivity.

It should be possible to determine secretion rates using as little as 0.5 microcuries of $7H^3$ progesterone which would allow 4 serial determinations.

The potential advantages of the radioactive method are therefore quite marked for the investigation of a number of pathological conditions. However, the previous rigorous treatment and examination of assumptions reveals that the method must be applied in a judicious manner.

The preceding examination of the validity of the secretion rate method has some more general applications. A comparison of the specific activities of various metabolites can been used to test the assumption that certain excreted compounds are all derived from the same hormone. Also if the secretion rate is calculated from the specific activity of one metabolite, then the excretion of another metabolite can be

estimated from a knowledge of its radioactivity alone. (See discussion on Peak X, page 134).

APPENDIX VIII

a) Method for Estimating the Disappearance of Tritiated Progesterone in Human Blood.

b) Constant infusion experiment.
(Experimental Male Subject *XII).

a) Disappearance of Tritiated Progesterone in Human Blood.

Extraction of Progesterone and assay of radioactivity.

1. Progesterone $7H^3$ is made up in a concentration of 11 µc/1 ml. which is 2.2 µc/0.2 ml.
2. To 11 ml. of saline is added 0.2 ml. equivalent to 2.2 µc of $7H^3$ Progesterone of which 10 ml. are injected into the antecubital vein (i.e. 2.0 µc).
3. Blood samples will be taken at 2.5, 5, 7.5, 10, 15, 20, 30 minutes after injection into syringes which have been wetted with less than .5 cc. of heparin. (Dry skin surfaces after cleaning skin area with alcohol). The amount of plasma obtained by the individual withdrawals are 5, 5, 10, 10, 20, 20, 20 ml. each. (Additional 50, 70 minute samples may be necessary, requiring 40 and 100 ml. plasma respectively).

4. The blood samples are transferred to vacuum tubes and spun for 15 minutes in the International Centrifuge at 2500 rpm.
5. Aliquots of 65-75 counts per minute C^{14} Progesterone + 200 gamma cold Progesterone (previously purified through a column) are put in 50 ml. centrifuge tubes and taken to dryness.
6. An equal aliquot of C^{14} Progesterone is taken and put into a scintillator vial as experimental standard (C^{14}) it is dried down and 10 ml. of 2% ethanol P.P.O. and P.O.P.O.P. is added.
7. Measure the proper amount of plasma into the centrifuge tubes containing the internal Standard C^{14} + carrier = cold Progesterone, shake carefully and pour into 60 ml. separatory funnels.
8. Rinse each tube with 1.3 volume of Ethyl acetate, which has just previously been prepared by shaking with an excess

of sodium bicarbonate, filtered, then washed with an equal volume of distilled water and filtered again through sodium sulfate anhydrous (Na_2SO_4) to eliminate all the water.

9. Invert separatory funnel gently, recollect solutions into proper 50 ml. centrifuge tubes and centrifuge for 10 minutes at 2500 rpm.

10. Avoiding replacement of the mixture into the separatory funnels, use Pasteur pipettes to take up the upper layer of ethyl acetate into 100 ml. round bottom glasses, or 300 ml. round bottom flasks respectively. Repeat the extractions with 1.3 volume ethyl acetate three times.

11. The final total extracts, 5.2 volume each, are washed with 1/5 volume of distilled water into clean 125 ml. separatory funnels using the water for rinsing the round bottom glasses into the separatory funnels. It is not

necessary to centrifuge this step.
After removal of the lower (water) layer, bring extract back into round bottom flask and rinse separatory funnel with 1-2 ml. ethyl acetate.

12. The ethyl acetate extractions are taken to dryness in vacuum.

13. The dried extracts are transferred to the column as follows -
Take up residue in CH_3OH : $CHCl_3$ equal parts four times 2 ml. volume and transfer to 10 ml. beakers, dry down under N_2, add 300 mg. celite and mix all together carefully with the appropriate amounts of stationary phase (.15 ml.). Residual celite is washed out of beaker with less than 1 ml. of eluent phase.
The column is a 60 cm. celite column.
Stationary phase - 80% methanol.
Mobile phase - 224 trimethyl pentane.

14. 0.5 ml. of the three (five) tubes, assumed as including the peak tubes (# 11, 12, 13, 14, 15) taken to dryness under Nitrogen and transferred in .1 ml. of methanol to filter paper for a 1½ - 2 hour run in a mixture of equal parts of 90% Ligroine and Methanol. Viewed under U-V lamp and peak tube determined. (Note correction for values in these tubes).

15. Pool peak tube and one on either side into a scintillator vial. If two tubes show U-V, take the third as the next tube more polar (i.e. # 11 + 12, then take also # 13).

16. Add also the three tubes prior to the peak tubes and the three following into scintillator vials. Dry in vacuum.

17. Add 10 ml. 2% ethanol - PoPoP to each and let sit in refrigerator overnight.

18. All samples are counted for 4 hours,

i.e. 8 X ½ hours = 4 X ½ hour on tap 3 and 4 X ½ hour on tap 8.

19. The counter is then set up as follows: Preset on 100000 (10^5)

1. Machine blank
2. Reagent Blank + 10 ml. PoPoP + 2% Ethanol
3. 7H^3 injection solution = permanent standard about 3000 cts/mins.
4. C^{14} permanent standard about 3000 cts/mins.
5. C^{14} internal standard about 70 cts/mins.
6. C^{14} internal standard in column eluent.
7. Column Blank 1
8. Column Blank 11
9. Column Blank 111

20. Samples: each extraction 2½' - 30' giving 3 vials. All results must be expressed as ± error: i.e.

$$\frac{100}{\sqrt{\text{total cts of sample}}} + \frac{100}{\sqrt{\text{total cts of blank}}}$$

The tap on the machine should be set so that there are minimal counts across from C^{14} - $7H^{3}$, but C^{14} efficiency must be at least 35% and $7H^{3}$ at least 12%.

($7H^{3}$ 1/300 value at C^{14} tap
C^{14} 1/5 value at $7H^{3}$ tap)

The values for radioactivity present specifically as progesterone and fully corrected for recovery, in plasma taken at the stated time intervals after injection, are expressed as per cent injected dose per litre and are graphed semi-logarithmically against time of collections. From these graphs the transport and metabolism rate constants and volumes of distribution of progesterone are calculated. (See Appendix IX).

b) Constant Infusion Experiments.

(Experimental Male Subject *XII)

From results obtained from the previous male subjects, the metabolic clearance rate could be expected to be some 3000 litres per day and the time of equilibration (T_{EQ}) to be approximately 10 minutes with a concentration of radioactivity on the plasma (x^1) of some 1.5% of the injected dose per litre.

An initial priming injection of 0.5 microcurie of $7H^3$ progesterone was planned thus leaving 1.5 microcuries for constant infusion to be commenced at T_{EQ} (10 minutes from zero time) so that a total of 2 microcuries of tritiated progesterone would be administered.

Since $\frac{r}{x_c^1} = M = 3000$ litres per day

where r = rate of infusion of radioactive steroid.

x^1 = fraction of injected radioactivity as concentration (equal to that in plasma) at time t.

x_c^1 = The constant level of x^1 obtained during the constant infusion (see Appendix IX).

If the above constant infusion lasts for 100 minutes from zero time plus 10 minutes,

then $$\frac{r_1}{x_c} = \frac{\text{21.6 microcuries per day}}{\text{0.0075 microcuries per litre}}$$

$$= \text{2880 litres per day}$$

Therefore infusion of 1.5 microcuries of tritiated progesterone over a period of 100 minutes and commencing 10 minutes after a priming injection of 0.5 microcurie is within the theoretical requirements and should result in a constant level of radioactivity in the plasma if the metabolic clearance rate calculations are valid.

The subject for the constant infusion experiment was managed in a fashion similar to the other experimental subjects. The

procedure was commenced at 8 a.m. with the patient fasting and having emptied the bladder.

The $7H^3$ progesterone was as usual (2.2 microcuries per 0.2 ml. benzene). An initial priming injection of 0.5 microcurie was administered intravenously (0.05 ml. $7H^3$ progesterone solution to 11 ml. normal saline; 10 ml. injected via antecubital vein; zero time at 5 ml.).

The constant infusion of $7H^3$ progesterone was commenced at zero time plus 10 minutes. The infusion solution was 0.15 ml. of $7H^3$ progesterone diluted in 42.0 ml. normal saline (containing 1.65 microcurie of radioactivity). The Harvard Apparatus Infusion Machine with "pentothal" adapter (polyethylene) and 50 ml. "luer-lock" syringe was used (about 1.5 ml. residue in tubing).

The infusion solution was injected at a rate of 0.382 ml. per minute for 100

minutes.

Thus 38.2 ml. of this $7H^3$ progesterone solution was injected, this amount containing 1.5 microcuries of radioactivity ($38.2 \div 42 \times 1.55 = 1.5$).

Blood samples were withdrawn in heparinized syringes at zero time plus 10, 85, 95 and 105 minutes in sufficient volume to obtain 20 ml. plasma for each sample. These samples were extracted and assayed for radioactivity as previously described for the single injection experiments.

The content of radioactivity as progesterone was calculated for each sample making full correction for loss by the method. Where in the single injection experiments this was calculated as per cent injected dose per litre of plasma, for the constant infusion experiment the results were calculated as micromicrocuries per litre of plasma. (See Figure 19, page 115).

APPENDIX IX

Theory of Calculating Metabolism
Rate Constants and volumes of
Distribution of Progesterone.

The study of the disappearance of the radioactivity in plasma after the injection of labelled progesterone should elucidate the transport and metabolism of this steroid in man. In particular this would allow the calculation of the metabolic clearance rate in various clinical conditions. This value, together with a concomitant determination of the secretion rate estimated from the specific activity of a urinary metabolite, would enable the mean plasma concentration of progesterone to be calculated. This was particularly important as practical direct methods had yet to be reported for the analysis of progesterone in peripheral blood in such low concentration as is present in males and castrate females.

The values for the radioactivity present specifically as progesterone and fully corrected for recovery, in plasma taken 2½, 5, 5½, 7, 10, 15, 20 and 30 minutes after the intravenous injection of 2

microcuries of $7H_3$ progesterone are obtained. They are plotted as logarithm of per cent dose injected per litre of plasma against time after injection. An example of the disappearance curve for experimental subject *VII is shown in Figure 18, page 111. Figure 41 shows some hypothetical radioactive concentrations in plasma and tissue at various times after injection. The initial rapid drop in plasma radioactive concentration is considered to be the result of the equilibration of the radioactivity throughout the body. After this the curve becomes less steep and is indicative of the metabolic clearance of the labelled steroid.

From the characteristics of these disappearance curves the concentration of radioactivity in plasma as a function of time may be expressed as

$$x^1 = Ae^{-\alpha T} + Be^{-\beta T}$$

where x^1 = per cent injected radioactive dose

Model for disappearance of Plasma Radioactivity.

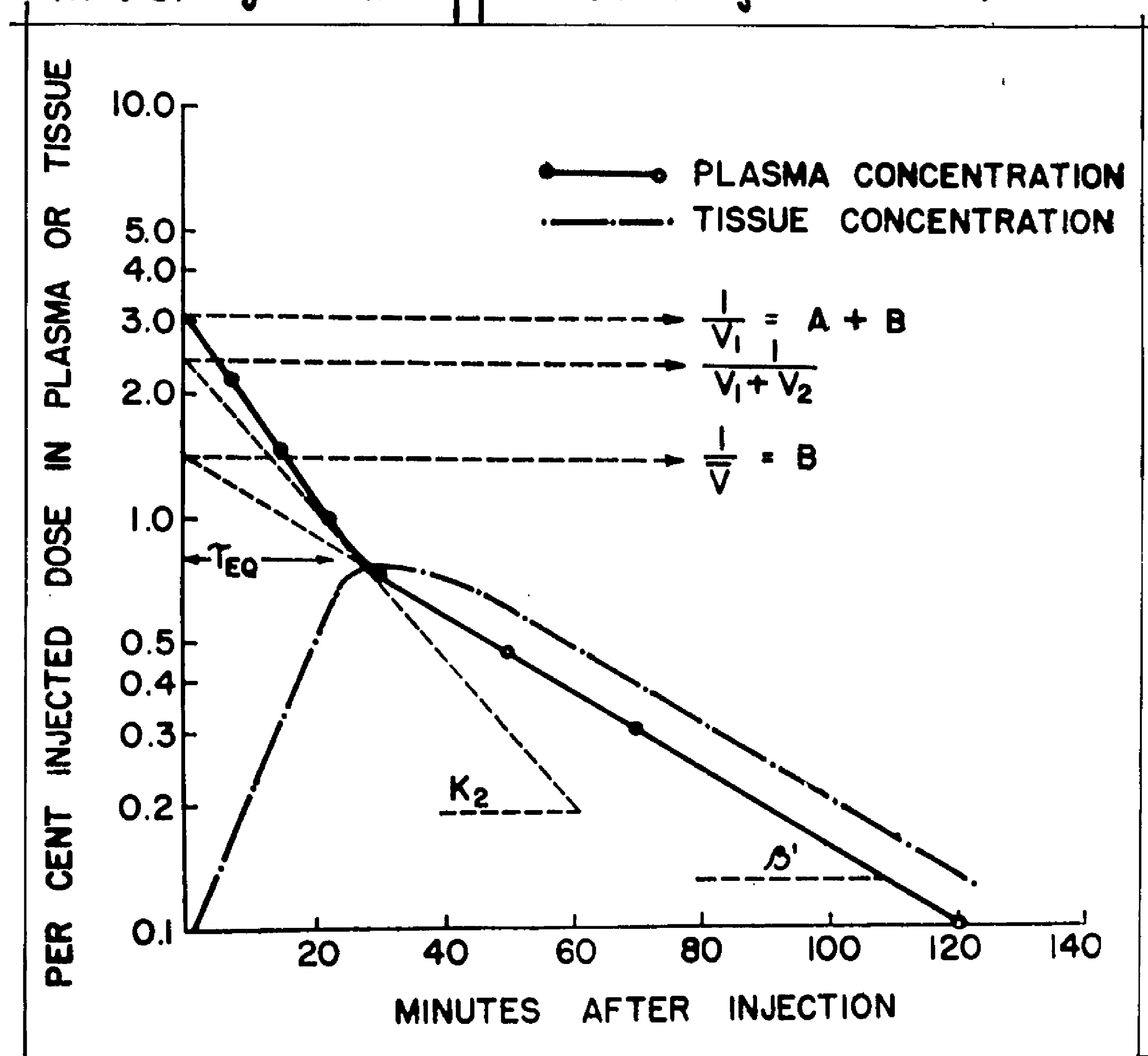


FIG. 41.

per litre of plasma as progesterone at time T.

B is the intercept on the ordinate of the extrapolated part of the curve (semilog plot, Figure 41). A + B is the corresponding intercept from the earlier part of the curve. β is the slope of the final part of the curve. $\beta = 1000/T_{\frac{1}{2}}$ (min) (units per day). Alpha is calculated as follows: the latter part of the curve is extrapolated (intercepting the ordinate at B(Figure 41), and the resulting calculated plasma concentrations subtracted from the measured values. These corrected concentrations are plotted semilogarithmically and the slope is α.

Discussion.

Theoretical. A reasonable model for the transport and metabolism of a steroid in man consists of an inner pool which, for steroids weakly bound to plasma proteins, probably includes the plasma and extracellular volumes, and an outer pool (Figure 40, page 311). Transport between the spaces can be

described by a rate constant K_1. If the liver volume is relatively small, and transport from plasma to metabolic cells proceeds at a reasonable rate, it can be considered as part of the inner pool as regards metabolic events. However, any reversible spread of steroid into liver tissue after the first few minutes after injection would be considered as transport into the outer pool. This seems reasonable in view of the fact that molecules much larger than steroids such as albumin are metabolized in the liver and must be transported into the cells. The over-all metabolism of this pool, total volume V_1, can be described by the rate constant K_2. The total volume of the outer pool is V_2. In the present treatment, metabolism in the outer pool is taken as being negligible. This must be assumed, because analyses in the outer pool are at present impossible to

obtain and otherwise, solutions of any mathematical treatment would be indeterminate. However, the liver is efficient in metabolizing progesterone (Samuels & Weist 1952). Therefore, this assumption is probably reasonable as a first approach for this hormone. If all radioactivity is measured as progesterone, then: K_1 = fraction of injected dose per milliliter of plasma transported to the outer pool in unit time; K_2 = fraction of injected dose per milliliter of plasma metabolized in the inner pool per unit time; x^1 = fraction of injected dose per milliliter of plasma at time T; z^1 = fraction of injected dose per milliliter of outer pool at time T.

$$V_1 \frac{dx^1}{dT} = - V_1 K_1 x^1 - V_1 K_2 x^1 + V_1 K_1 z^1$$

$$V_2 \frac{dz^1}{dT} = V_1 K_1 x^1 - V_1 K_1 z^1$$

$$\text{and } x^1 = Ae^{-\alpha T} + e^{-\beta T}$$

$$z^1 = D\,(e^{-\alpha T} - e^{-\beta T})$$

where $A = \left\{ \dfrac{\dfrac{K_1 V_1}{V_2} - \alpha}{\beta - \alpha} \right\} \times \dfrac{1}{V_1}$;

$$B = \left\{ \dfrac{\beta - \dfrac{K_1 V_1}{V_2}}{\beta - \alpha} \right\} \times \dfrac{1}{V_1}$$

and $D = \left\{ \dfrac{K_1}{V_2} \right\} \times \dfrac{1}{\beta - \alpha}$

$$\frac{K_1 V_1 \times K_2}{V_2} = \alpha\beta \; ;$$

$$K_1 + K_2 + \frac{K_1 V_1}{V_2} = \alpha + \beta$$

and $K_2 = \dfrac{\alpha\beta (A + B)}{A\beta + B\alpha}$;

$$V_1 + V_2 = \frac{\beta^2 A + B\alpha^2}{(A\beta + B\alpha)^2}$$

$$K_1 = V_2 \; (A\beta + B\alpha).$$

Figure 41 shows some hypothetical

radioactive concentrations in plasma and tissue at various times after injection. As has been pointed out by several investigators in other fields, Campbell, Cuthbertson, Matthews & McFarlane (1956), Dominguez (1956), Skinner, Clark, Baker & Shipley (1959), Veall and Vetter (1958), Zilversmit, Enterman & Fishler (1943), the radioactive concentration in the outer pool is lower than that in the inner pool for some time after injection. These concentrations then become equal when that in the outer pool is maximal (Zilversmit et al 1943). From this time on, the radioactive concentration in the outer pool is greater than in the inner. The final slope of the plasma concentration (β) is a result of a combination of the effects of metabolism and transport. It will be a flatter slope than would be expected from the effects of metabolism alone ($\beta < K_2$) because continuous transport occurs from the outer pool into the inner

pool. The slope of the curve will only be equal to K_2 when the outer and inner radioactive concentrations are equal (at time T_{Eq}). Similarly, the intercept on the ordinate from the extrapolated later curve (B) gives a volume ($\bar{V} = 1/B$) which is not the true volume of distribution. This volume multiplied by the radioactive concentration in the plasma will give the total radioactivity as the hormone at any particular time. However, the radioactive concentration in the outer pool is greater than in the inner, therefore the volume $\bar{V}$ is greater than the more meaningful volume of distribution ($V_1 + V_2$). This volume ($V_1 + V_2$) multiplied by the blood concentration of steroid will be equal to the total body content of nonisotopic hormone. $V_1 + V_2$ can be calculated from the expressions given. It is also the reciprocal of the intercept on the ordinate obtained by extrapolating, using the slope K_2, from the radioactive

concentration at the equilibrium time as shown in Figure 41 (page 346).

As has been stated, the slope (β) of the later part of the curve for the disappearance of $7H^3$ progesterone does not represent metabolism alone. A more valid method of comparison of the metabolism of steroid would be similar to that adopted by Pearlman (1957a). Thus Secretion Rate (P) = M x i where M is the metabolic clearance rate of the plasma and i is the plasma concentration (See Materials and Methods, page 47) and $M = V_1 \times K_2$. The metabolic clearance rate (M) seems a reasonable measure of the over-all metabolism of a steroid for the purposes of comparison.

The M value is dependent upon whether a single or double-compartment model is used for the calculation. If M_1 is the value for the single-compartment model and M is the value for the two-compartment model, then

$$M_1 = \beta \times \bar{V} = \frac{\beta}{B}$$

$$M = V_1 K_2 = \frac{\alpha\beta (A + B)}{A\beta + B\alpha} \times \frac{1}{A + B}$$

$$M = \frac{\alpha\beta}{A\beta + B\alpha} = \frac{\beta}{B} \times \frac{1}{1 + \frac{A}{B} \times \frac{\beta}{\alpha}}$$

$$M = M_1 \times \frac{1}{1 + \frac{A}{B} \times \frac{\beta}{\alpha}}$$

A knowledge of the metabolic clearance rate can be used to calculate the mean plasma concentration from the secretion rate of the hormone obtained from the specific activity of a urinary metabolite.

$$P = M \times i$$

$$i = \frac{P}{M}$$

However, although the two-compartment model has the advantage of giving determinate values for the volumes of distribution and also for K_1 and K_2, it is entirely possible

that a more complex model is appropriate. It could be that there are additional compartments (such as the cerebrospinal fluid) into which the steroid slowly diffuses. In this case, the curve would change in slope with time even after 50 minutes. There is no evidence that this occurs from the data obtained but because of the low plasma radioactive concentrations it is impossible to test this critically. If such diffusion did occur, the calculated K_2 values and turnover rate would be too high. Also, if metabolism occurred in the outer pool, the turnover rate calculated from the single-injection data would again be inaccurate, and the β value would more truly reflect the over-all metabolic processes.

The validity of the calculations for a two-compartment system have been tested independently by the results observed from a constant infusion experiment. After the initial priming dose, steroid has been

continuously infused. The radioactivity in the plasma measured specifically as the steroid remained constant. (See Appendix VIII).

Considering x_c^1 = the final constant radioactivity of the steroid per liter of plasma and r = rate of infusion of radioactivity as steroid per day, the

$$\frac{r}{x_c^1} = \text{metabolic clearance rate M.}$$

During the constant infusion of the radioactive steroid for 100 minutes after a priming dose, the radioactivity was found to be constant within the limits of experimental error.

It therefore appears that the value of the metabolic clearance rate calculated from the data obtained after a single injection of radioactive hormone

$$M_2 = V_1 K_2 = \frac{\alpha\beta}{A\beta + B\alpha}$$

is valid (See Materials and Methods, pages 49-58). It should now be possible by using

the methods described here to compare the
mean concentrations and over-all rates of
metabolism of progesterone in various clinical
conditions.

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PROGESTERONE SECRETION AND METABOLISM IN THE HUMAN MALE AND CASTRATE FEMALE.

by

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VOLUME II

DETAILED RESULTS.

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Abbreviations used in Enumerating Results.

AB : Acid blank.

BC : Background Correction.

BE : Benzene Extract.

CCC : Corrected for counts carried over when counting at two different HVT with mixture of isotopes.

c.p.m. : Counts per minute.

E.A.S. : Experimental standard Pregnanediol diacetate sample in gamma.

EB : Experimental blank.

EE : Ether extract.

EIS : Experimental Injection Solution.

ES : Experimental Standard.

ESP : Experimental standard pregnanediol standard sample in gamma.

ETS : Experimental standard pregnanetriol standard sample in gamma.

H^3IS : H^3 internal standard (Tritiated pregnanediol).

HVT : High Voltage tap. (Spectrometry).

MB : Machine Blank. (Spectrometry).

$MC^{14}S$: Machine C^{14} Standard (Spectrometry).

MH^3S : Machine H^3 standard (Spectrometry).

Pd. : Pregnanediol.

Pd.g. : Tube content of Pregnanediol in gamma.

QF : Quenching factor = Mean TBC ÷ Mean H^3IS.

SA : Specific Activity - counts per minute per milligram.

SB : Scintillation blank.

SD : Standard Deviation.

SD±% : Coefficient of variation.

TBC : Total background correction = cpm urine plus internal standard minus cpm urine.

UB : Urine blank.

Peak X : Peak of radioactivity in tubes following column front.

Remnant : Steroid (measured by weight or radioactivity) in washings from container from which extract had been put on a chromatographic column.

Gamma : Micrograms.

$<$: Less than.

$>$: More than.

μ : Microns.

APPENDIX X.

DETAILED RESULTS.

A. METHOD PROOF.

B. PROOF OF PURITY OF PREGNANEDIOL
RECOVERED BY METHOD.

C. EXPERIMENTAL SUBJECTS.

RESULTS.

A. METHOD PROOF.

a) Column Recovery of Pregnanediol.

b) Column Recovery of Pregnanetriol.

c) Column Recovery of H^3 Pregnanediol.

d) Recovery of H^3 Pregnanediol internal standard from urine.

e) Recovery of Pregnane-3α, 17α, 20α-triol from female urine.

a) Column Recovery of Pregnanediol.

To determine the efficiency of the chromatographic column for recovery of 5β-pregnane-$3\alpha, 20\alpha$-diol and the tubes in which it is recovered. 50 gamma pregnanediol put on column on Experiments 1-4, 100 gamma in Experiment 5. Column front approximately tube 5. Tubes 1-4 used to make experimental blank and pregnanediol standards. Total contents of tubes dried down in Experiments 1-4, 2.0 ml. aliquots taken from tubes in Experiment 5.

Experiment 1.

Column I. Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
EB	.003.	.003	.001	0	100% Reference.
25 ESP	.126	.220	.138	.087	
50 ESP	.217	.378	.227	.156	
100 ESP	.522	.944	.560	.403	25 gamma = .092.
Tube 6	.050	.033	.022	-	
7	0	0	0	0	
8	.005	.005	.002	.003	
9	.028	.024	.020	0	
10	.024	.026	.019	.005	
11	.105	.172	.109	.065)	39.2 gamma = 78.4%
12	.109	.190	.112	.079)	
13	.985	.888	.825	-	
14	0	0	0	0	
15	.006	.009	.006	.003	
16	0	0	0	0	
17	.025	.025	.025	0	
AB	.000	.000	.000	.000	

Experiment 2.

Column I. Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
EB	.004	.005	.006	0	100% Reference.
25 ESP	.144	.240	.145	.096	
50 ESP	.276	.463	.284	.183	
100 ESP	.576	.950	.589	.367	25 gamma = .092
Tube 6	.013	.010	.006	.001	
7	0	0	0	-	
8	.014	.010	.008	-	
9	.016	.010	.005	0	
10	0	0	0	-	
11	.002	0	.002	-	
12	.018	.029	.015	.012)	
13	.123	.201	.128	.075)	39.4 gamma = 78.8%
14	.087	.140	.087	.053)	
15	.020	.034	.019	.014)	
16	.004	.006	.006	-	
17	.002	.004	.006	0	
AB	.000	.000	.000	.000	

Experiment 3.

Column I. Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol Gamma
EB	.000	.002	.002	0	100% Reference.
25 ESP	.089	.158	.093	.067	
25 ESP	.091	.155	.090	.065	
50 ESP	.197	.350	.203	.150	
50 ESP	.198	.355	.206	.153	
100 ESP	.405	.753	.422	.340	
100 ESP	.440	.752	.412	.326	25 gamma = .079
Tube 10	.197	.160	.133	-	
11	.025	.022	.013	-	
12	.128	.190	.118	.067)	39.3 gamma = 78.6
13	.097	.152	.093	.057)	
14	.033	.030	.029	-	
15	.204	.160	.136	-	
16	.022	.017	.015	-	
AB	.000	.000	.000	.000	

Experiment 4.

Column II. Sample.	Wavelength Microns 390	425	460	Allen Correction	Pregnanediol Gamma.
EB	.001	.003	.002	.001	100% Reference.
25 ESP	.118	.188	.120	.069	
25 ESP	.153	.250	.153	.097	
50 ESP	.234	.413	.252	.170	
50 ESP	.290	.474	.290	.184	
100 ESP	.528	.911	.536	.379	
100 ESP	.510	.882	.520	.367	25 gamma = .090
Tube 7	.006	.001	.007	-	
8	.004	.010	.008	.004	
9	.012	.005	.015	-	
10	.002	.016	.023	-	
11	.000	.001	.003	-	
12	.059	.110	.069	.054)	41.4 = 82.8%
13	.192	.285	.188	.095)	
14	.045	.050	.053	-	
15	.009	.013	.013	-	
16	.030	.025	.008	-	
AB	.000	.000	.000	.000	

Experiment 5.

Column I. Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol Gamma.
EB	.002	.001	.001	.000	100% Reference.
25 ESP	.086	.158	.082	.074	
25 ESP	.080	.156	.076	.078	
25 ESP	.138	.218	.132	.073	
50 ESP	.216	.345	.201	.136	
50 ESP	.240	.376	.210	.151	
50 ESP	.272	.426	.264	.158	25 gamma = .0744
Tube 10	.005	.005	.003	-	
11	.057	.073	.039	.024)	30.9 = 77.3%
12	.140	.195	.114	.068)	
13	.022	.020	.008	-	
14	.026	.010	.117	-	
AB	.000	.000	.000	.000	

(2 ml. aliquots from tubes).

b) Column Recovery of Pregnanetriol.

To determine the efficiency of the chromatographic column for recovery of pregnanetriol and the tubes in which it is recovered. 50 gamma pregnane 3-17α -20 triol put on column as Experiments 1 and 2, 100 gamma pregnane 3, 17, 20 triol on Experiment 3, and 100 gamma pregnane-3α,17α,20α -triol in Experiments 4 and 5.

Column front approximately tube 5. Tubes 1-4 used to make experimental blank and pregnanediol standards.

Total contents of tubes dried down.

Experiment 1.

Column I. Sample.	Wavelength Microns. 410	440	470	Allen Correction	Pregnanetriol Gamma.
25 ETS	.215	.310	.073	.166	
25 ETS	.209	.320	.078	.176	
50 ETS	.427	.631	.153	.341	
50 ETS	.412	.628	.155	.344	
100 ETS	.840	1.29	.315	.712	25 gamma = .174
Tube 20	.003	.004	.003	.001	
21	.006	.006	.003	.001	
22	.000	.004	.003	.002	
23	.002	.005	.003	.002	
24	.007	.029	.009	.011)	
25	.030	.045	.013	.024)	
26	.045	.062	.026	.027)	
27	.023	.043	.010	.027)	
28	.020	.050	.010	.035)	36.2 = 72.4%
29	.067	.094	.040	.041)	
30	.032	.051	.012	.029)	
31	.047	.058	.034	.018)	
32	.042	.045	.035	.006)	
33	.017	.021	.005	.010)	
34	.048	.070	.043	.024)	
35	.060	.043	.028	—	
36	.051	.054	.047	.005	
37	.037	.038	.029	.005	
EB	.001	.001	.000	.000	100% reference.
AB	.000	.000	.000	.000	

Experiment 2.

Column II. Sample.	Wavelength Microns. 410	440	470	Allen Correction	Pregnanetriol gamma.
25 ETS	.205	.316	.094	.167	
25 ETS	.184	.330	.115	.181	
50 ETS	.330	.538	.133	.307	
50 ETS	.493	.704	.281	.317	
100 ETS	.930	1.41	.327	.757	
100 ETS	.812	1.310	.339	.735	25 gamma = .176
Tube 22	.038	.024	.021		
23	.031	.028	.026	.000	
24	.036	.009	.000	–	
25	.008	.004	.000	–	
26	.009	.013	.005	.006	
27	.015	.030	.002	.021	
28	.043	.100	.034	.061	
29	.031	.067	.001	.051	
30	.034	.057	.004	.038	
31	.033	.045	.012	.022	38.9 = 77.8%
32	.033	.038	.023	.010	
33	.035	.041	.005	.021	
34	.038	.025	.007	–	
35	.031	.041	.000	.025	
36	.032	.037	.008	.017	
37	.023	.026	.011	.009	
38	.028	.010	.005	–	
AB	.000	.000	.000	.000	
EB	.002	.002	.001	.000	100% Reference.

Experiment 3.

Column I. Sample.	Wavelength Microns. 410	440	470	Allen Correction	Pregnanetriol Gamma.
EB	.154	.136	.116	.001	100% Reference
20 ETS	.070	.095	.026	.047	
40 ETS	.133	.202	.056	.107	
20 ETS	.058	.085	.018	.047	
40 ETS	.127	.170	.051	.081	
20 ETS	.057	.083	.021	.044	
40 ETS	.113	.160	.043	.082	20 gamma = .045
Tube 24	.067	.055	.048	-	
25	.068	.054	.049	-	
26	.049	.037	.030	-	
27	.085	.081	.045	-	
28	.138	.153	.073	.048)	
29	.126	.158	.063	.064)	66.2 = 66.2%
30	.097	.110	.050	.037)	
31	.060	.054	.031	-	
32	.053	.042	.030	-	
33	.060	.043	.033	-	
34	.074	.062	.046	-	
35	.143	.109	.079	-	
AB	.000	.000	.000	.000	

Experiment 4.

Column I Sample.	Wavelength Microns. 410	440	470	Allen Correction	Pregnanetriol Gamma.
EB	.004	.005	.006	.000	100% Reference
25 ETS	.045	.075	.017	.044	25 gamma = .0443
50 ETS	.099	.158	.039	.089	
Tube 31	.042	.042	.029	-	
32	.089	.103	.038	.039	
33	.093	.126	.048	.050	74.5 gamma = 74.5%
34	.076	.108	.045	.046	
35	.053	.071	.029	.030	
36	.042	.042	.026	-	
AB	.000	.000	.000	.000	

Experiment 5.

Column II. Sample.	Wavelength Microns. 410	440	470	Allen Correction	Pregnanetriol Gamma.
EB	.006	.006	.003	.000	100% Reference
25 ETS	.060	.089	.028	.045	25 gamma = .0440
50 ETS	.109	.168	.052	.087	
Tube 34	.048	.041	.036	-	
35	.046	.046	.032	-	
36	.058	.074	.036	.027)	
37	.074	.096	.036	.041)	66.4 = 66.4%
38	.078	.106	.038	.048)	
39	.070	.077	.024	.030)	
AB	.000	.000	.000	.000	

c) Column recovery of tritiated pregnanediol.

A known amount of Radioactive pregnanediol added
c) Column recovery of tritiated pregnanediol.
to column -

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml.

Scintillation fluid.

Experimental Blank - Tube 1

Counting: High voltage tap 7

Time: in minutes.

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental Blank - from tube 1

Experimental standards : 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1 and 2.

Experiment 1.

100 gamma pregnanediol and 26300 cpm H^3 pregnanediol added to Column I.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
1.0 ml. H^3IS	10001	0.38	26340	26302		
1.0 ml. H^3IS	10001	0.38	26340	26302		
MH_3S	10000	.067	14800			
SB	382	10	38			
EB	494	10	49			
Tube 11	10000	13.67	732	683	7.6)	78.7%
12	10000	8.08	1238	1189	12.7)	
13	7416	30	247	198	3.7)	

Column I Sample.	Wavelength Microns. 390	425	460	Allen Correction	Recovery.
25 ESP	.114	.193	.120	.076	25 gamma = .076
50 ESP	.332	.470	.293	.153	
AB	0	0	0		
EB	.011	.011	.008	.001	100% Reference.
Tube 11	.015	.013	.009	-	
12	.055	.090	.054	.035)	
13	.110	.160	.094	.058)	80.5 gamma = 80.5%
14	.022	.024	.016	.005)	

Experiment 2.

100 gamma pregnanediol and 26300 cpm H^3 pregnanediol added to Column II.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
1.0 ml. H^3IS	10001	0.38	26340	26302		
1.0 ml. H^3IS	10001	0.38	26340	26302		
MH_3S	10000	0.67	14800			
SB	382	10	38			
EB	530	10	53			
Tube 12	10000	26.70	374	321	4.3	
13	10000	6.21	1610	1557	16.3	81.7%
14	9740	30	325	272	4.1	

Column II. Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Recovery.
25 ESP	.123	.193	.129	.069	
50 ESP	.208	.353	.194	.152	25 gamma = 0.76
25 ESP	.160	.228	.136	.077	
50 ESP	.363	.502	.342	.158	
AB	0	0	0		
EB	.015	.028	.018	.009	100% Reference.
Tube 11	.010	.000	.000	–	
12	.089	.091	.063	.015	
13	.204	.353	.160	.071	79 gamma = 79%
14	.081	.084	.067	.010	

Experiment 3.

100 gamma pregnanediol and 21500 cpm H^3 pregnanediol added to Column III.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
MH^3S	74461	5	14892			
0.5 ml. H^3IS	53148	5	10630	10601)	21500 cpm	
0.5 ml. H^3IS	54605	5	10920	10891)	added.	
EB	866	30	29			
SB	58	2	29			
Tube 9	81	2	40	9		
10	65	2	32	1		
11	78	2	39	8		
12	1442	30	48	19		
13	10693	20	535	506	5.3)	79.5%
14	12268	10	1227	1198	11.0	
15	3556	30	119	90		
16	85	2	42	12		

Sample.	Wavelength Microns. 390	425	460	Allen Correction	Recovery.
EB	.019	.015	.013	000	100% Reference.
25 ESP	.266	.322	.204	.087	25 gamma = .086
50 ESP	.432	.596	.416	.172	
Tube 12	.018	.018	.014	-	
13	.138	.170	.114	.044)	32.56 = 81.4%
14	.152	.207	.126	.068)	gamma.
15	.018	.018	.014	-	

Experiment 4.

50 gamma pregnanediol and 21900 cpm H^3 pregnanediol added to Column IV.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
0.5 ml. H^3IS	109504	10	10950	10912)	21900 cpm	
0.5 ml. H^3IS	109899	10	10990	10952)	added	
SB	945	25	38			
EB	960	25	38			
Tube 7	37	1	37			
8	7891	25	315	277	3.8)	86.8%
9	41595	25	1663	1625	8.3)	
10	2780	25	113	75		
11	1010	25	40	2		

Sample	Wavelength Microns. 390	425	460	Allen Correction.	Recovery.
AB	0	0	0	0	
EB	.004	.004	.004	0	100% Reference.
ESP 25	.132	.199	.119	.074	25 gamma = .076
50 ESP	.249	.395	.235	.153	
25 ESP	.158	.231	.140	.082	
50 ESP	.249	.388	.229	.149	
Tube 7	.018	.018	.014	.002	
8	.027	.033	.023	.008)	39.5 gamma = 79%
9	.103	.131	.080	.040)	
10	.009	.010	.008	.002	
11	.039	.034	.031	.000	
12	.041	.031	.024	.000	

d) Recovery of H^3 pregnanediol internal standard from urine.

Experiment 1.   Column II.

Female Urine: Collected during a phase of amenorrhoea.

860 ml. output per day.

500 ml. extracted by method 3 b after 21000 cpm H^3 pregnanediol added.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Front : tube 5.

Counting: High voltage tap 7.

Time: In minutes.

Spectrophotometry:

Sample: 1.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental standards : 25 and 50 gamma standard pregnanediol plus 1.0 ml. from tube 1 and 2.

Experiment 1. Column II.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Total.
MH^3S	26660	2	13330			
SB	864	30	29			
EB	944	30	31			
Tube 12	1435	30	48	17		170
13	27073	30	902	871	5.5	8710) 64%
14	15204	30	507	476	4.2	4760)recovery.
15	2941	30	98	67		670
1.0 ml. H^3IS	210642	10)	21034	21004 cpm. added.		
1.0 ml. H^3IS	210038	10)				

Experiment 1. Column II.

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
EB	.032	.033	.032	0	100% Reference.
AB	0	0	0	0	
25 ESP	.086	.158	.082	.074	
50 ESP	.216	.345	.201	.136	
25 ESP	.080	.156	.076	.078	
50 ESP	.240	.376	.210	.151	
25 ESP	.138	.218	.132	.073	
50 ESP	.272	.426	.264	.158	25 gamma = .0744
Tube 12	.124	.095	.073	-	
13	.230	.298	.190	.088	29.6
14	.171	.188	.125	.040	13.4
15	.112	.095	.072	-	

Daily output of Pregnanediol = 0.578 mgm. (corrected for recovery).

Experiment 2. Column I.

Male Urine: (Same subject for Experiments 2, 3, 4 and 5).

1200 ml. output per day.

500 ml. extracted by method 3 (b) after

21500 cpm. H^3 pregnanediol added.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml.

Scintillation fluid.

Experimental blank : from tube 1.

Front : tube 5.

Counting: High voltage tap 7.

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental standards: 25 and 50 gamma

standard pregnanediol plus 2.0 ml. from

tubes 1 and 2.

Experiment 2. Column I.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	
MH^3S	27634	2	13717			
MB	52	2	26			
SB	146	5	29			
EB	871	30	29			
0.5 ml. H^3IS	53148	5	10630	10604	} 21,500 cpm added.	
0.5 ml. H^3	54605	5	10920	10894	}	
Tube 10	70	2	35	6		
11	80	2	40	11		
12	11736	20	587	558	5.5	} 68.9% recovery.
13	9534	10	953	924	9.8	}
14	6678	30	223	194		
15	1436	30	48	29		
Remnant	267	2	133	104		
0.1 ml. B.E.	1311	20	66	37	4.1	

Benzene Extract (B.E.) = 49 ml. = 18.30 cpm = 84.3% recovery.

Experiment 2. Column I.

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	.000	.000	.000	.000	
EB	.014	.013	.008	.002	100% Reference.
25 ESP	.174	.233	.147	.073	
50 ESP	.276	.430	.265	.160	25 gamma = .078
Tube 12	.219	.226	.165	.034)	30.8
13	.365	.385	.281	.062)	
14	.196	.176	.136	-	
15	.258	.237	.147	-	

Daily output of pregnanediol = 0.1848 mgm. = 0.2682 mgms. (corrected).

Experiment 3. Column II.

Male Urine:

770 ml. output per day

500 ml. extracted by method 3 (b)

after 21340 cpm H^3 pregnanediol added.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml.

Scintillation fluid.

Experimental Blank: from tube 1.

Front: tube 5.

Counting: High voltage tap 7.

Time: in minutes.

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental standards: 25 and 50 gamma standard pregnanediol plus 2.0 ml. from tubes 2 and 3.

Experiment 3. Column II.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
MH^3S	26969	2	13485			
1.0 ml. H^3IS	108504	5	21700	21670)		21340 cpm added.
1.0 ml. H^3IS	105503	5	21100	21070)		
SB	154	5	31			
EB	169	5	34			
Tube 11	54	2	27			
12	5989	10	599	565	8.1)	1495 = 70.1%
13	9643	10	964	930	10.1)	
14	124	2	62	28		
B.E. (0.1 ml.)	703	10	70	40	2.8	

Benzene Extract (B.E.) = 48.5 ml. = 19400 cpm = 90.9% recovery.

Experiment 3. Column II.

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.022	.018	.015	.000	100% Reference.
25 ESP	.201	.278	.194	.082	25 gamma = .079
50 ESP	.326	.475	.313	.155	
50 ESP	.301	.451	.283	.159	
Tube 11	.226	.165	.126	-	
12	.233	.248	.181	.041)	29.95
13	.371	.380	.283	.053)	
14	.256	.230	.174	-	

Daily output of Pregnanediol = 0.1145 mg. = 0.1635 mgm. (Corrected).

Experiment 4. Column IV.

Male Urine:

890 ml. output per day

500 ml. extracted by method 3 (b)

after 25800 cpm. H^3 pregnanediol added.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml.

Scintillation fluid.

Experimental Blank: from tube 1.

Front: tube 5.

Counting: High voltage tap 7.

Time: in minutes.

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental standards: 25 and 50 gamma

standard pregnanediol plus 2.0 ml. from

tubes 2 and 3.

Experiment 4. Column IV.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
1.0 ml. H^3IS	10000	3.87	25840	25802		
1.0 ml. H^3IS	10000	3.89	25840	25802		
MH^3S	140019	10	14002			
SB	382	10	38			
EB	492	10	49			
Tube 11	1019	10	102	52		
12	40000	80	1250	1201	4.6)	61.3%
13	4296	10	430	381	7)	
Remnant	200	1	200	162		
B.E. 0.1 ml.	4981	60	83	45	2.3	

Benzene Extract (B.E.) = 48.0 ml. = 21600 cpm = 83.5%

Experiment 4. Column IV.

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	0	0	0		
EB	.003	.003	.004	.000	100% Reference.
25 ESP	.070	.159	.082	.083	
50 ESP	.238	.414	.235	.178	
25 ESP	.123	.201	.111	.084	
50 ESP	.215	.386	.205	.176	25 gamma = .087
Tube 11	.199	.163	.144	–	
12	.275	.317	.214	.073)	26.7
13	.392	.405	.378	.020)	
14	.964	.810	.590		

Daily output of pregnanediol = 0.1188 mgm. = 0.1938 cpm (corrected).

Experiment 5. Column I.

Male Urine:

870 ml. output per day.

500 ml. extracted by method 3 (b)

after 25800 cpm H^3 pregnanediol added.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml.

Scintillation fluid.

Experimental Blank: from tube 1.

Front: tube 5.

Counting: High voltage tap 7.

Time: in minutes.

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental standards: 25 and 50 gamma

standard pregnanediol plus 2.0 ml. from

tubes 2 and 3.

Experiment 5. Column I.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	B.C.	SD±	Recovery.
1.0 ml. H^3IS	10000	3.87	25840	25802		
1.0 ml. H^3IS	10000	3.87	25840	25802		
MH^3S	140019	10	14002			
SB	382	10	38			
EB	530	10	53			
Tube 11	4335	10	433	380	7)	
12	10000	8.32	1201	1148	4.4)	70.3%
13	3400	10	340	287	6.6)	
Remnant	166	1	166	128		
B.E. 0.1 ml.	4979	60	83	45	2.3	

Benzene Extract (B.E.) = 48.0 ml. = 21600 cpm = 83.5%

Experiment 5. Column I.

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol gamma.
AB	.000	.000	.000	.000	
EB	.000	.000	.000	.000	100% Reference.
25 ESP	.092	.188	.108	.084	
50 ESP	.231	.406	.233	.176	25 gamma = .087
Tube 12	.225	.236	.217	.016)	
13	.382	.410	.282	.078)	30.0
14	.200	.215	.191	.010)	

Daily output of pregnanediol = 0.1305 mg. = 0.1842 mgm. (corrected).

e) Recovery of Pregnane-3α,17α,20α-triol from female urine.

Urine and Column as for Experiment 1.

Spectrophotometry.

Sample: Total content of tubes dried down. Experimental blank : Tube 3. Experimental Standards : 20 and 40 gamma pregnane-3α,17α,20α-triol plus 5 ml. mobile phase.

Spectrophotometry:

Column II. Sample.	Wavelength Microns. 410	440	470	Allen Correction.	Pregnanetriol gamma.
EB	.134	.126	.106	.001	
AB	.000	.000	.000	.000	
20 ETS	.070	.095	.026	.047	
40 ETS	.133	.202	.056	.107	
20 ETS	.058	.085	.018	.047	
40 ETS	.127	.170	.051	.081	
20 ETS	.057	.083	.021	.044	
40 ETS	.113	.160	.043	.082	20 gamma = .045
Tube 24	.413	.378	.305	-	
25	.443	.398	.319	-	
26	.480	.404	.337	-	
27	.383	.308	.251	-	
28	.335	.275	.229	-	
29	.327	.274	.220	-	
30	.260	.214	.165	-	
31	.355	.294	.210	-	
32	.403	.304	.242	-	
33	.496	.367	.274	-	
34	.590	.600	.332	.139)	530
35	1.225	1.417	.503	.553)	
36	.506	.396	.309	-	

Daily output of pregnanetriol (corrected for pregnanediol recovery rate) (64%) = 0.825 mgm.

RESULTS.

B. PROOF OF PURITY OF PREGNANEDIOL RECOVERED BY THE METHOD.

a) Absorption spectra of pregnanediol.

b) Formation of pregnanediol diacetate.

c) Counter current distribution of pregnanediol diacetate.

d) Infrared analysis of pregnanediol and pregnanediol diacetate.

a) <u>Absorption Spectra of Experimental Sample.</u>

<u>Standard Samples</u> (a) 10 (b) 25 gamma 5β pregnane- 3α 20α -diol (c) 50 gamma 5β pregnanediol as used in Experimental Subject VII, Day 3.

<u>Experimental Sample</u> (d) Experimental Subject VII. Day 3 urine extract 2.0 ml. from tube 12 from Column as prepared for and used in the secretion rate experiment. (77.3 gamma).

<u>Experimental Blank:</u>

1) For samples (a) and (b) - acid blank

2) For samples (c) and (d) - Experimental blank as used in Experiment VII, day 3.

Absorption Spectra.

Wavelength Microns.	10 gamma	25 gamma	50 gamma	P.VII Day 3 T.12	AB	EB
320	.082	.154	.285	.558	.000	
330	.048	.114	.290	.548		
340	.043	.106	.283	.550		
350	.032	.103	.276	.562		
360	.032	.101	.260	.546		
370	.031	.100	.235	.530		
375	.032	.100	.224	.512		
380	.032	.101	.215	.498		
385	.037	.105	.218	.498		
390	.039	.115	.230	.514	.000	.058
395	.044	.124	.248	.542		
400	.049	.138	.274	.580		
405	.054	.154	.314	.616		
410	.059	.171	.334	.660		
415	.065	.182	.363	.697		
420	.066	.185	.375	.710		
425	.069	.195	.377	.712	.000	.064
430	.068	.193	.375	.700		
435	.067	.193	.370	.687		
440	.066	.190	.360	.670		
445	.063	.178	.337	.624		
450	.057	.162	.300	.563		
455	.048	.139	.252	.510		
460	.039	.119	.210	.440	.000	.053
465	.031	.101	.170	.393		
470	.031	.087	.143	.352		
480	.027	.076	.118	.308		
490	.027	.072	.111	.282		
500	.026	.066	.093	.248		
Allen Correction.	.030	.078	.157	.235		

b) Acetylation Experiment.

Samples. 1) Preacetylation experimental sample (Pre ES).

Approximately 2.5 ml. from tube 11 and 2.5 ml. from tube 12 from Experimental Subject I, day 1 (extraction method 3) pooled, plus 300 gamma standard pregnanediol, dried down and taken up in 8 ml. methanol.

0.5 ml. for spectrometry.

0.5 ml. for spectrophotometry.

11) Postacetylation experimental sample (Post ES) 2.0 ml. total volume.

0.2 ml. for spectrometry.

1.0 ml. for spectrophotometry.

111) Postacetylation control sample (Post CS) 2.0 ml. total volume.

0.2 ml. for spectrometry.

1.0 ml. for spectrophotometry.

SPECTROMETRY.

HVT 3	Counts.	Time.	cpm.	B.C.	CCC.	Total
MC14S	28806	2	14403			
MB	60	5	12			
SB	62	5	12			
Post ES	279	5	56	44	35	350
Post CS	301	5	62	50		500

HVT 7	Counts.	Time.	cpm.	B.C.	CCC.	Total
MH3S	27863	2	13932			
MB	151	5	30			
SB	151	5	30			
Pre ES	17064	10	1706	1676		
Post ES	11773	10	1177	1147	1134	11340

SPECTROPHOTOMETRY.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.	Total gamma.
AB	000	000	000	000	100% Reference	
25 ESP	.127	.215	.133	.085		
25 ESP	.127	.215	.130	.087		
25 ESP	.116	.201	.126	.080		
50 ESP	.236	.434	.266	.183		
50 ESP	.240	.431	.266	.178		
50 EB	.239	.430	.268	.185	25 gamma = .089	
Pre ES	.151	.240	.140	.094	26.4	422.4

Sample	390	425	460	Allen Correction	Pregnanediol diacetate gamma	Total gamma
AB	000	000	000	000	100% Reference	
25 EDS	.137	.212	.113	.087		
50 EDS	.248	.420	.232	.180	25 gamma = .089	
Post ES	.702	1.040	.674	.352	98.9	197.8
Post GS	.745	1.307	.809	.530	148.9	297.8

<u>Specific Activities (Counts per minute per milligram).</u>

(1)	Tube 11	252900	
(2)	Tube 12	279200	
(3)	Mean Tube 11, 12.	266050	
(4)	Pre ES	63485	422.4 = 122.4 gamma plus 300 gamma standard pregnanediol.
(5)	Pre ES (Corrected).	219085	122.4 gamma
(6)	Post ES	57331	197.8 gamma = 57.3 + 140.5 gamma from standard.
(7)	Post ES (Corrected).	197906	59.3 gamma
(8)	Correction of (6) for M. Wt.	72237	x 1.26 = ratio of Molecular weight (M.Wt.) of Pregnanediol Diacetate
(9)	Correction of (7) for M. Wt.	249362	

Comparisons:

(1) and (2) ± 4.9% (3) and (5) ± 9.7%
(4) and (8) ± 6.4% (3) and (9) ± 3.2%

c) <u>Counter Current Distribution Experiment.</u>

<u>Sample:</u>

1) Post acetylation experimental sample 2.0 ml. 0.8 ml. for Counter Current Distribution 79.12 gamma : 4536 cpm H^3 : 140 cpm C^{14} :

11) Post acetylation Control Sample 2.0 ml. 0.8 ml. for Counter Current Distribution. 119.12 gamma : 200 cpm. C^{14}.

Spectrometry:

1) Experimental samples:

Contents of the eight tubes after counter current distribution dried down: plus 10 ml. Scintillation fluid.

11) Control samples:

Contents of eight tubes after counter current distribution dried down: plus 10 ml. Scintillation fluid.

111) Experimental blank: 10 ml. mobile phase and 10 ml. stationary phase dried down: plus 10 ml. Scintillation fluid.

IV) Counting HVT 3 and 7

Time: in minutes.

SPECTROMETRY.

HVT 3	Counts.	Time.	cpm.	BC.	Reduced.
MB	29	2	15		
$MC^{14}S$	29108	2	14504		
EB	380	30	13		
Control Tube 1	399	30	13		
2	362	30	12		
3	475	30	16	3	.043
4	592	30	20	7	.101
5	1328	30	44	31	.449
6	2471	30	82	69	1.00
7	2123	30	71	58	.841
8	794	30	26	13	.188
Total				181	

SPECTROMETRY.

HVT 3	Counts.	Time.	cpm.	BC.	CCC.	Reduced.
Experimental Tube 1	578	30	19	6	6	.187
2	517	30	17	4	4	.125
3	533	30	18	5	4	.125
4	686	30	23	10	8	.250
5	1235	30	41	28	22	.688
6	1655	30	55	42	32	1.00
7	1534	30	51	38	29	.906
8	668	30	22	9	6	.187
Total					111	

SPECTROMETRY.

HVT 7		Counts.	Time.	cpm.	BC.	CCC.	Reduced.
MB		52	2	30			
MH3S		28077	2	14038			
EB		942	30	31			
Experimental Tube	1	2360	30	77	48	46	.037
	2	2633	30	88	57	56	.045
	3	4198	30	140	109	108	.088
	4	10648	30	355	324	321	.259
	5	12191	15	813	782	775	.629
	6	12771	10	1277	1246	1234	1.00
	7	11746	10	1175	1144	1134	.915
	8	10908	30	364	333	331	.268
Total						4005	

d) Infrared Analysis.

1) Preparation of sample for analysis.

Subject (Y). M.J. :

Expected date of delivery 27.5.61

Urine collection 25.5.61

Total daily output 800 ml.

Made up to 2000 ml. with deionised water.

500 ml. extracted by Method 4 after 80,000 opm H^3 pregnanediol internal standard added.

Subject (Z). D.J. :

Expected date of delivery 22.5.61

Urine collection 25.5.61

Normal pregnancy except for slightly excessive weight gain.

Total daily output 2100 ml.

525 ml. extracted by Method 4, after 80,000 cpm. H^3 pregnanediol internal standard added.

Spectrometry: Sample: 0.2 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: From tube 2.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrophotometry: Sample: 0.2 ml. from tubes

Experimental blank: from tube 2

Experimental standards: 25, 50 and 100 gamma standard pregnanediol plus 0.2 ml. from tubes 1 and 2.

Columns: Subject Y - Column I: Front; tubes 5

Subject Z - Column II: Front; tube 5

Siphons: Measured at 5.4 ml. for each emptying.

Benzene Extract: Subject Y and Z - total for each : 73 ml.

Portion (a) 25 ml.

(b) 23 ml.

(c) 23 ml.

Remnant: A considerable amount of radioactivity remained after transfer of the samples to the Column. The recovery results were also calculated for this factor.

SPECTROMETRY.

Sample.	Counts.	Time.	cpm.	
MB	906	30	30	
MH^3S	446642	30	14888	
SB	900	30	30	
H^3IS	100001	12.5	80000	) cpm added to urine
H^3IS	100001	12.5	80000	) as internal standard.

SPECTROMETRY.

Sample Y (a).	Count.	Time.	cpm.	BC.	Recovery.
EB	922	30	31	X	
Tube 9	972	30	32	1	
10	2799	30	93	62	) 467 off column
11	3783	30	126	95	) 12609 from portion
12	4177	30	139	108	) 36820 per day
13	3615	30	121	90	) 46%
14	3225	30	108	77	) 50.1% corrected
15	1983	30	66	35	) for remnant.
Remnant	68565	30	2285	2254	
0.2 ml. B.E.	5734	30	191	161	= 73.5%

SPECTROMETRY.

Sample Y (b).	Count.	Time.	cpm.	BC.	Recovery.
EB	910	30	30	X	
Tube 10	994	30	33	3	) 289 off column
11	3351	30	112	82	) 7803 from portion
12	5369	30	179	149	) 31%
13	1893	30	63	33	) 37.9% corrected
14	1337	30	45	15	) for remnant.
15	1195	30	40	10	)
Remnant	122882	30	4096	4066	

SPECTROMETRY.

Sample Y (c).	Counts.	Time.	cpm.	BC.	Recovery.
EB	896	30	30	X	
Tube 9	910	30	30	-	
10	950	30	32	2	
11	1830	30	61	31	) 348 off column
12	3630	30	121	91	) 9396 from portion
13	4558	30	152	122	) 37.2%
14	2864	30	95	65	) 42.7% corrected
15	1672	30	56	26	) for remnant.
16	1300	30	43	13	)
17	1100	30	37	7	
Remnant	81211	30	2707	2677	

SPECTROMETRY.

Sample Z (a).	Counts.	Time.	cpm.	BC.	Recovery.
EB	939	30	31	X	
Tube 9					
10	1132	30	38	7)	337 off column
11	7459	30	249	218)	9099 from portion
12	4512	30	150	119)	26570 per day
13	1190	30	40	9	33.2%
Remnant	162335	30	5411	5380	41.3% corrected for remnant.
0.2 ml. B.E.	4867	30	162	132	60.2%

SPECTROMETRY.

Sample Z (b).	Counts.	Time.	cpm.	BC.	Recovery.
EB	905	30	30	X	
Tube 10	929	30	31	1	
11	1061	30	35	5	
12	3923	30	131	101)	213 off column
13	3852	30	128	98)	5751 from portion
14	1329	30	44	14)	22.8%
15	960	30	32	2	31% corrected
Remnant	183869	30	6129	6099	for remnant.

SPECTROMETRY.

Sample Z (c)	Counts.	Time.	cpm.	BC.	Recovery.
EB	962	30	32	X	
Tube 9	958	30	32	-	
10	949	30	32	-	
11	1012	30	34	2	
12	2743	30	91	59)	329 off column
13	6073	30	202	170)	8883 from portion
14	3592	30	118	86)	35.2%
15	1390	30	46	14)	42.9% corrected
16	1000	30	33	1	for remnant.
Remnant	119673	30	3989	3959	

SPECTROPHOTOMETRY.

Sample Y (a).	Wavelength Microns. 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	042	038	021	001	100% Reference
25 ESP	133	229	133	090)	
50 ESP	231	424	243	187)	25 gamma = .088
100 ESP	444	789	456	339)	
Tube 10	079	128	087	045)	
11	053	112	065	053)	.283 = 80.4 gamma
12	061	129	077	060)	= 6.34 mgm total
13	071	138	079	063)	= 13.8 mgm corrected
14	185	118	073	039)	= 55.2 mgm/day
15	015	042	023	023)	

SPECTROPHOTOMETRY.

Sample Y (b)	Wavelength Microns. 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	012	008	009	002	100% Recovery
25 ESP	123	212	133	094	
50 ESP	236	411	245	171	25 gamma = .088
100 ESP	474	542	502	354	
Tube 10	015	023	014	008	.175 = 49.7
11	079	134	083	053	
12	135	229	137	063	= 13.6 mgm corrected total
13	028	045	028	017	= 54.4 mgm per day
14	037	044	030	020	
15	040	044	029	014	

SPECTROPHOTOMETRY.

Sample Y (c).	Wavelength Microns. 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	013	014	014	000	100% Reference.
25 ESP	125	215	127	089)	
50 ESP	211	389	231	168 }	25 gamma = .084
100 ESP	451	792	470	338)	
Tube 10	020	024	020	004	
11	040	065	042	024)	.201 = 62.8
12	077	127	081	048)	
13	104	166	098	065)	= 138 mgm corrected total
14	069	100	063	034)	= 55.2 mgm per day
15	023	047	033	019)	
16	012	020	006	011)	
17	005	007	004	002	

SPECTROPHOTOMETRY.

Sample Z (a)	Wavelength Microns. 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	051	044	037	000	100% Reference
Tube 10	-	-	-	-	.115 = 32.67 gamma
11	080	160	090	075)	= 2.643 total gamma
12	009	058	028	040)	= 7.8 mgm corrected
13	-	-	-	-	= 31.2 mgm/day
25 ESP	137	225	132	090 }	
50 ESP	218	388	218	170 }	25 gamma = .088
100 ESP	417	779	429	356 }	

SPECTROPHOTOMETRY.

Sample Z (b)	Wavelength Microns. 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	002	001	001	000	100% Reference
Tube 11	015	014	007	-	
12	040	076	043	034)	167 = 19
13	030	058	030	028)	= 7.4 mgm corrected
14	028	035	031	005)	= 29.6 mgm per day
25 ESP	116	204	125	093)	25 gamma = .088
50 ESP	229	406	239	172)	

SPECTROPHOTOMETRY.

Sample Z (c)	Wavelength Microns 390	425	460	Correction	Recovery.
AB	0	0	0	0	
EB	011	008	006	000	100% Reference
25 ESP	114	187	116	079)	
50 ESP	236	440	250	197 }	25 gamma = .084
100 ESP	462	785	480	314)	
Tube 11	013	017	017	002	
12	042	066	048	021)	.114 = 33.9
13	104	158	102	055)	
14	046	077	044	030)	= 8.2 mgm corrected total
15	007	016	011	008)	= 32.8 mgm per day
16	-	002	005	-	

Recovery %	Patient Y Portion A	Patient Y Portion B	Patient Y Portion C	MEAN.	Patient Z Portion A	Patient Z Portion B	Patient Z Portion C	MEAN.
Total procedure	46	31	37.2	38	33.2	22.8	35.2	30.4
From Column	62.6	42.2	50.6	51.8	55.2	37.9	58.3	50.5
Total Procedure Corrected for Remnant	50.1	37.9	42.7	43.6	41.3	31.0	42.9	38.4
From Column Corrected for Remnant	68.2	51.6	58.1	59.3	68.6	51.5	71.3	63.8
Mgm. Pregnanediol per day	55.2	54.4	55.2	54.9	31.2	29.6	32.8	31.2

	Patient Y	Patient Z
Extraction Recovery	73.5%	60.2%

Total procedure recovery for these six experiments

34.2% SD 7.62 SE 3.1

Corrected for remnant: 41.0% SD 6.6 SE 2.7

Column recovery for these six experiments

51.1% SD 9.53 SE 3.9

Corrected for remnant: 61.5% SD 8.95 SE 3.6

	Patient Y Portion			Patient Z Portion			Total
	A	B	C	A	B	C	
mgm. Pregnanediol available for further study.	2.02	1.24	1.57	0.82	0.47	0.85	6.97

RESULTS.

C. EXPERIMENTAL SUBJECTS.

a) Trial of Method.

b) Normal Males.

c) Castrate Females.

RESULTS.

C. EXPERIMENTAL SUBJECTS.

a) Trial of Method.

1) Subject *I

11) Subject *II

111) Paper chromatography.

SUBJECT *I RESULTS.

Patient A.B. Height 6 ft. 1 in.

Weight 195 lbs.

Normal Male Volunteer.

Urine Day 1 : 1210 ml.

Day 2 : 1110 ml.

Experiment 1 (*I): 500 ml. Day 1 urine extracted by Method 1 : 100 gamma standard pregnanediol added to extract as carrier; put on Column II : Front ; tube 6.

Spectrometry: Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: 0.5 ml. from tube 3 plus 10 ml. Scintillation fluid.

Counting: High Voltage tap 7.

Time: in minutes.

Experiment 1 (*I) Spectrometry.

Sample.	Counts.	Time.	cpm.	BC.	Total.
MH^3S	30641	3	15320		
MB	56	2	28		
0.1 ml. EIS.	8688129	10	868813		
EB	125	5	25		
Tube 6	336	5	67	42	
7	927	"	185	160	
8	168	"	34	9	
9	188	"	38	13	
10	263	"	53	28	
11	466	"	93	68	
12	13811	"	2762	2737	27370
13	2671	"	534	519	5190 = 32560
14	205	"	41	16	
15	226	"	45	20	
16	186	5	37	12	
17					

Experiment 1 (*I) Spectrometry.

Sample.	Counts.	Time.	Corrected for time and background.	
Tube 18	145	5	4	
19	56	2	3	
20	140	5	3	
21	188	"	13	
22	150	"	5	
23	155	"	6	
24	168	"	9	
25	172	"	9	
26	183	"	12	
27	188	"	13	
28	165	"	8	
29	146	5	4	
30	145	"	4	
31	181	"	11	}
32	184	"	12	}
33	165	"	8	}
34	215	"	18	} = 73
35	220	"	19	} = 730 total
36	150	"	5	} = 1766 per day
37	131	"	1	
38	169	"	9	
39	193	"	14	

Experiment 1 (*I).

Spectrophotometry: Sample 2.0 ml. from tubes.

Experimental blank: from tube 1.

Experimental Standard: 25 and 50 gamma standard pregnanediol plus 2.0 ml. from tubes 1 and 2.

Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol gamma.
AB	000	000	000	000	
EB	.004	.002	.000	.000	100% Reference.
25 ESP	.116	.185	.112	.071	
25 ESP	.149	.194	.111	.064	
50 ESP	.229	.383	.225	.156	
50 ESP	.267	.419	.250	.160	25 gamma = .075
Tube 11	.090	.080	.064	-	
12	.320	.465	.292	.159	53.0 } = 150 total
13	.112	.119	.083	.022	7.0 } = 70 (corrected for 80% recovery of carrier)
14	.058	.056	.046	-	
Remnant	.254	.221	.166	-	

Experiment 2 (*I).

Urine from Experiment I re-extracted by Method 2 (100 gamma standard pregnanediol added to extract as carrier). Extract put on Column I:

Front: tube 5.

Spectrometry: Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 3

Counting: High Voltage tap 7

Time: in minutes.

Sample.	Counts.	Time.	cpm.	BC.	Total
MH^3S	30641	2	15320		
MB	56	2	28		
EB	140	5	28		
Tube 5	2139	5	428	400	
6	999	"	200	172	
7	286	"	57	29	
8	387	"	77	49	
9	2727	"	545	517	
10	2179	"	436	408	
11	8126	"	1625	1597	15970
12	4619	"	924	896	8960 = 26320
13	835	"	167	139	1390
14	295	"	59	31	
15	312	"	62	34	
16	259	"	52	24	
17	210	"	42	14	

Experiment 2 (*I).

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: 2.0 ml. from tube 1.

Experimental standard: 2.0 ml. from tubes 1 and 2 plus 25 and 50 gamma standard pregnanediol.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	000	000	000	000	
EB	.004	.002	.000	.000	100% Reference.
25 ESP	.148	.215	.134	.075	
50 ESP	.238	.381	.222	.151	25 gamma = .075
Tube 9	1.35	1.39	1.42	.000	
10	1.105	1.095	1.00	-	
11	1.03	1.160	1.010	.140	46.7 }
12	.778	.818	.736	.0610	20.0 } gamma = 194.25 total
13	.526	.550	.506	.034	11.0 } = 114.25 Corrected
Remnant	2.15	1.480	1.414	-	for 80% recovery of Carrier.

Experiment 3 (*I).

500 ml. Day 1 urine extracted by Method 3 (100 gamma standard pregnanediol added to extract as carrier). Extract put on Column I: Front: tubes 6.

Spectrometry:

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 3

Counting: High voltage tap 7

Time: in minutes.

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
MH^3S	31608	2	15804				
MB	51	2	36				
0.1 ml. EIS	8688239	10	868824				
EB	1286	30	43				
Tube 5	425	5	85	39			
6	10724	20	536	493	5.2		
7	453	5	91	45			
8	714	5	143	97			
9	11818	35	338	295			
10	10360	20	518	475			
11	11610	5	2322	2276	22	90	252900)
12	18204	5	3641	3595	27	128.75	279200)± 4.94%
13	14556	15	970	937			
14	722	5	144	98			
15	502	"	100	54			
16	434	"	87	41			
17	338	"	68	22			

Experiment 3 (*I) Spectrometry.

Sample.	Counts.	Time.	Corrected for time and background.
Tube 18	313	5	17
19	372	"	28
20	301	"	14
21	237	"	1
22	236	"	1
23	202	"	-
24	224	"	-
25	185	"	-
26	248	"	4
27	199	"	-
28	173	"	39
29	200	5	-
30	186	"	-
31	184	"	-
32	183	"	-
33	204	"	-
34	154	"	5
35	286	"	11
36	394	"	33
37	407	"	35
38	457	"	45
39	407	"	35

(Tubes 36–39, bracketed:) 148 = 1480 total
= 3581 per day

Experiment 3 (*I).

Spectrophotometry:

Sample: 2.0 ml. from tubes.

Experimental blank: 2.0 ml. from tube 1.

Experimental standards: 25 and 50 gamma standard pregnanediol plus 2.0 ml. from tube 1 and 2.

Sample Day 1.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	000	000	000	000	
EB	006	008	007	002	100% Reference
25 ESP	.110	.181	.105	.074	25 gamma = .068
25 ESP	.110	.176	.105	.071	
25 ESP	.115	.165	.117	.059	
50 ESP	.185	.297	.179	.115	
50 ESP	.218	.385	.215	.169	
50 ESP	.220	.340	.204	.128	
Tube 9	.309	.266	.206	–	
10	.454	.290	.218	–	
11	.457	.501	.354	.095	36) = 218.75 total
12	.471	.561	.371	.140	51.5) = 138.75 Corrected
13	.325	.315	.235	–	) for 80% recovery of
Remnant	.110	.117	.092	.016	Carrier.

Experiment 4 (*I).

Urine from experiment 3 re-extracted by Method 2. (100 gamma standard pregnanediol added to extract as carrier). Extract put on Column I: Front: tube 5.

Spectrometry and Spectrophotometry samples as in Experiment 3.

Spectrometry:

Sample.	Counts.	Time.	Corrected for time and background.
MH^3S	28029	2	14015
MB	58	2	29
EB	284	5	57
Tube 5	526	5	48
6	427	5	28
7	325	5	8
8	328	5	9
9	327	5	8
10, 11	412	5	15
12	330	5	8
13	320	5	7
14, 15	263	5	-
16	195	5	-
17	182	5	-

Experiment 4 (*I).

Spectrophotometry.

Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	000	000	000	000	
EB	.008	.008	.004	.002	100% Reference.
25 ESP	.127	.215	.133	.085	25 gamma = .089
25 ESP	.127	.215	.130	.087	
25 ESP	.116	.201	.126	.080	
50 ESP	.236	.434	.266	.183	
50 ESP	.240	.431	.266	.178	
50 ESP	.239	.430	.268	.185	
Tube 9	.818	.770	.701	-	
10, 11	1.742	1.812	1.625	.128	36
12	.572	.664	.549	.103	28.9 } = 200.5 total
13	.509	.560	.500	.055	15.4 } = 120.5 after Correction for 80% recovery of Carrier.

Experiment 5. (*I).

500 ml. day 2 urine extracted by Method 3. (100 gamma standard pregnanediol added to extract as carrier). Extract put on Column II: Front: tube 6.

Spectrometry and Spectrophotometry samples as in Experiment 3.

Spectrometry:

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
MH^3S	31608	2	15804				
MB	51	2	26				
EB	995	30	33				
Tube 6	360	5	72	41	1.8		
7	7179	60	120	87			
8	211	5	42	11			
9	246	5	47	16			
10	438	5	88	57			
11	10704	25	428	395	4.3	41.25	95758) ± 4.1%
12	14448	15	963	930	8.1	90	103333)
13	10415	55	189	156			
14	374	5	75	44			
15	245	5	49	18			
16	196	5	39	8			
17	168	5	34	3			

Experiment 5 (*I) Spectrometry.

Sample.	Counts.	Time.	Corrected for time and background.	
Tube 18	200	5	9	
19	192	"	7	
20	173	"	4	
21	160	"	1	
22	182	"	5	
23	164	"	2	
24	156	"	-	
25	151	"	-	
26	150	"	-	
27	146	"	-	
28	183	"	6	
29	167	5	2	
30	170	"	3	
31	192	"	7	}
32	239	"	17	}
33	224	"	14	} 92 = 920 total
34	275	"	14	} = 2042 per day
35	195	"	8	}
36	214	"	12	}
37	207	"	10	}
38	217	"	12	}
39	198	"	9	

Experiment 5 (*I).

Spectrophotometry:

Sample. Day 2	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	000	000	000	000	
EB	008	006	006	-	100% Reference
25 ESP	.114	.185	.110	.073	25 gamma = .068
50 ESP	.219	.347	.213	.131	
Tube 11	.504	.508	.421	.045	16.5
12	.510	.534	.361	.098	36.0
13	.283	.276	.256	-	-
Remnant	.021	.025	.019	.005	2

Experiment 6 (*I).

Estimations of Counts in Urine:

Sample: 0.4 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid plus 6 ml. absolute ethanol.

Experimental blank : 0.4 ml. tritium free urine as above.

Internal standard : 0.2 ml. standard H^3 pregnanediol.

Counting : High Voltage tap 7. Automatic.

Time: In minutes.

Spectrometry a) Standards.

Sample.	Counts.	Time.	cpm.	Mean.	BC
MB	943	30	31		
MH^3S	409999	30	13667		
SB	1089	30	36		
UB	2307	60	38.5)	38	
UB	2250	60	37.5)		
0.2 ml. H^3IS	100001	20.93			
0.2 ml. H3IS	100002	20.34	)	4854	4820
0.2 ml. H3IS	100002	20.51			

Experiment 6 (*I).

Spectrometry b) Urine Samples.

Sample.	Counts.	Time.	cpm.	BC	Mean.	SD±%
0.4 ml. Day 1	5320	60	88.7	50	43.5	2.3
0.4 ml. Day 1	4498	60	75	37		
0.4 ml. Day 2	2946	60	49.1	11		
0.4 ml. Day 2	2943	60	49	11	11	7.7

Spectrometry c) Urine Samples plus internal standard.

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.4 ml. Day 1	38263	30	1275	1186	
0.4 ml. Day 1	42041	30	1401	1326	3.84
0.4 ml. Day 2	42292	30	1410	1361	
0.4 ml. Day 2	42788	30	1426	1377	3.52

Gamma pregnanediol recovered per day. (Corrected for 80% recovery of carrier pregnanediol).

	Day 1	Day 2	Mean
Gamma	335.8	113.8	224.8

Counts recovered as pregnanediol from Column. (*I)

	Day 1	Day 2	Total
cpm	142078	29415	171493
% Injection Solution.			10.9

Counts recovered as Peak X from Column (*I).

	Day 1	Day 2	Total
cpm	11930	1931	13861
% Injection Solution.			0.88

Counts recovered from urine (*I)

	Day 1	Day 2	Total
cpm	505296	107448	612744
% Injection Solution.			38.8

(*I).

Experimental Injection Solution.

$$0.2 \text{ ml.} = 1,737,600 \text{ cpm.}$$

$$\frac{10}{11} = 1,580,000 \text{ cpm.}$$

$$= \text{total radioactivity injected.}$$

Secretion Rate of Progesterone (*I).

$$= \frac{\text{Total radioactivity injected}}{\text{Specific activity of Pregnanediol}}$$

$$= 158000 \div \frac{171493}{224.8} \; \frac{\text{cpm.}}{\text{gamma.}}$$

$$= \frac{158000 \times 224.8}{171493}$$

$$= 2.071 \text{ mgm. per day.}$$

SUBJECT *II RESULTS.

Patient L.S. : Height 6 ft.

Weight 170 lbs.

Normal Male Volunteer.

Urine: Day 1 1125 ml.

Day 2 1140 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by Method 3. 100 gamma standard pregnanediol added to extracts put on Columns.

Columns: Day 1 Column II Front : tube 5

Day 2 Column III Front : tube 5

Spectrophotometry: Column Eluate (*II).

Sample: 2.0 ml. from tubes dried down

Experimental blank: 2.0 ml. from tube 1

Experimental standard : 25 and 50 gamma standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry. Column Eluate (*II).

Sample. Day 1	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol gamma.
AB	.000	.000	.000	000	
EB	.012	.012	.008	002	100% Reference
25 ESP	.091	.181	.117	.077	
25 ESP	.104	.184	.112	.076	
50 ESP	.268	.433	.274	.162	
50 ESP	.235	.405	.264	.155	25 gamma = .078
Tube 11	.192	.151	.124	-	
12	.340	.427	.293	.111	34.3
13	.330	.408	.289	.101	32.4
14	.228	.220	.189	-	-

Spectrophotometry. Column Eluate (*II).

Sample. Day 2	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol gamma.
AB	000	000	000	000	
EB	.002	.004	.008	-	100% Reference
25 ESP	.138	.214	.142	.074	
50 ESP	.227	.400	.253	.160	25 gamma = .078
Tube 14	.074	.069	.058	-	
15	.271	.386	.260	.121	38.8
16	.292	.375	.263	.098	31.4
17	.123	.103	.078	-	

Spectrometry: a) Column Eluate. (*II).

Sample: 0.5 ml. from tubes, 1.0 ml. from ether extract, and 0.1 ml. from benzene extract, plus 10 ml. Scintillation fluid to each.
Experimental blank: from tube 1
Counting: High Voltage tap 7
Time: in minutes.

Sample. Day 1	Count.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
MB	48	2	24				
MH^3S	28625	2	14312				
0.2 ml. EIS	8211099	10	821110				
SB	136	5	27				
EB	933	30	31				
EE (1.0 ml.)	466	5	93	66			
BE (0.1 ml.)	555	5	111	84			
Tube 5	169	5	34	3			
6	7504	30	250	219	3.6		
7	11816	30	394	363	3.8		
8	296	5	59	28			
9	405	5	81	50			
10	1060	5	212	181			
11	923	5	185	154			
12	13044	10	1304	1273	11.4	85.75	147954) ± 1.6%
13	12693	10	1269	1238	11.3	81.0	152839)
14	1347	5	269	238			
15	458	5	92	61			
Remnant	358	5	72	43			

Ether Extract (EE) 790 ml. = 52140 cpm.
Benzene Extract (BE) 49 ml. = 41160 cpm.

Spectrometry a) Column Eluate (*II).

Sample. Day 2	Count.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
MB	48	2	24				
MH3S	29350	2	14675				
SB	4161	5	32				
EB	957	30	32				
EE (1 ml.)	217	5	43	11	3.86		
BE (0.1 ml.)	237	5	47	15	4		
Tube 5	175	5	35	3			
6	169	5	34	2			
7	3226	30	108	76	2.2		
8	176	5	35	3			
9	149	5	30	-			
10	148	5	30	-			
11	164	5	33	1			
12	271	5	54	22			
13	285	5	57	25			
14	172	5	34	2			
15	7529	30	250	218	3.1	97.0	22,474) ± 4.4%
16	6744	30	225	193	2.9	78.5	24,586)
17	217	5	43	11			
18	180	5	36	4			
Remnant	334	5	67	23			

Ether extract (EE) 775 ml. = 8525 cpm.
Benzene extract (BE) 49 ml. = 7350 cpm.

<u>Spectrometry b) Urine. (*II).</u>

Sample: 0.4 ml. urine (after thoroughly shaking up specimen) plus 10 ml.

Scintillation fluid plus 6 ml. absolute ethanol.

Experimental blank: 0.4 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

<u>Spectrometry b) Urine. (*II).</u>

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	943	30	31		
MH^3S	409999	30	13667		
SB	1089	30	36		
0.4 ml. UB	2307	60	38.5)	38	
0.4 ml. UB	2250	60	37.5)		
0.2 ml. H^3IS	100001	20.93)			
0.2 ml. H^3IS	100002	20.34)		4854	4820
0.2 ml. H^3IS	100001	20.51)			

Spectrometry b) Urine. (*II).

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.4 ml. Day 1	4471	60	74.5	36		
0.4 ml. Day 1	3537	60	59	21	28.5	3.3
0.4 ml. Day 2	2492	60	41.5	3.5		
0.4 ml. Day 2	2726	60	45.4	7.4	5.4	15.8

Spectrometry c) Urine plus internal standard. (*II).

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.4 ml. Day 1	44460	30	1482	1406	
0.4 ml. Day 1	43752	30	1458	1399	3.44
0.4 ml. Day 2	43847	30	1462	1420	
0.4 ml. Day 2	45167	30	1506	1461	3.35

Total Counts recovered from Column as Pregnanediol (*II).

	Day 1	Day 2	Total
cpm	56498	9471	65969
Per cent injected dose.			8.8

Total Counts recovered from Column as Peak X. (*II).

	Day 1	Day 2	Total
cpm	13095	1734	14829
Per cent injected dose.			1.99

Total Counts recovered from Urine. (*II).

	Day 1	Day 2	Total
cpm	275738	50200	325938
Per cent injected dose.			43.7

Counts recovered from Extracts.

	Day 1		Day 2	
	cpm	Ratio	cpm	Ratio
Ether Extract	52140	2.08	8525	2.07
Benzene Extract	41160	1.64	7350	1.79
Pregnanediol tubes	25110	1	4110	1

Total Pregnanediol Recovered per day. (*II).

(Corrected for 80% Column recovery of carrier pregnanediol).

	Day 1	Day 2	Mean
gamma	196	218	207

Injection Solution (*II) 0.2 ml. = 821000 cpm.

Total radioactivity injected = $\frac{10}{11}$ of 821000 cpm.

= 746000 cpm.

Secretion Rate of Progesterone (*II).

$$= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific activity of Pregnanediol}}$$

$$= 74600 \div \frac{65969}{207} \text{ gamma.}$$

$$= \frac{74600 \times 207}{65969}$$

= 2.341 mgm. per day.

Paper Chromatography Experiments.

Experiment a). To test the efficiency of the Bush System using the Column Solvent System to differentiate.

5β pregnane-3α, 20α-diol (pregnanediol).

5α pregnane-3α, 20α-diol (Allopregnanediol).

5β pregnane-3α,ol-20, one (Pregnaneolone)

pregnane- 3, 17α, 20 -triol (Pregnanetriol).

pregnane- 3β, 20α -diol.

Paper 1) 20 gamma of each steroid were pipetted on to the paper. The samples were "walked up" twice.

Paper 2) 25 gamma of pregnanediol, pregnaneolone and pregnanetriol as before.

Paper 3) 25 gamma of each steroid as before.

Paper 4) 25 gamma of pregnanediol and pregnaneolone, and 40 gamma pregnanetriol as before.

Rf = rate of movement of steroid in relation to the front.

= Distance from starting line to mid point of steroid spot in cms. ÷ Distance from starting line to front in cm.

Rf	Paper 1	Paper 2	Paper 3	Paper 4
Allopregnanediol	0.681		0.638	
Pregnane-3β-20α-diol	0.676		0.622	
Pregnanediol	0.678	0.673	0.610	0.610
Pregnaneolone.	0.852	0.848	0.810	0.800
Pregnanetriol.	0.393	0.413	0.287	0.313
			0.333	0.359
			0.354	0.378

Conclusions.

1) Separation of pregnanediol from allo-pregnanediol and pregnane-3β, 20α -diol was not satisfactory.

11) The pregnanetriol spot was larger than for the other steroids and suggested that the standard sample was a mixture of stereoisomers of pregnanetriol. This was more evident when larger amounts of the steroid sample were put on the paper. The results suggest that three isomers were present giving their different Rfs. in the pregnanetriol standard sample (Pregnane-3 , 17α, 20 -triol). Subsequently a pure sample of pregnane-3α, 17α, 20α, -triol was obtained for later column experiments.

<u>Experiment b).</u> Tube 6 from Column I,
Patient *I, Day 1. (Extraction Method 3).
Approximately 4.5 ml. from tube 6 dried down
= 4237 cpm. approximately.
50 gamma standard pregnaneolone added as
carrier and dried down.
1.0 ml. methanol added and sample divided
into two 0.5 ml. portions.
One 0.5 ml. sample portion, 25 gamma standard
pregnanediol and 25 gamma standard pregnane-
olone run on Bush paper chromatography system
after "walking up" twice.
Control strips and 0.5 cm. of sample strip
stained with phosphomolybdic acid.
Sample strip then cut into six portions which
were separately eluted in Methanol. The
Methanol eluates were then dried down in
Scintillation vials and 10 ml. Scintillation
fluid added.
Vials counted at high voltage tap 7.

	Pregnanediol Control	Pregnaneolone Control				Sample
Rf	0.490	0.771				0.771
Sample		Counts	Time	cpm	BC	Total $(=x\frac{5}{4})$
MH3S		28777	2	14388		
SB		136	5	27		
Segment A	(Start line–6.5 cms.)	290	5	58	31	39
B	(6.5 cms. – 13.0 cms.)	126	5	25	–	–
C	(13.0 cms.– 20.0 cms.)	156	5	31	4	5
D	(20.0 cms.– 24.0 cms.)	154	5	31	4	5
E	(24.0 cms.– 29.8 cms.)	7375	5	1475	1448	1811
F	(29.8 cms.– front)	377	5	75	48	60

Segment E = pregnaneolone spot plus 1 cm. on each side. (85.4% recovery).

Segment C = pregnanediol spot plus 1 cm. on each side.

Experiment c)   Tube 15 Column III Patient *II Day 2.

2.0 ml. from tube 15 dried down = approximately 908 cpm. and 38.8 gamma.

1.0 ml. Methanol added and sample divided into two 0.5 ml. portions.

Both 0.5 ml. sample portions and 25 gamma standard pregnanediol run on Bush System after walking up twice.

Control strip and 0.5 cm. sample strips stained with phosphomolybdic acid.

Sample strips then cut into five segments which were separately eluted in Methanol. Methanol eluates were dried down in Scintillation vials and 10 ml. Scintillation fluid added. Then the vials were counted at high voltage tap 7.

		Pregnanediol Control					Sample		Strips		
Rf				0.508			0.508		0.508		
Sample		Count.	Time.	cpm.	BC.	Total.	Count.	Time.	cpm.	BC.	Total.
MH^3S		28777	2	14388							
SB		134	5	27							
Segment	A	121	5	24			119	5	24		
	B	185	5	37	10	12	186	5	307	10	12
	C	1669	5	334	307	384	1759	5	352	325	406
	D	101	5	20			132	5	26		
	E	209	5	42	15	19	141	5	28	1	1

Segment A = start to 7.0 cm.
B = 7.0 cms. - 13.8 cm.
C = 13.8 cm. - 21.8 cm.
D = 21.8 cm. - 28.2 cm.
E = 28.2 cm. - front.

Segment C = pregnanediol spot plus 1 cm. on each side. (87% recovery).

Total = Correction for 0.5 cm. removed from strips for staining = cpm x 5/4.

Experiment d). Tube 7 Column III Patient *II Day 2.

Approximately 4.5 ml. from tube 7 dried down = 693 cpm. approximately.

50 gamma standard pregnaneolone added as carrier and dried down.

1.0 ml. Methanol added and sample divided into two 0.5 ml. portions.

One 0.5 ml. sample portion, 25 gamma standard pregnanediol and 25 gamma standard pregnaneolone were run on a Bush paper chromatography system after "walking up" twice.

Control strips and 0.5 cm. of sample strip stained with phosphomolybdic acid.

The sample strip was then cut into six segments which were separately eluted in Methanol. The Methanol eluates were dried down in Scintillation vials and 10 ml. Scintillation fluid added.

The vials were counted at high voltage tap 7.

	Pregnanediol Control.		Pregnaneolone Control.			Sample.
Rf	0.490		0.771			0.771
	Counts.	Time.	cpm.	BC.	Total (=cpm x $\frac{5}{4}$)	
MH^3S	28777	2	14388			
SB	1350	5	27			
Segment A (Start to 6.5 cm.)	131	5	26			
B (6.5 cms. - 13.0 cm.)	135	5	27			
C (13.0 cm. - 20.0 cm.)	139	5	27			
D (20.0 cm. - 24.0 cm.)	166	5	33	6	7	
E (24.0 cm. - 29.8 cm.)	1344	5	269	242	303	
F (29.8 cm. - Front)	175	5	35	8	10	

Segment E = pregnaneolone spot plus 1 cm. on each side. (87.4% recovery).

Segment C = pregnanediol spot plus 1 cm. on each side.

RESULTS.

C. Experimental Subjects.

b) Normal Males.

Subject *III

*IV

*V

*VI

*VII

*IX

*XII

SUBJECT #III RESULTS.

Patient R.W. : Height 6 ft.

Weight 180 lbs.

Normal Male Volunteer.

Urine Day 1 1325 ml.

2 1610 ml.

3 1990 ml.

4 1490 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (100 gamma standard pregnanediol added to extracts as carrier).

Columns	Day		Column		Front, Tube
	1	:	Column II	:	Front, Tube 6
	2	:	III	:	5
	3	:	III	:	6
	4	:	II	:	5

Spectrophotometry Column Eluate:

Sample: 2.0 ml. from tubes.

Experimental blank : 2.0 ml. from tube 1.

Experimental standard : 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1 and 2.

Spectrophotometry: Column Eluate. (*III).

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma
AB	0	0	0		
EB	.004	.003	.003	100% Reference	
25 ESP	.111	.195	.116	.081	
50 ESP	.231	.405	.240	.169	25 gamma = .083
Tube 8	1.090	1.025	.900	–	
9	.227	.210	.126	–	
10	.648	.850	.535	.259	78.0
Day 2					
AB	0	0	0		
EB	.002	.003	.004	0	100% Reference
25 ESP	.118	.202	.120	.083	
50 ESP	.253	.417	.253	.164	25 gamma = .082
Tube 9	.125	.120	.087	–	
10	.111	.094	.065	–	
11	.387	.610	.374	.230	70.0
12	.186	.166	.120	–	

Spectrophotometry: Column Eluate. (*III).

Day 3 Sample.	Wavelength 390	Microns. 425	460	Allen Correction.	Pregnanediol Gamma.
AB	000	000	000	000	
EB	000	000	000	"	100% Reference
25 ESP	.118	.200	.118	.082	
50 ESP	.235	.402	.231	.169	25 gamma = .084
Tube 12	.080	.073	.058	-	
13	.078	.079	.062	.009	2.68
14	.280	.385	.240	.125	37.2
15	.174	.177	.127	.027	8.04
Day 4					
AB	000	000	000	000	
EB	001	004	006	000	100% Reference
25 ESP	.125	.213	.136	.082	
50 ESP	.234	.382	.240	.145	25 gamma = .076
Tube 10	.252	.194	.143	-	
11	.348	.456	.336	.114	37.5
12	.355	.420	.275	.105	34.27

Spectrometry: a) Column Eluate. (*III).

Sample: 0.5 ml. from tubes, 1.0 ml. from ether extract, and 0.1 ml. from benzene extract, plus 10 ml. Scintillation fluid to each.

Experimental blank: from tube 1

Counting: High Voltage tap 7

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	53	2	27
MH^3S	28888	2	14444
0.5 ml. ES	111109	10	11111
0.2 ml. EIS	6902512	10	690251
SB	145	5	29

Spectrometry a) Column Eluate. (*III).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	813	30	27				
Tube 5	74	2	37	10			
6	11088	40	277	250	2.80		
7	5642	100	140	113	2.1		
8	385	2	192	165			
9	10171	30	339	312			
10	37212	20	1861	1834	10	105	174667
11	235	2	117	90			
12	83	2	41	14			
13	80	2	40	13			
14	55	2	27	-			
Remnant	950	2	475	446			
EE (1 ml.)	379	5	76	47	4.6		
BE (0.1 ml.)	498	5	100	71	5.1		

Ether Extract (EE) 805 ml. = 37835 cpm
Benzene Extract (BE) 48.5 ml. = 34435 cpm.
(Pd.g. corrected for carrier pregnanediol assuming 80% Column recovery).

Spectrometry a) Column Eluate. (*III).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	815	30	27				
Tube 5	68	2	34	7			
6	5358	60	89	62	1.55		
7	71	2	35	8			
8	51	2	25	-			
9	154	2	77	50			
10	98	2	49	22			
11	10867	30	362	335	3.6	95	40526
12	121	2	60	33			
13	65	2	32	5			
14	63	2	31	4			
15	53	2	27	-			
Remnant	340	2	170	141			
EE (1 ml.)	1113	30	37	8	2.65		
BE (0.1 ml.)	1239	30	41	12	2.68		

Ether Extract (EE) 845 ml. = 6760 cpm.
Benzene Extract (BE) 49 ml. = 5880 cpm.
(Pd.g. corrected for carrier pregnanediol assuming 80% Column recovery).

Spectrometry a) Column Eluate. (*III).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	936	30	31				
Tube 6	64	2	32	1			
7	2936	60	49	18	1.36		
8	67	2	33	2			
9	64	2	32	1			
10	70	2	35	4			
11	76	2	38	7			
12	99	2	49	18			
13	2221	60	37	6	1.3	2.2	27273)
14	4697	40	1117	86	1.97	31.0	27724)± 2.1%
15	2254	45	50	19	1.45	6.7	28358)
16	57	2	28	-			
Remnant	84	2	42	13			

(Pd.g. corrected for carrier pregnanediol assuming 80% Column recovery).

Spectrometry a) Column Eluate. (*III).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Qt.C.	Pd.g.	S.A.
Day 4 EB	900	30	30					
Tube 5	63	2	31	1				
6	2416	60	40	9	1.3			
7	66	2	33	3				
8	65	2	32	1				
9	63	2	31	1				
10	88	2	44	14				
11	3927	60	65	35	1.44	51.54	6803)	± 3.1%
12	3825	60	64	34	1.44	47.0	7234)	
13	62	2	31	1				
14	66	2	33	2				
Remnant	66	2	33	4				

(Pd.g corrected for carrier pregnanediol assuming 80% Column recovery).

Spectrometry: b) Urine. (*III).

Sample: 0.4 and 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid plus 6 ml. absolute ethanol.

Experimental blank: 0.4 and 0.6 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine.

Sample.	Counts.	Time.	cpm.	Mean.	BG.
MB	933	30	31		
MH^3S	419969	30	13999		
SB	1022	30	34		
0.4 ml. UB	2307	60	38.5	38	
0.4 ml. UB	2250	60	37.5		
0.6 ml. UB	2311	60	38.5	38	
0.6 ml. UB	2270	60	37.8		
0.2 ml. H^3IS	100001	20.93		4854	4820
0.2 ml. H^3IS	100002	20.34			
0.2 ml. H^3IS	100001	20.51			

Spectrometry b) Urine. (*III).

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.4 ml. Day 1	3301	60	55	17		
0.4 ml. Day 1	3276	60	54.6	16.6	16.8	5.3
0.4 ml. Day 2	2522	60	42	4		
0.4 ml. Day 2	2543	60	42.4	4.2	4.1	20
0.6 ml. Day 3	2516	60	41.9	4		
0.6 ml. Day 3	2391	60	39.9	2	3.0	26
0.4 ml. Day 4	2344	60	39.1	1		
0.4 ml. Day 4	2397	60	39.9	2	1.5	53

Spectrometry c) Urine plus internal standard. (*III).

Sample.	Counts.	Time.	cpm.	TBC	QF.
0.4 ml. Day 1	44646	30	1488	1433	
0.4 ml. Day 1	45080	30	1503	1448	3.35
0.4 ml. Day 2	44766	30	1492	1450	
0.4 ml. Day 2	46112	30	1537	1495	3.27
0.6 ml. Day 3	43588	30	1453	1411	
0.6 ml. Day 3	45960	30	1532	1492	3.32
0.4 ml. Day 4	45317	30	1511	1472	
0.4 ml. Day 4	42632	30	1421	1381	3.38

Total Count recovered from Column as Pregnanediol. (*III).

	Day 1	Day 2	Day 3	Day 4	Total
cpm	48601	10787	4418	2056	65862
Per cent	73.8	16.4	6.7	3.1	100
Per cent Injected Dose.					10.5

Total Counts recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	9620	1996	716	269	12601
Per cent	76.4	15.8	5.7	2.1	100
Per cent Injected Dose.					2.01

Total Counts recovered from urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	186428	53963	33034	18866	292291
Per cent	63.8	18.5	11.3	6.5	100
Per cent Injected Dose.					46.6

Total Pregnanediol recovered per day.

	Day 1	Day 2	Day 3	Day 4	Mean.
gamma	278.25	305.90	158.4	297.1	260

(Corrected for carrier pregnanediol assuming 80% Column recovery rate).

Ratio of $\frac{\text{\% Recovery of injected dose as pregnanediol}}{\text{\% Recovery of injected dose as peak X}} = \frac{5.22}{1}$

Ratio of $\frac{\text{\% Recovery of injected dose in urine}}{\text{\% Recovery of injected dose in pregnanediol}} = \frac{4.44}{1}$

Ratio of $\frac{\text{\% Recovery of injected dose in urine}}{\text{\% Recovery of injected dose as peak X}} = \frac{23.2}{1}$

	Day 1		Day 2	
	cpm.	Ratio.	cpm.	Ratio.
Ether extract	37835	2.06	6760	2.02
Benzene extract	34435	1.88	5880	1.76
Pregnanediol tubes	18340	1	3350	1

Injection Solution (*III) 0.2 ml. = 690251 cpm.

Total radioactivity injected = $\frac{10}{11}$ of 690251 cpm = 627500 cpm.

Secretion Rate of Progesterone (*III).

$$= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$$

$$= 627500 \div \frac{65862}{260} \text{ gamma}$$

$$= \frac{627500 \times 260}{65862}$$

= 2.477 mgm. per day.

SUBJECT *IV RESULTS.

Patient M.S. : Height 5 ft. 7 ins.

Weight 185 lbs.

Normal Male Volunteer.

Urine	Day	
	1	630 ml.
	2	880 ml.
	3	700 ml.
	4	740 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b) (100 gamma standard pregnanediol added to Day 2 extract as carrier).

Columns:	Day	Column	Front; Tube
	1	II	5
	2	III	6
	3	II	5
	4	I	5

Spectrophotometry Column Eluate:

Sample: 2.0 ml. from tubes.

Experimental blank: 2.0 ml. from tube 1.

Experimental standard: 25, 50 and 100 gamma of standard pregnanediol plus 2.0 ml. from tubes 2 and 3.

Spectrophotometry: Column Eluate. (*IV).

Day 1 Sample.	Wavelength Microns. 390	 425	 460	Allen Correction.	Pregnanediol Gamma
AB	0	0	0		
EB	.002	.003	.004	000	100% Reference.
25 ESP	.162	.275	.193	.097	
50 ESP	.317	.542	.371	.198	25 gamma = .098
Tube 10	.210	.203	.150	–	
11	.320	.207	.203	–	
12	.750	1.180	.750	.430	108.7
13	.310	.384	.263	.098	25.0
Day 2					
AB	0	0	0		
EB	.001	.001	.002	000	100% Reference.
25 ESP	.144	.225	.143	.081	25 gamma = .081
50 ESP	.253	.426	.275	.162	
Tube 12	.244	.232	.174	–	
13	.502	.400	.291	–	
14	.442	.641	.438	.201	62.0
15	.756	1.173	.770	.410	126.54

Spectrophotometry: Column Eluate. (*IV).

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma
AB	0	0	0		
EB	.037	.048	.050	.004	100% Reference.
25 ESP	.143	.241	.160	.089	25 gamma = .082
50 ESP	.269	.440	.285	.163	
100 ESP	.462	.821	.529	.325	
Tube 11	.464	.406	.310	–	
12	.651	.747	.550	.147	44.82
13	.940	1.268	.840	.368	112.2
14	.381	.436	.315	.088	26.82
Day 4					
AB	0	0	0		
EB	.011	.012	.013	.000	100% Reference.
25 ESP	.113	.191	.122	.073	25 gamma = .077
50 ESP	.268	.415	.276	.143	
100 ESP	.427	.770	.466	.323	
Tube 9	.407	.327	.248	–	
10	.709	.980	.649	.301	97.72
11	.551	.754	.520	.218	70.8
12	.387	.383	.309	.035	11.3

<u>Spectrometry. a) Column Eluate.</u> (*IV).

Sample : 0.5 ml. from tubes, 0.5 ml. from ether extract, and 0.1 ml. from benzene extract, plus 10 ml. Scintillation fluid to each.

Experimental blank: from tube 1.

Counting: High Voltage tap 7.

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	1411	5	28
MH^3S	27458	2	13729
0.2 ml. EIS	3650494	5	730098
0.5 ml. ES	108417	10	10842
SB	641	20	32

Spectrometry a) Column Eluate. (*IV)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	861	30	29				
Tube 5	74	2	37	8			
6	19622	60	327	298	2.53		
7	12508	30	417	388	3.84		
8	130	2	65	36			
9	170	2	85	56			
10	11655	30	388	357			
11	12418	30	414	385			
12	41120	10	4112	4083	20	271.75	150,248)
13	15534	15	1036	1007	8.4	62.5	161,120)± 3.6%
14	302	2	151	122			
15	148	2	74	45			
Remnant	1921	2	960	928			
EE (0.5 ml.)	189	2	94	62	7		
BE (0.1 ml.)	845	4	211	179	7.3		

Ether Extract (EE) 785 ml. = 97340 cpm.
Benzene Extract (BE) 49 ml. = 87710 cpm.

Spectrometry a) Column Eluate. (*IV).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	914	30	30				
Tube 6	62	2	31	1			
7	3923	30	131	101	2.34		
8	1304	20	65	35	1.78		
9	74	2	37	7			
10	65	2	32	1			
11	90	2	45	15			
12	268	2	134	104			
13	95	2	47	17			
14	9107	30	303	273	3.33	129	21,163) ± 4.8%
15	12820	20	641	611	5.7	262	23,320)
16	135	2	67	37			
17	92	2	46	16			
Remnant	289	2	145	113			
EE (0.5 ml.)	90	2	45	13	3.7		
BE (0.1 ml.)	102	2	51	19	5.2		

Ether Extract (EE) 810 ml. = 21060 cpm.
Benzene Extract (BE) 49.5 ml. = 9405 cpm.

Pd.g. corrected for carrier pregnanediol assuming 80% Column recovery.

Spectrometry a) Column Eluate. (*IV).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	832	30	28				
Tube 5	62	2	31	3			
6	3559	60	59	31	1.38		
7	4513	60	75	47	1.48		
8	58	2	29	1			
9	79	2	39	11			
10	126	2	63	35			
11	146	2	73	45			
12	10019	60	167	139	1.92	112.05	12,405)
13	20575	60	343	315	2.6	280.5	11,229) ± 4.7%
14	6219	60	104	76	1.2	67.05	11,343)

Spectrometry a) Column Eluate. (*IV).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	717	30	25				
Tube 5	64	2	32	7			
6	3402	60	57	32	1.34		
7	2009	60	334	8	1.18		
8	69	2	34	9			
9	124	2	62	37			
10	8606	60	143	118	1.8	244.3	4830)
11	6702	60	112	87	1.64	177.0	4915) ± 0.75%
12	2334	60	39	14	1.22	28.25	4956)
13	54	2	27	2			

Spectrometry. b) Urine. (*IV)

Sample : 0.3 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid plus 6 ml. absolute ethanol.

Experimental blank: 0.3 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine. (*IV)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	933	30	31		
MH^3S	419969	30	13999		
SB	1022	30	34		
0.3 ml. UB	2312	60	38.5)	38	
0.3 ml. UB	2291	60	38.1)		
0.2 ml. H^3IS	100001	20.93			
0.2 ml. H^3IS	100002	20.34	)	4854	4820
0.2 ml. H^3IS	100001	20.51			

Spectrometry b) Urine. (*IV).

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.3 ml. Day 1	4006	60	66.7	28.7		
0.3 ml. Day 1	3861	60	64.3	26.3	27.5	3.3
0.3 ml. Day 2	2681	60	44.7	6.7		
0.3 ml. Day 2	2668	60	44.5	6.5	6.6	12.4
0.3 ml. Day 3	2518	60	42	4.0		
0.3 ml. Day 3	2496	60	41.6	3.6	3.8	21.2
0.3 ml. Day 4	2466	60	41.0	3.0		
0.3 ml. Day 4	2524	60	42.0	4.0	3.5	23.1

Spectrometry c) Urine plus internal standard.

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.3 ml. Day 1	14107	10	1411	1344	
0.3 ml. Day 1	13498	10	1350	1286	3.67
0.3 ml. Day 2	14595	10	1460	1415	
0.3 ml. Day 2	14573	10	1457	1412	3.41
0.3 ml. Day 3	11936	10	1194	1152	
0.3 ml. Day 3	11676	10	1168	1126	4.23
0.3 ml. Day 4	11990	10	1199	1158	
0.3 ml. Day 4	12266	10	1227	1185	4.12

Total Counts Recovered from Column as Pregnanediol. (*IV)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	64134	15558	7420	3241	90353
Per cent	71.0	17.2	8.2	3.6	100
Per cent Injected Dose.					13.6

Total Counts Recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	8669	2394	1092	592	12747
Per cent	68.0	18.8	8.6	4.6	100
Per cent Injected Dose.					1.92

Total Counts Recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	211942	66018	37506	35509	351035
Per cent	60.4	18.8	10.7	10.1	100
Per cent Injected Dose.					52.9

Total Pregnanediol Recovery per day.

	Day 1	Day 2	Day 3	Day 4	Total
gamma	421.2	688.8	644.8	665.3	605

(Day 2 corrected for carrier pregnanediol assuming 80% Column recovery).

$$\text{Ratio of } \frac{\%\text{ Recovery of injected dose as pregnanediol}}{\%\text{ Recovery of injected dose as peak X}} = \frac{7.08}{1}$$

$$\text{Ratio of } \frac{\%\text{ Recovery of injected dose in urine}}{\%\text{ Recovery of injected dose in pregnanediol}} = \frac{3.9}{1}$$

$$\text{Ratio of } \frac{\%\text{ Recovery of injected dose in urine}}{\%\text{ Recovery of injected dose as peak X}} = \frac{27.6}{1}$$

	Day 1		Day 2	
	cpm	Ratio	cpm	Ratio
Ether extract	97340	1.91	21060	2.38
Benzene extract	87710	1.72	9405	1.06
Pregnanediol tubes	50900	1	8840	1

Injection Solution (*IV): 0.2 ml. = 730098 cpm

Total radioactivity injected $= \frac{10}{11}$ of 730098 = 664000 cpm.

Secretion Rate of Progesterone (*IV).

$$= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$$

$$= 664000 \div \frac{90353}{605} \text{ gamma}$$

$$= \frac{664000 \times 605}{90353}$$

= 4.446 mgm. per day.

Subject *V Results.

Patient: R.S.: Height 5 ft. 9½ ins.

Weight 190 lbs.

Normal male volunteer.

Urine: Day 1 : 960 ml.

Day 2 : 1410 ml.

Day 3 : 850 ml.

Day 4 : 840 ml.

Extraction: 500 ml. aliquots daily urine output extracted by method 3 (b).

Columns: Day 1 : Column II : Front; tube 6

Day 2 : Column II : Front; tube 5

Day 3 : Column II : Front; tube 5

Day 4 : Column III : Front; tube 6

Spectrophotometry: Column Eluate.

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental Blank: 2.0 ml. from tube 1.

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry: Column Eluate. (*V).

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	000	000	000	000	
EB	.026	.023	.019	.000	100% Reference.
25 ESP	.133	.208	.132	.075	
25 ESP	.112	.190	.120	.074	
25 ESP	.124	.204	.126	.079	
50 ESP	.258	.416	.250	.162	
50 ESP	.230	.385	.238	.151	
50 ESP	.210	.357	.207	.148	25 gamma = .077
Tube 9	.770	.530	.414	-	
10	.938	1.069	.730	.235	76.3
11	.733	.840	.590	.179	58.1
12	.522	.485	.368		

Spectrophotometry: Column Eluate. (*V).

Day 2	Wavelength Microns.			Allen	Pregnanediol
Sample.	390	452	460	Correction.	Gamma.
AB	000	000	000	000	
EB	.054	.053	.052	.001	100% Reference.
25 ESP	.124	.196	.124	.072	
25 ESP	.246	.314	.232	.075	
50 ESP	.300	.433	.267	.149	25 gamma = .074
50 ESP	.400	.542	.384	.150	
Tube 11	.268	.226	.178	-	
12	.390	.303	.231	-	
13	.324	.420	.303	.107	36.2
14	.190	.214	.176	.031	10.46

Spectrophotometry: Column Eluate. (*V).

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.058	.047	.039	.000	100% Reference.
25 ESP	.248	.303	.207	.075	
50 ESP	.321	.480	.310	.164	25 gamma = .079
Tube 9	.555	.445	.338	-	
10	.660	.520	.420	-	
11	.805	.990	.715	.230	72.8
12	.361	.304	.232	-	

Day 4	390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.058	.046	.038	.000	100% Reference.
25 ESP	.250	.304	.210	.074	
50 ESP	.343	.478	.297	.158	25 gamma = .077
Tube 10	.440	.352	.259	-	
11	.650	.600	.446	-	
12	.812	1.025	.695	.272	88.3
13	.382	.303	.230	-	

Spectrometry c) Column Eluate. (*V).

Sample: 0.5 ml. from tubes, 0.5 ml. from ether extract, 0.1 ml. from benzene extract, plus 10 ml. Scintillation Fluid to each.

Experimental Blank: from tube 1.

Counting: High Voltage tap 7.

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	52	2	26
MH^3S	68060	5	13612
0.2 ml. ES	8536	2	4268
0.5 ml. ES	21501	2	10750
0.2 ml. EIS	3650061	5	730012
SB	561	20	28

Spectrometry a) Column Eluate. (*V)

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 1 EB	954	30	32				
Tube 6	14801	40	370	338	3.2		
7	18042	40	451	419	3.5		
8	238	2	119	87			
9	741	2	370	338			
10	21633	10	2163	2131	15	190.75	111701) ± 2.4%
11	17352	10	1735	1702	13	145.25	117177)
12	562	2	281	249			
13	248	2	124	92			
14	126	2	63	31			
15	86	2	43	11			
Remnant	407	2	200	172			
EE (0.5 ml.)	158	2	79	51	6.4		
BE (0.1 ml.)	342	2	171	143	9.3		

Ether Extract (EE) 800 ml. = 81600 cpm.
Benzene Extract (BE) 48 ml. = 68640 cpm.

Spectrometry a) Column Eluate. (*V)

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 2 EB	912	30	30				
Tube 5	71	2	35	5			
6	88	2	44	14			
7	2040	25	82	52	2.1		
8	60	2	30	-			
9	60	2	30	-			
10	81	2	40	10			
11	93	2	46	16			
12	2160	30	72	42			
13	11041	60	184	154	2	90.5	17017) ± 3.5%
14	4322	60	72	42	1.5	26.15	16016)
15	66	2	33	3			
Remnant	141	2	70	42			

Spectrometry a) Column Eluate. (*V).

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 3 EB	955	30	32				
Tube 5	72	2	36	4			
6	1500	25	60	28	1.42		
7	2349	60	39	7	1.28		
8	63	2	31	-			
9	2568	60	43	11			
10	3834	60	64	32			
11	8271	60	138	106	1.82	182	5824
12	79	2	39	7			
13	56	2	28	-			
14	68	2	34	2			
15	49	2	25	-			
Remnant	390	2	195	167			

Spectrometry a) Column Eluate. (*V).

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 4 EB	906	30	30				
Tube 6	66	2	33	3			
7	1260	25	50	20	1.73		
8	71	2	35	5			
9	67	2	33	3			
10	74	2	37	7			
11	2517	60	42	12			
12	4538	60	76	46	1.5	220.75	2084
13	67	2	33	3			
14	66	2	33	3			
15	48	2	24	-			
Remnant	95	2	47	19			

<u>Spectrometry b) Urine.</u> (*V)

Sample: 0.3 and 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.3 and 0.6 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High voltage tap 7 (automatic).

Time: in minutes.

Spectrometry b) Urine. (*V)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	933	30	31		
MH^3S	419969	30	13666		
SB	1022	30	34		
0.3 ml. UB	2291	60	38.1)	38	
0.3 ml. UB	2312	60	38.5)		
0.6 ml. UB	2311	60	38.5)	38	
0.6 ml. UB	2270	60	37.8)		
0.2 ml. H^3IS	100001	20.93			
0.2 ml. H^3IS	100002	20.34	)	4854	4820
0.2 ml. H^3IS	100001	20.51			

Spectrometry b) Urine. (*V)

Sample.	Counts.	Time.	cpm.	BC.	Mean.	Sd±%
0.3 ml. Day 1	3603	60	60	22		
0.3 ml. Day 1	3765	60	62.75	24.75	23.4	3.4
0.6 ml. Day 2	2807	60	46.8	8.8		
0.6 ml. Day 2	2810	60	46.8	8.8	8.8	9.5
0.6 ml. Day 2	2812	60	46.9	8.9		
0.6 ml. Day 2	2797	60	46.6	8.6	8.7	9.6
0.6 ml. Day 2	2808	60	46.8	8.8		
0.6 ml. Day 2	2799	60	46.6	8.6	8.7	9.6

Spectrometry c) Urine plus internal standard. (*V)

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.3 ml. Day 1	13644	10	1364	1304	
0.3 ml. Day 1	13850	10	1385	1322	3.67
0.6 ml. Day 2	14753	10	1475	1428	
0.6 ml. Day 2	15139	10	1514	1467	3.33
0.6 ml. Day 3	14870	10	1487	1440	
0.6 ml. Day 3	14288	10	1429	1382	3.42
0.6 ml. Day 4	14749	10	1475	1428	
0.6 ml. Day 4	14468	10	1447	1400	3.41

Total Counts recovered from Columns as Pregnanediol. (*V)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	73594	5527	1802	771	81694
Per cent	90.1	6.75	2.2	0.95	100
Per cent Injected Dose.					12.3

Total Counts recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	14534	1861	595	420	17410
Per cent	83.5	10.7	3.4	2.4	100
Per cent Injected Dose.					2.62

Total Counts recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	274330	68864	42152	41534	426880
Per cent	66.3	16.1	9.9	9.7	100
Per cent Injected Dose.					64.3

Total Pregnanediol Recovered per Day. (*V)

	Day 1	Day 2	Day 3	Day 4	Mean.
gamma	645	329	309.4	370.6	413.6

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose as Pregnanediol}}{\text{\% Recovery of injected dose as Peak X}} = \frac{4.62}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose as Urine.}}{\text{\% Recovery of injected dose as Pregnanediol}} = \frac{5.23}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose in Urine.}}{\text{\% Recovery of injected dose as Peak X}} = \frac{24.2}{1}$$

	Day 1	
	cpm.	Ratio.
Ether Extract	81600	2.12
Benzene Extract	68640	1.79
Pregnanediol	38330	1

Injection Solution : 0.2 ml. = 730012 cpm.

Total Radioactivity injected = $\frac{10}{11}$ of 730012 cpm = 664000 cpm.

Secretion Rate of Progesterone =

$$= \frac{\text{Total Radioactivity Injected (cpm).}}{\text{Specific Activity of Pregnanediol.}}$$

$$664000 \div \frac{81694}{413.6} \text{ gamma}$$

$$= \frac{66400 \times 413.6}{81694}$$

= 3.362 mgm. per day.

Subject *VI Results.

Patient: M.I. : Height 5 ft. 8 ins.

Weight 159 lbs.

Normal male volunteer.

Urine: Day 1 : 725 ml.

Day 2 : 1860 ml.

Day 3 : 2480 ml.

Day 4 : 1290 ml.

Extraction : 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1: Column II : Front; tube 5

Day 2: Column I : Front; tube 5

Day 3: Column II : Front; tube 6

Day 4: Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental Blank: 2.0 ml. from tube 1.

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry - Column Eluate. (*VI).

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.024	.022	.019	.000	100% Reference.
25 ESP	.111	.195	.115	.082	
50 ESP	.235	.400	.247	.159	25 gamma = .080
Tube 10	.512	.348	.250		
11	.501	.586	.400	.136	42.5
12	.808	1.221	.769	.432	135.0
13	.274	.291	.212	.048	15
Day 2					
AB	0	0	0	0	
EB	.024	.028	.019	.006	100% Reference.
25 ESP	.133	.230	.145	.091	25 gamma = .085
50 ESP	.243	.406	.243	.163	
Tube 10	.430	.325	.243		
11	.510	.748	.482	.252	74.12
12	.299	.374	.261	.094	27.65
13	.276	.270	.196		

Spectrophotometry - Column Eluate. (*VI).

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.123	.164	.139	.033	100% Reference.
25 ESP	.340	.427	.298	.108	
25 ESP	.157	.240	.141	.091	25 gamma = .082
50 ESP	.367	.482	.305	.146	
50 ESP	.270	.398	.233	.147	
Tube 9	.294	.190	.129	-	
10	.482	.516	.316	.117	35.67
11	.360	.412	.248	.108	32.93
12	.149	.090	.066	-	
Day 4.					
AB	0	0	0	0	
EB	.027	.024	.022	.000	100% Reference.
25 ESP	.085	.153	.074	.084	25 gamma = .082
50 ESP	.225	.370	.190	.163	
Tube 10	.543	.420	.312	-	
11	.553	.632	.433	.139	42.38
12	1.045	1.485	.920	.503	153.35

Spectrometry a) Column Eluate. (*VI)

Sample: 0.5 ml. from tube plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	809	25	32
MH^3S	464914	30	15497
0.2 ml. ES	155101	30	5170
0.5 ml. ES	389102	30	12970
0.2 ml. EIS	8581366	10	858137
SB	859	25	34

Spectrometry a) Column Eluate. (*VI).

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 1 EB	959	25	38				
Tube 5	43	1	43	5			
6	20101	30	670	632	6.1		
7	17522	30	584	546	4.6		
8	97	1	97	59			
9	157	1	157	119			
10	404	1	404	366			
11	44881	30	1496	1458	7.2	106.25	137224)
12	129002	30	4300	4262	12	337.5	126282) ± 4.2%
13	16354	30	545	507	4.50	37.5	135200)
14	102	1	102	64			

Spectrometry a) Column Eluate. (*VI).

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 2 EB	909	25	36				
Tube 5	36	1	36	-			
6	3214	30	107	71	7		
7	2552	30	85	49	6.5		
8	46	1	46	10			
9	53	1	53	17			
10	98	1	98	62			
11	14251	30	475	439	4.2	185.3	23691) ± 4.2%
12	6423	30	214	178	2.9	69.1	25760)
13	54	1	54	18			
14	26	1	26	-			

Spectrometry a) Column Eluate. (*VI).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	2710	50	54				
Tube 6	3459	50	69	15	1.55		
7	2739	50	55	1	1.48		
8	26	1	26	-			
9	2910	50	58	4			
10	7310	50	146	92	2	89.2	10314) ± 0.5%
11	6895	50	138	84	1.9	82.3	10207)
12	2920	50	58	4			
13	57	1	57	3			
14	45	1	45	-			
Remnant	57	1	57	21			

Spectrometry a) Column Eluate. (*VI).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	1911	50	38				
Tube 5	41	1	41	3			
6	2729	50	54	16	1.3		
7	2483	50	50	12	1.3		
8	38	1	38	-			
9	38	1	40	2			
10	2570	50	51	13			
11	3591	50	72	34	1.5	106	3204) ± 4.2%
12	7549	50	151	113	1.9	383.4	2947)
13	1998	50	40	2			
14	40	1	40	2			
Remnant	80	1	80	44			

<u>Spectrometry b) Urine.</u> (*VI).

Sample: 0.3, 0.4, 0.6 ml. urine (after thoroughly shaking specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.3, 0.4, 0.6 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine. (*VI).

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	933	60	30		
MH^3S	419969	60	15666		
SB	1022	60	34		
0.3 ml. UB	2291	60	38.1	38	
0.3 ml. UB	2312	60	38.5		
0.4 ml. UB	2307	60	38.5	38	
0.4 ml. UB	2250	60	37.5		
0.6 ml. UB	2311	60	38.5	38	
0.6 ml. UB	2270	60	37.8		
0.2 ml. H^3IS	100001	20.93		4854	4820
0.2 ml. H^3IS	100002	20.34			
0.2 ml. H^3IS	100001	20.51			

Spectrometry: b) Urine. (*VI).

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.3 ml. Day 1	4053	60	67.5	29.5		
0.3 ml. Day 1	4067	60	67.8	29.8	29.5	3.2
0.4 ml. Day 2	2751	60	45.9	8		
0.4 ml. Day 2	2824	60	47.1	9	8.5	9.9
0.6 ml. Day 3	2393	60	39.9	2		
0.6 ml. Day 3	2388	60	39.8	2	2	40
0.6 ml. Day 4	2452	60	40.9	3		
0.6 ml. Day 4	2406	60	40.1	2	2.5	33

Spectrometry c) Urine plus Internal Standard. (*VI).

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.3 ml. Day 1	14966	10	1497	1429	
0.3 ml. Day 1	15144	10	1514	1446	3.37
0.4 ml. Day 2	13999	10	1400	1354	
0.4 ml. Day 2	43731	30	1458	1411	3.49
0.6 ml. Day 3	42524	30	1417	1377	
0.6 ml. Day 3	44359	30	1479	1439	3.42
0.6 ml. Day 4	44181	30	1473	1432	
0.6 ml. Day 4	43216	30	1441	1401	3.4

Total Counts Recovered from Column as Pregnanediol. (*VI).

	Day 1	Day 2	Day 3	Day 4	Total
cpm	90292	22952	8730	3793	125767
Per cent	71.8	18.3	6.9	3.0	100
Per cent Injected Dose.					16.1

Total Counts Recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	17081	4464	793	727	23065
Per cent	74.1	19.4	3.4	3.1	100
Per cent Injected Dose.					2.96

Total Counts Recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	240253	137942	28272	27412	433879
Per cent	55.4	31.8	6.5	6.3	100
Per cent Injected Dose.					55.6

Total Pregnanediol Recovered per Day.

	Day 1	Day 2	Day 3	Day 4	Total
gamma	697.8	946.4	850.6	1262.7	939.4

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose as pregnanediol}}{\text{\% Recovery of injected dose as Peak X}} = \frac{5.44}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose in Urine.}}{\text{\% Recovery of injected dose as Pregnanediol}} = \frac{3.54}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of injected dose in Urine.}}{\text{\% Recovery of injected dose in Peak X}} = \frac{18.8}{1}$$

Injection Solution : 0.2 ml. = 858139 cpm.

Total Radioactivity Injected = $\frac{10}{11}$ of 858139 cpm = 780,000 cpm.

Secretion rate of Progesterone.

$$= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$$

$$= 780000 \div \frac{125767}{939.4} \quad \text{gamma}$$

$$= \frac{780000 \times 939.4}{125767}$$

= 5.826 mgm. per day.

SUBJECT *VII RESULTS.

Patient: F.S. : Height 5 ft. 8 ins.

Weight 160 lbs.

Normal Male Volunteer.

Urine: Day 1 : 1170 ml.

Day 2 : 1000 ml.

Day 3 : 1030 ml.

Day 4 : 970 ml.

Extraction: 500 ml. aliquots daily urine output extracted by method 3 (b).

Columns: Day 1 : Column II : Front; tube 5

Day 2 : Column I : Front; tube 5

Day 3 : Column II : Front; tube 5

Day 4 : Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental Blank: 2.0 ml. from tube 1.

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry - Column Eluate. (*VII).

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.042	.036	.032	-	100% Reference.
25 ESP	.074	.138	.082	.060)	25 gamma = .068
50 ESP	.192	.337	.194	.144)	
Tube 10	.169	.137	.099	-	
11	.244	.304	.207	.079	
12	.349	.442	.290	.123	
13	.100	.093	.071	-	
Day 2					
AB	0	0	0		
EB	.070	.062	.058	-	100% Reference.
25 ESP	.092	.158	.088	.068)	75 gamma = .075
50 ESP	.256	.411	.251	.158)	
Tube 9	.410	.340	.254	-	
10	.320	.275	.182	-	
11	.470	.639	.404	.202	67.3
12	.271	.304	.193	.072	24.0

Spectrophotometry - Column Eluate. (*VII).

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.039	.034	.030	.000	
25 ESP	.134	.193	.113	.070	25 gamma = .076
50 ESP*	.230	.377	.210	.157	
Tube 11	.406	.330	.250	-	
12*	.514	.712	.440	.235	77.3
13	.440	.513	.348	.119	39.14
14	.270	.269	.196	-	
Day 4.					
AB	0	0	0	0	
EB	.058	.064	.053	.008	
25 ESP	.133	.200	.117	.075	25 gamma = .077
50 ESP	.247	.390	.220	.156	
Tube 10	.378	.240	.176	-	
11	.440	.480	.313	.104	33.8
12	.520	.620	.398	.161	52.3
13	.330	.310	.222		

* Samples for pregnanediol spectrum.

Spectrometry a) Column Eluate. (*VII).

Sample: 0.5 ml. from tubes, 0.1 ml. from benzene extract, plus 10 ml. Scintillation fluid to each.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	950	30	32
MH^3S	459082	30	15303
0.2 ml. ES	159345	30	5312
0.5 ml. ES	403084	30	13436
0.2 ml. EIS	8377759	10	837776
SB	1184	30	39

Spectrometry a) Column Eluate. (*VII).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	1116	30	37				
Tube 5	49	1	49	12			
6	24495	30	816	779	5.3		
7	12293	30	410	373	3.9		
8	87	1	81	44			
9	116	1	116	79			
10	318	1	318	271			
11	49846	30	1662	1615	7.5	72.5	222621) ± 1.6%
12	77526	30	2584	2547	8.2	113	225398)
13	163	1	163	126			
14	72	1	72	35			
B.E. 0.1 ml.	5853	30	195	156	2.8		
Remnant	190	1	190	151			

Benzene Extract (BE) 48.5 ml. = 75560 cpm.

Spectrometry a) Column Eluate. (*VII).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	1182	30	39				
Tube 5	51	1	51	12			
6	7370	30	246	207	3.1		
7	2456	30	82	43	2.0		
8	75	1	72	36			
9	31	1	31	-			
10	97	1	97	58			
11	151	1	151	112			
12	21114	30	704	665	5.2	168.25	39525) ± 2.2%
13	7972	30	266	227	3.2	60	37833)
14	69	1	69	30			
15	43	1	43	4			
BE (0.1 ml.)	2127	30	71	32	1.9		
Remnant	61	1	61	22			

Benzene Extract (BE) 48.5 ml. = 15520 cpm.

Spectrometry a) Column Eluate. (*VII).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.	
Day 3 EB	1572	30	52					
Tube 5	54	1	54	2				
6	3691	30	123	71	2.4			
7	2759	30	92	40	2.2			
8	63	1	63	11				
9	57	1	57	5				
10	81	1	81	29				
11	90	1	90	38				
12	6533	30	218	166	3	183.25	9053)	
13	4166	30	139	87	2.5	97.85	8891)±	.9%
14	2005	30	67	15				
Remnant	94	1	94	55				

Spectrometry a) Column Eluate. (*VII).

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	1468	30	49				
Tube 5	52	1	52	3			
6	2530	30	84	35	2.2		
7	1981	30	66	17	2		
8	64	1	64	15			
9	65	1	65	16			
10	69	1	69	20			
11	2451	30	82	33	2.1	84.5	3905) ± 2%
12	2936	30	98	49	2.3	130.75	3826)
13	70	1	70	21			
14	57	1	57	8			
15	57	1	57	8			
Remnant	49	1	49	10			

Spectrometry: b) Urine. (*VII)

Sample: 0.3 and 0.4 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6.0 ml. absolute Ethanol.

Experimental Blank: 0.3 and 0.4 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (automatic).

Time: in minutes.

Spectrometry b) Urine. (*VII)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	933	30	31		
MH^{3}S	419969	30	13666		
SB	1022	30	34		
0.3 ml. UB	2291	60	38.1	38	
0.3 ml. UB	2312	60	38.5		
0.4 ml. UB	2307	60	38.5	38	
0.4 ml. UB	2250	60	37.5		
0.2 ml. H^{3}IS	100001	20.93			
0.2 ml. H^{3}IS	100002	20.34		4854	4820
0.2 ml. H^{3}IS	100002	20.51			

Spectrometry b) Urine. (*VII)

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.3 ml. Day 1	3659	60	61.0	23		
0.3 ml. Day 1	3684	60	61.4	23	23	3.9
0.3 ml. Day 2	2839	60	47.3	9		
0.3 ml. Day 2	2877	60	47.9	10	9.5	8.8
0.4 ml. Day 3	2659	60	44.3	6.3		
0.4 ml. Day 3	2546	60	42.4	4.4	5.3	15.5
0.4 ml. Day 4	2445	60	40.7	2.7		
0.4 ml. Day 4	2496	60	41.6	3.6	3.2	25.3

Spectrometry e) Urine plus internal standard. (*VII).

Sample.	Counts.	Time.	cpm.	T.B.C.	Q.F.
0.3 ml. Day 1	48324	30	1611	1550	
0.3 ml. Day 1	48397	30	1613	1552	3.1
0.3 ml. Day 2	45692	30	1523	1466	
0.3 ml. Day 2	46071	30	1536	1488	3.26
0.4 ml. Day 3	42913	30	1430	1386	
0.4 ml. Day 3	42778	30	1426	1384	3.48
0.4 ml. Day 4	41505	30	1384	1343	
0.4 ml. Day 4	41070	30	1369	1327	3.6

Total Counts recovered from Column as Pregnanediol. (*VII).

	Day 1	Day 2	Day 3	Day 4	Total
cpm	97391	17840	5212	1591	122034
Per cent	79.8	14.6	4.3	1.3	100
Per cent Injected Dose.					16

Total Counts recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	26957	5000	2287	1009	35253
Per cent	76.5	14.2	6.5	2.8	100
Per cent Injected Dose.					4.63

Total Counts recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	278070	103233	47493	27936	456732
Per cent	60.9	22.6	10.4	6.1	100
Per cent Injected Dose.					59.9

Total Pregnanediol recovered per Day.

	Day 1	Day 2	Day 3	Day 4	Mean
gamma	434	456.5	579	417.6	471.8

(*VII)

$$\text{Ratio of } \frac{\%\ \text{Recovery of Injected Dose as Pregnanediol}}{\%\ \text{Recovery of Injected Dose as Peak X}} = \frac{3.46}{1}$$

$$\text{Ratio of } \frac{\%\ \text{Recovery of Injected Dose in Urine.}}{\%\ \text{Recovery of Injected Dose in Pregnanediol}} = \frac{3.74}{1}$$

$$\text{Ratio of } \frac{\%\ \text{Recovery of Injected Dose in Urine.}}{\%\ \text{Recovery of Injected Dose as Peak X}} = \frac{12.9}{1}$$

	Day 1		Day 2	
	cpm	Ratio	cpm	Ratio
Benzene Extract	75560	1.82	15520	1.74
Pregnanediol	41620	1	8920	1

Injection Solution: 0.2 ml. = 837776 cpm.

Total Radioactivity injected = $\frac{10}{11}$ of 837776 = 762000

Secretion Rate of Progesterone = $\frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol.}}$

$$= 76200 \div \frac{122034}{471.8} \text{ gamma}$$

$$= \frac{762000 \times 471.8}{122034}$$

= 2.946 mgm. per day.

SUBJECT *IX RESULTS.

Patient: J.G. : Height 5 ft. 11 ins.

Weight 136 lbs.

Normal Male Volunteer.

Urine: Day 1 : 615 ml.

Day 2 : 750 ml.

Day 3 : 750 ml.

Day 4 : 1370 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1 : Column IV : Front; tube 5

Day 2 : Column I : Front; tube 5

Day 3 : Column IV : Front; tube 5

Day 4 : Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental Blank: 2.0 ml. from tube 1.

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry - Column Eluate. (*IX)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.023	.023	.017	.003	100% Reference.
25 ESP	.115	.188	.114	.073)	25 gamma = .074
50 ESP	.199	.349	.198	.150)	
Tube 10	.422	.263	.186	-	
11	.418	.340	.208	-	
12	.498	.628	.396	.181	61.14
13	.304	.272	.198	-	
Day 2					
AB	0	0	0	0	
EB	.007	.010	.009	.002	100% Reference.
25 ESP	.093	.163	.090	.072)	25 gamma = .074
50 ESP	.215	.360	.203	.151)	
Tube 10	.340	.228	.168	-	
11	.443	.511	.335	.122	41.2
12	.330	.332	.230	.052	17.6
13	.320	.278	.207	-	

Spectrophotometry - Column Eluate. (*IX)

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.091	.094	.093	.002	100% Reference
25 ESP	.104	.174	.102	.071	25 gamma = .071
50 ESP	.196	.336	.190	.143	
Tube 10	.436	.295	.220	-	
11	.370	.330	.220	-	
12	.474	.520	.344	.111	39.1
13	.259	.179	.134	-	

Day 4	390	425	460	Allen Correction	Pregnanediol Gamma
AB	0	0	0	0	
EB	.038	.039	.034	.003	
25 ESP	.105	.173	.098	.072	25 gamma = .068
50 ESP	.172	.305	.172	.133	
Tube 10	.185	.120	.091	-	
11	.221	.199	.146	-	
12	.240	.264	.170	.059	21.7
13	.230	.177	.125	-	

Spectrometry a) Column Eluate. (*IX)

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	992	30	33
MH3S	470141	30	15671
0.5 ml. ES	383971	30	12799
0.2 ml. ES	152309	30	5077
0.2 ml. EIS	8636344	10	863634
SB	1561	30	52

Spectrometry a) Column Eluate. (*IX)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	1689	30	56				
Tube 5	82	1	82	26			
6	28021	30	934	878	5.75		
7	9632	30	321	265	3.55		
8	115	1	115	59			
9	255	1	255	199			
10	464	1	464	408			
11	40384	30	1346	1290			
12	126359	30	4212	4156	12	153	278000
13	665	1	665	609			
14	110	1	110	44			
Remnant	716	1	716	664			

Spectrometry a) Column Eluate. (*IX)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	1536	30	51				
Tube 5	68	1	68	17			
6	6662	30	222	171	3		
7	2433	30	81	30	2.1		
8	56	1	56	5			
9	122	1	122	71			
10	147	1	147	96			
11	17719	30	591	540	4.63	103	52427) ± 2.9%
12	8851	30	295	244	3.4	44	55459)
13	93	1	93	42			
14	70	1	70	19			
Remnant	120	1	120	68			

Spectrometry a) Column Eluate. (*IX)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	1735	30	58				
Tube 5	67	1	67	9			
6	3599	30	120	62	2.4		
7	2942	30	98	40	2.2		
8	70	1	70	12			
9	98	1	98	40			
10	3481	30	116	58			
11	6058	30	202	144			
12	10774	30	359	301	3.7	97.75	20793
13	81	1	81	23			
14	53	1	53	-			
Remnant	81	1	81	29			

Spectrometry a) Column Eluate. (*IX)

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 4 EB	1579	30	53				
Tube 5	58	1	58	5			
6	2584	30	86	33	2.1		
7	1862	30	62	9			
8	73	1	73	20			
9	68	1	68	15			
10	65	1	65	12			
11	2399	30	80	25			
12	3421	30	114	61	2.4	54.25	11061
13	1737	30	58	5			
14	54	1	54	1			
Remnant	66	1	66	14			

Spectrometry b) Urine. (*IX)

Sample: 0.3 and 0.4 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6.0 m.l. absolute Ethanol.

Experimental Blank: 0.3 and 0.4 ml. tritium from urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (automatic).

Time: in minutes.

Spectrometry b) Urine. (*IX)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	1839	60	31		
MH^3S	200002	1.34	14179		
SB	1757	60	29		
0.3 ml. UB	2828	60	47.1	49	
0.3 ml. UB	3080	60	51.3		
0.4 ml. UB	3006	60	50.1	49	
0.4 ml. UB	2884	60	48		
0.2 ml. H^3IS	94673	20	4733	4733	4700
0.2 ml. H^3IS	94668	20	4733		

Spectrometry b) Urine. (*IX)

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±
0.3 ml. Day 1	4443	60	74	25		
0.3 ml. Day 1	4333	60	72	23	24	4.2
0.3 ml. Day 2	3278	60	54.8	5.8		
0.3 ml. Day 2	3248	60	54.1	5	5.4	17
0.3 ml. Day 3	3014	60	50.1	1		
0.3 ml. Day 3	3048	60	50.8	2	1.5	60.6
0.4 ml. Day 4	2960	60	49.3	0.3		
0.4 ml. Day 4	3003	60	50	1.0	0.65	138.6

Spectrometry c) Urine plus internal standard. (*IX)

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.3 ml. Day 1	27331	20	1366	1292	
0.3 ml. Day 1	26703	20	1335	1263	3.66
0.3 ml. Day 1	26275	20	1314	1259	
0.3 ml. Day 1	25563	20	1278	1224	3.72
0.3 ml. Day 1	25444	20	1272	1222	3.82
0.3 ml. Day 1	25810	20	1290	1239	
0.4 ml. Day 1	25152	20	1258	1209	
0.4 ml. Day 1	24857	20	1243	1193	3.92

Total Counts recovered from Columns as Pregnanediol. (*IX)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	51119	11760	4515	1671	69065
Per cent	74.0	17.0	6.5	2.4	100
Per cent Injected Dose.					8.8

Total Counts recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	14059	3015	1530	904	19508
Per cent	72.1	15.5	7.8	4.6	100
Per cent Injected Dose.					2.48

Total Counts recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	180072	51165	14325	8727	254289
Per cent	70.8	20.2	5.6	3.4	100
Per cent Injected Dose.					32.4

Total Pregnanediol recovered per day.

	Day 1	Day 2	Day 3	Day 4	Total
gamma	188	220.5	147	148.5	176

(*IX)

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered as Pregnanediol}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{3.55}{1}$$

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered in Urine}}{\text{\% Injected Dose Recovered in Pregnanediol}} = \frac{3.68}{1}$$

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered in Urine}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{13.1}{1}$$

Injection Solution : 0.2 ml. = 863434 cpm.

Total Radioactivity Injected = $\frac{10}{11}$ of 863434 = 785000

$$\text{Secretion Rate of Progesterone} = \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific activity of Pregnanediol}}$$

$$= 785000 \div \frac{69065}{176} \text{ gamma}$$

$$= \frac{785000 \times 176}{69065}$$

$$= 2.002 \text{ mgm. per day.}$$

SUBJECT *XII RESULTS.

Patient: Height 5 ft. 6½ ins.

Weight 157 lbs.

Normal Male Volunteer.

Urine: Day 1 : 440 ml.

Day 2 : 710 ml.

Day 3 : 510 ml.

Day 4 : 575 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1 : Column II : Front; tube 5

Day 2 : Column IV : Front; tube 5

Day 3 : Column II : Front; tube 5

Day 4 : Column IV : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental Blank: 2.0 ml. from tube 1.

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1, 2 and 3.

Spectrophotometry - Column Eluate. (*XII)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.010	.008	.006	.000	100% Reference.
25 ESP	.154	.233	.134	.089)	
50 ESP	.260	.429	.254	.172)	25 gamma = .087
25 ESP	.130	.219	.130	.089)	
50 ESP	.225	.407	.240	.174)	
Tube 12	.190	.178	.152	-	
13	.560	.796	.498	.267	76.7
14	.380	.491	.318	.142	40.8
15	.190	.170	.150	-	

Day 2

Sample.	390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.015	.014	.014	.000	100% Reference.
25 ESP	.118	.200	.119	.082)	25 gamma = .084
50 ESP	.283	.439	.258	.169)	
Tube 11	.151	.146	.099	-	
12	.430	.612	.394	.200	59.53
13	.326	.405	.254	.115	34.26
14	.125	.122	.104	-	

Spectrophotometry - Column Eluate. (*XII).

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol Gamma
AB	0	0	0		
EB	.020	.018	.015	.000	100% Reference.
25 ESP	.081	.156	.077	.077)	25 gamma = .079
50 ESP	.276	.431	.264	.161)	
Tube 11	.359	.321	.245	-	
12	.655	.956	.621	.318	100.6
13	.540	.752	.502	.231	73.1
14	.274	.294	.248	.033	10.4

Day 4

Sample	390	425	460	Allen Correction	Pregnanediol Gamma
AB	0	0	0		
EB	.025	.024	.024	.001	100% Reference.
25 ESP	.115	.185	.103	.076)	25 gamma = .081
50 ESP	.262	.420	.244	.167)	
Tube 12	.301	.271	.209	-	
13	.550	.730	.450	.230	71
14	.350	.385	.240	.090	27.8
15	.276	.216	.183	-	

Spectrometry a) Column Eluate. (*XII)

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	837	30	28
MH^3S	450913	30	15030
0.2 ml. ES	141758	30	4725
0.2 ml. EIS	8470109	10	847011
SB	921	30	31

Spectrometry a) Column Eluate. (*XII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	918	30	31				
Tube 5	34	1	34	3			
6	10230	30	341	310	3.52		
7	21895	30	730	699	5.07		
8	58	1	58	27			
9	79	1	79	48			
10	86	1	86	55			
11	141	1	141	110			
12	5815	30	197	166			
13	137618	30	4587	4556	14.7	191.75	237601) ± 4.1%
14	79548	30	2652	2621	9.45	102	257941)
15	7878	1	263	232			
Remnant	1948	1	1948	1917			

Spectrometry a) Column Eluate. (*XII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	928	30	31				
Tube 5	31	1	31	-			
6	6771	30	226	195	2.93		
7	2011	30	67	36	1.81		
8	31	1	31	-			
9	30	1	30	-			
10	65	1	65	34			
11	2218	30	74	43			
12	26538	30	885	854	5.5	148.8	57392) ± 0.8%
13	15943	30	531	500	4.3	85.7	58308)
14	3369	30	112	81			
Remnant	958	1	958	927			

Spectrometry a) Column Eluate. (#XII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	1221	30	41				
Tube 5	41	1	41	-			
6	6025	30	201	160	2.82		
7	1300	30	43	2			
8	43	1	43	2			
9	41	1	41	-			
10	91	1	91	50			
11	1959	30	65	24			
12	20491	30	683	642	4.9	251.6	25513)
13	14528	30	484	443	4.2	182.8	24234) ± 2.1%
14	3172	30	106	65	2.2	26.1	24904)
Remnant	783	1	783	752			

Spectrometry a) Column Eluate. (*XII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	1304	30	43				
Tube 5	43	1	43	-			
6	2183	30	73	30	1.95		
7	1070	30	36	-			
8	41	1	41	-			
9	41	1	41	-			
10	43	1	43	-			
11	44	1	44	1			
12	1396	30	47	4			
13	6897	30	230	187	3.0	177.5	10535) ± 1.2%
14	3531	30	118	75	2.3	69.5	10791)
15	1154	30	38	-			
Remnant	120	1	120	89			

<u>Spectrometry b) Urine.</u> (*XII)

Sample : 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute Ethanol.

Experimental Blank: 0.6 ml. tritium from urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High voltage tap 7

Time: in minutes.

Spectrometry b) Urine. (*XII)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	837	30	28		
MH^3S	450913	30	15030		
SB	1787	60	30		
0.6 ml. UB	1898	60	31.6)	31.5	
0.6 ml. UB	1891	60	31.5		
0.2 ml. H^3IS	514003	30	17133)	17226	17200
0.2 ml. H^3IS	519586	30	17320		

Spectrometry b) Urine. (*XII)

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.6 ml. Day 1	5424	60	90.4	59		
0.6 ml. Day 1	5161	60	86	54.5	56.7	1.6
0.6 ml. Day 2	2619	60	43.6	12		
0.6 ml. Day 2	2766	60	46	14.5	13.25	6.0
0.6 ml. Day 3	2384	60	40	8.5		
0.6 ml. Day 3	2382	60	40	8.5	8.5	8.9
0.6 ml. Day 4	2055	60	34	2.5		
0.6 ml. Day 4	2069	60	34.5	3	2.75	26.5

Spectrometry c) Urine plus internal standard. (*XII)

Sample.	Counts.	Time.	cpm.	TBC.	QF.
0.6 ml. Day 1	112771	30	3759	3669	
0.6 ml. Day 1	114500	30	3817	3731	4.65
0.6 ml. Day 2	122837	30	4095	4051	
0.6 ml. Day 2	120654	30	4022	3976	4.3
0.6 ml. Day 3	106661	30	3555	3515	
0.6 ml. Day 3	110073	30	3669	3629	4.76
0.6 ml. Day 4	112064	30	3735	3701	
0.6 ml. Day 4	116060	30	3869	3835	4.5

Total Counts Recovered from Columns as Pregnanediol. (*XII)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	71700	19227	11730	4002	106659
Per cent	67.2	18.0	11.0	3.8	100
Per cent Injected Dose.					13.85

Total Counts Recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	10090	3280	1632	345	15347
Per cent	65.7	21.4	10.6	2.3	100
Per cent Injected Dose.					1.99

Total Counts Recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	193347	67420	34391	11859	307017
Per cent	63.0	22.0	11.2	3.8	100
Per cent Injected Dose.					39.8

Total Pregnanediol Recovered per day.

	Day 1	Day 2	Day 3	Day 4	Total
gamma	294	333	470	284	345.25

(*XII)

$$\text{Ratio of } \frac{\text{\% Recovery of Injected Dose as Pregnanediol}}{\text{\% Recovery of Injected Dose as Peak X}} = \frac{6.96}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of Injected Dose in Urine}}{\text{\% Recovery of Injected Dose as Pregnanediol}} = \frac{2.9}{1}$$

$$\text{Ratio of } \frac{\text{\% Recovery of Injected Dose in Urine}}{\text{\% Recovery of Injected Dose as Peak X}} = \frac{20}{1}$$

Injection Solution : 0.2 ml. = 847011 cpm.

Total Radioactivity injected = $\frac{10}{11}$ of 847011 cpm.

= 770000 cpm.

Secretion Rate of Progesterone = $\frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$

$= 770000 \div \frac{106659}{345.25}$ gamma.

$= \frac{770000 \times 345.25}{106659}$

= 2.495 mgm. per day.

RESULTS.

C. Experimental Subjects.

c) Castrate Females.

Subject *VIII

*X

*XI

*XIII

Subject *VIII Results.

Patient P.S. : Height 5 ft. 4 ins.

Weight 136 lbs.

Age 37 years.

6 days after panhysterectomy.

Urine: Day 1 : 1600 ml.

Day 2 : 1575 ml.

Day 3 : 910 ml.

Day 4 : 2100 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1 : Column IV : Front; tube 5

Day 2 : Column I : Front; tube 5

Day 3 : Column IV : Front; tube 4

Day 4 : Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes.

Experimental blank: 2.0 ml. from tube 1.

Experimental standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1 and 2.

Spectrophotometry - Column Eluate. (*VIII)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol Gamma
AB	0	0	0		
EB	.142	.093	.045	.000	100% Reference.
25 ESP	.316	.375	.245	.094	
50 ESP	.380	.531	.317	.182	
25 ESP	.185	.269	.178	.088	
50 ESP	.265	.465	.300	.183	25 gamma = .091
Tube 12	.101	.070	.051	-	
13	.105	.146	.077	.055	15.1
14	.085	.105	.045	.040	10.9
15	.058	.057	.036	-	

Spectrophotometry - Column Eluate. (*VIII).

Day 2 Sample.	Wavelength Microns. 390	425	460	Allen Correction	Pregnanediol Gamma.
AB	0	0	0		
EB	.065	.054	.044	.000	100% Reference.
25 ESP	.080	.135	.062	.064	
50 ESP	.164	.320	.178	.149	25 gamma = .071
25 ESP	.115	.184	.104	.074	
50 ESP	.266	.397	.248	.140	
Tube 11	.045	.026	.013	-	
12	.053	.071	.022	.034	12
13	.075	.078	.047	.017	6
14	.025	.013	.004	-	

Spectrophotometry - Column Eluate. (*VIII).

Day 3 Sample.	Wavelength Microns. 390	 425	 460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.044	.053	.061	.000	100% Reference.
25 ESP	.169	.216	.121	.091	
50 ESP	.185	.327	.175	.147	25 gamma = .073
Tube 10	.096	.051	.022	-	
11	.122	.130	.084	.027	9.2
12	.157	.164	.090	.041	14.0
13	.171	.112	.088		

Day 4					
AB	0	0	0		
EB	.035	.018	.001	.000	100% Reference.
25 ESP	.123	.190	.111	.073	
50 ESP	.215	.362	.215	.147	25 gamma = .073
Tube 10	.077	.062	.042	-	
11	.064	.065	.044	.011	3.4
12	.066	.079	.052	.020	6.2
13	.042	.028	.013	-	

Spectrometry a) Column Eluate. (*VIII).

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	976	30	33
MH_3S	154509	10	15451
0.5 ml. ES	128318	10	12832
0.2 ml. ES	51604	10	5160
0.2 ml. EIS	8431638	10	843164
SB	1567	30	52

<u>Spectrometry a) Column Eluate.</u> (*VIII).

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 1 EB	1604	30	53				
Tube 5	61	1	61	8			
6	72	1	72	19			
7	3244	30	108	55	1.95		
8	1824	30	61	8			
9	78	1	78	25			
10	73	1	73	20			
11	86	1	86	33			
12	118	1	118	65			
13	33689	30	1123	1070	6.25	37.75	28344) ± 1.73%
14	23963	30	799	746	6.71	27.25	27376)
15	163	1	163	110			
Remnant	233	1	233	181			

Spectrometry a) Column Eluate. (*VIII)

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 2 EB	1693	30	56				
Tube 5	44	1	44	–			
6	2061	30	69	13	2.04		
7	1827	30	61	5	1.97		
8	1630	30	54	–			
9	43	1	43	–			
10	74	1	74	18			
11	97	1	97	41			
12	10917	30	364	308	5.55	30.0	10,267) ± 1.99%
13	6134	30	204	148	2.95	15.0	9,867)
14	69	1	69	13			
Remnant	97	1	97	45			

Spectrometry a) Column Eluate. (*VIII)

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 3 EB	1989	30	66				
Tube 4	52	1	52	-			
5	2218	30	74	8			
6	2304	30	77	11	2.19		
7	2086	30	70	4	2.12		
8	63	1	63	-			
9	62	1	62	-			
10	2038	30	68	2			
11	5374	30	179	113	2.86	23	4909) ± 0.27%
12	7098	30	237	171	3.18	35	4886)
13	2654	30	88	22			
14	65	1	65	-			
Remnant	90	1	90	38			

Spectrometry a) Column Eluate. (*VIII)

Sample.	Counts.	Time.	cpm.	BC.	Sd±	Pd.g.	S.A.
Day 4 EB	1705	30	57				
Tube 5	1643	30	55	–			
6	1854	30	62	5	1.98		
7	1718	30	57	–			
8	54	1	54	–			
9	66	1	66	9			
10	1853	30	62	5			
11	2647	30	88	31	2.19	8.5	3641) ± 2.21%
12	3342	30	111	54	2.37	15.5	3483)
13	1822	30	61	4			
14	52	1	52	–			
Remnant	55	1	55	3			

Spectrometry b) Urine. (*VIII)

Sample: 0.3 and 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.3 and 0.6 ml. tritium free urine.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine. (*VIII)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	933	30	31		
MH^3S	419969	30	13999		
SB	1022	30	34		
0.3 ml. UB	2291	60	38.1	38	
0.3 ml. UB	2312	60	38.5		
0.6 ml. UB	2311	60	38.5	38	
0.6 ml. UB	2270	60	37.8		
0.2 ml. H^3IS	100001	20.93			
0.2 ml. H^3IS	100002	20.34		4854	4820
0.2 ml. H^3IS	100001	20.51			

Spectrometry b) Urine. (*VIII)

Sample.	Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.6 ml. Day 1	3896	60	65	27		
0.6 ml. Day 1	3934	60	65	27	27	3.4
0.6 ml. Day 2	2949	60	49	11		
0.6 ml. Day 2	2959	60	49	11	11	7.7
0.3 ml. Day 3	2638	60	44	6		
0.3 ml. Day 3	2644	60	44	6	6	13.7
0.6 ml. Day 4	2519	60	42	4		
0.6 ml. Day 4	2544	60	42	4	4	20.0

Spectrometry c) Urine plus Internal Standard. (#VIII)

Sample.	Counts.	Time.	cpm.	TBC	QF
0.6 ml. Day 1	43639	30	1455	1390	
0.6 ml. Day 1	44994	30	1500	1435	3.4
0.6 ml. Day 2	46491	30	1550	1501	
0.6 ml. Day 2	44942	30	1498	1449	3.27
0.3 ml. Day 3	54760	30	1525	1481	
0.3 ml. Day 3	46527	30	1551	1507	3.22
0.6 ml. Day 4	44304	30	1477	1435	
0.6 ml. Day 4	45735	30	1525	1483	3.3

Total Counts Recovered from Column as Pregnanediol. (*VIII)

	Day 1	Day 2	Day 3	Day 4	Total
cpm.	58112	14364	5169	3570	81215
Per cent	71.5	17.7	6.4	4.4	100
Per cent Injected Dose.					10.6

Total Counts Recovered from Column as Peak X. (*VIII)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	1760	567	273	210	2810
Per cent	62.6	20.2	9.7	7.4	100
Per cent Injected Dose.					0.37

Total Counts Recovered from Urine. (*VIII)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	244800	94421	58604	46200	444025
Per cent	55.1	21.3	13.2	10.4	100
Per cent Injected Dose.					57.9

Total Pregnanediol Recovered per day. (*VIII)

	Day 1	Day 2	Day 3	Day 4	Mean.
gamma	208	141.75	105.6	100.8	139.0

Ratio of $\frac{\text{\% Recovery of injected dose as pregnanediol}}{\text{\% Recovery of injected dose as peak X}} = \frac{28.6}{1}$

Ratio of $\frac{\text{\% Recovery of injected dose in urine}}{\text{\% Recovery of injected dose as pregnanediol}} = \frac{5.46}{1}$

Ratio of $\frac{\text{\% Recovery of injected dose in urine}}{\text{\% Recovery of injected dose as peak X}} = \frac{156.6}{1}$

Experimental Injection Solution: 0.2 ml. = 843164 cpm.

Total Radioactivity Injected: $\frac{10}{11}$ of 843164 cpm. = 766500 cpm.

Secretion Rate of Pregnanediol $= \dfrac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific activity of pregnanediol}}$.

$= 766500 \div \dfrac{81215}{139}$ gamma

$= \dfrac{766500 \times 139}{81215}$

= 1.312 mgm. per day. (#VIII)

SUBJECT *X RESULTS.

Patient J.T. : Height : 5 ft. 2 ins.

Weight : 125 lbs.

Age : 40 years.

26 days after panhysterectomy.

Urine: Day 1 : 1285 ml.

Day 2 : 1480 ml.

Day 3 : 1700 ml.

Day 4 : 1240 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1 : Column II : Front; tube 5

Day 2 : Column I : Front; tube 5

Day 3 : Column II : Front; tube 5

Day 4 : Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 2.0 ml. from tubes

Spectrophotometry: Column Eluate.

Experimental Blank: 2.0 ml. from tube 1

Experimental Standard: 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1 and 2.

Spectrophotometry. Column Eluate. (*X)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.012	.010	.008	000	100% Reference.
25 ESP	.120	.208	.118	.089	
50 ESP	.208	.370	.200	.166	25 gamma = .085
Tube 7	.656	.576	.410	–	
8	.380	.420	.220	.120	35.30
9	.202	.176	.115	–	
10	.162	.141	.080	–	
Day 2					
AB	0	0	0		
EB	.021	.022	.018	.002	100% Reference.
25 ESP	.085	.165	.094	.075	
50 ESP	.210	.396	.227	.178	25 gamma = .084
Tube 10	.281	.388	.255	.120	35.7
11	.154	.171	.128	.030	8.9

Spectrophotometry. Column Eluate. (*X)

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.007	.004	.002	.001	100% Reference.
25 ESP	.096	.171	.090	.078	
50 ESP	.182	.328	.174	.150	25 gamma = .076
Tube 10	.272	.379	.226	.130	42.8
11	.115	.102	.061	—	
Day 4					
AB	0	0	0	0	
EB	.033	.027	.023	.001	100% Reference.
25 ESP	.100	.172	.082	.081	
50 ESP	.200	.340	.180	.150	25 gamma = .077
Tube 11	.189	.264	.164	.087	28.2
12	.194	.265	.166	.085	27.6

Spectrometry a) Column Eluate. (*X)

Sample: 0.5 ml. from tube plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MH^3S	449391	30	14980
MB	879	30	29
0.2 ml. ES	146639	30	4888
0.5 ml. ES	369164	30	12305
0.2 ml. EIS	7069342	10	706934
SB	848	30	28

Spectrometry a) Column Eluate. (*X)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	917	30	31				
Tube 5	52	1	52	21			
6	8586	30	286	255	3.25		
7	3173	30	106	75			
8	166575	30	5553	5522	13.6	88.25	625,711
9	30384	30	1013	982			
10	2509	30	87	56			
11	484	1	48	17			
12	31	1	31	-			
13	69	1	69	38			
14	64	1	64	33			
15	30	1	30	-			
Remnant	1283	1	1283	1255			

Spectrometry a) Column Eluate. (*X)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	913	30	30				
Tube 5	32	1	32	2			
6	2992	30	100	70	2.08		
7	30	1	30	-			
8	30	1	38	8			
9	4578	30	153	123			
10	37886	30	1263	1233	6.51	89.25	138151) ± 3.95%
11	9408	30	314	284	3.24	22.25	127641)
12	42	1	42	12			
13	35	1	35	5			
14	30	1	30				
15	32	1	32	2			
Remnant	201	1	201	173			

Spectrometry a) Column Eluate. (*X)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	982	30	33				
Tube 5	30	1	30	-			
6	1594	30	53	20	1.7		
7	898	30	30	-			
8	30	1	30	-			
9	1263	30	42	9			
10	13676	30	456	423	4.04	107	39,532
11	2052	30	68	35			
12	30	1	30	-			
13	31	1	31	-			
14	31	1	31	-			
15	34	1	34	1			
Remnant	77	1	77	49			

<u>Spectrometry a) Column Eluate.</u> (*X)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	925	30	31				
Tube 5	30	1	30	–			
6	1259	30	42	11	1.56		
7	997	30	33	2	1.45		
8	31	1	31	–			
9	30	1	30	–			
10	31	1	31	–			
11	4315	30	144	113	2.49	70.5	16,028) ± 1.98%
12	4374	30	146	115	2.43	69.0	16,667)
13	1305	30	44	13			
14	30	1	30	–			
15	35	1	35	4			
Remnant	47	1	47	19			

<u>Spectrometry b) Urine.</u> (*X)

Sample: 0.4 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.4 ml. tritium free urine, as above.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrophotometry b) Urine. (*X)

Sample.	Counts.	Time.	cpm.	Mean.	BG.
MB	1839	60	31		
MH^3S	200002	1.34	14179		
SB	1757	60	29		
0.4 ml. UB	1811	60	30	30.5	
0.4 ml. UB	1874	60	31		
0.2 ml. H^3IS	91164	20	4558	4611	4580
0.2 ml. H^3IS	93281	20	4664		

Spectrophotometry b) Urine. (*X)

Sample.		Counts.	Time.	cpm.	BC.	Mean.	SD±%
0.4 ml.	Day 1	3528	60	58.5	28		
	Day 1	3542	60	59	28	28	3.1
	Day 2	2268	60	38	7		
	Day 2	2210	60	37	7	7	10.7
	Day 3	2078	60	34.6	4.6		
	Day 3	2123	60	35.4	4.4	4.5	14.2
	Day 4	2058	60	34.3	4.3		
	Day 4	2111	60	35.2	4.2	4.25	17.4

Spectrophotometry c) Urine plus Internal Standard. (*X)

Sample.		Counts.	Time.	cpm.	TBC.	QF.
0.4 ml.	Day 1	24500	20	1225	1166	
	Day 1	26177	20	1309	1250	3.8
	Day 2	26382	20	1319	1281	
	Day 2	26463	20	1323	1286	3.57
	Day 3	28663	20	1433	1398	
	Day 3	28439	20	1422	1387	3.29
	Day 4	28076	20	1404	1370	
	Day 4	28142	20	1407	1372	3.34

Total Counts Recovered from Column as Pregnanediol. (*X)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	141915	44903	14382	5654	206854
Per cent	68.6	21.7	7.0	2.7	100
Per cent Injected Dose.					30.2

Total Counts Recovered from Column as Peak X. (*X)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	6553	2072	680	273	9578
Per cent	68.4	21.6	7.1	2.9	100
Per cent Injected Dose.					1.49

Total Counts Recovered from Urine. (*X)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	341810	92463	62921	44004	541198
Per cent	63.1	17.1	11.6	8.1	100
Per cent Injected Dose.					84.2

Total Pregnanediol Recovered per day. (*X)

	Day 1	Day 2	Day 3	Day 4	Mean
gamma	227	330	364	346	314

Ratio of $\frac{\text{\% Injected Dose Recovered as Pregnanediol}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{20.3}{1}$

Ratio of $\frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Pregnanediol}} = \frac{2.79}{1}$

Ratio of $\frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{56.5}{1}$

Experimental Injection Solution (*X)

0.2 ml. = 706934 cpm.

Total Radioactivity Injected

$\frac{10}{11}$ of 706934 = 642500 cpm.

Secretion Rate of Progesterone (*X) $= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$

$= 642500 \div \frac{206854}{314}$ gamma

$= \frac{642500 \times 314}{206854}$

= 0.975 mgm. per day.

Subject *XI Results.

Patient H.F. : Height 5 ft. 5 ins.

Weight 250 lbs.

Age 46 years.

10 days after panhysterectomy.

Urine: Day 1 : 1625 ml.

Day 2 : 2710 ml.

Day 3 : 2450 ml.

Day 4 : 2225 ml.

Extraction: 500 ml. aliquots of daily urine output extracted by method 3 (b).

Columns: Day 1 : Column II: Front; tube 5

Day 2 : Column I : Front; tube 5

Day 3 : Column II: Front; tube 5

Day 4 : Column I : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: Day 1, 2.0 ml. from tubes.

Days 2, 3, and 4, 3.0 ml. from tubes.

Experimental blank: Day 1; 2.0 ml. from tube 1

Days 2, 3 and 4; 3.0 ml. from tube 1.

Experimental Standard: Day 1; 25 and 50 gamma of standard pregnanediol plus 2.0 ml. from tubes 1 and 2. Days 2, 3 and 4; 25 and 50 gamma of

Spectrophotometry - Column Eluate. (*XI)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.026	.025	.018	.003	100% Reference.
25 ESP	.202	.339	.192	.142	
50 ESP	.192	.326	.178	.141	25 gamma = .071
Tube 11	.025	.017	.009	-	
12	.053	.066	.043	.018	6.34
13	.077	.085	.061	.016	5.63
14	.046	.032	.021	-	
Day 2					
AB	0	0	0		
EB	.057	.054	.052	.000	100% Reference.
25 ESP	.104	.164	.082	.071	
25 ESP	.102	.163	.080	.072	25 gamma = .071
Tube 12	.090	.109	.062	.033	11.62
13	.106	.107	.072	.018	6.34

Spectrophotometry - Column Eluate. (*XI)

Day 3 Sample.	Wavelength Microns. 390	 425	 460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0	0	
EB	.038	.030	.021	000	100% Reference.
25 ESP	.097	.185	.115	.079)	25 gamma = .079
50 ESP	.179	.352	.212	.157)	
Tube 11	.144	.107	.083	-	
12	.112	.104	.070	-	
13	.120	.190	.126	.067	21.2
14	.072	.093	.068	.023	7.3
Day 4					
AB	0	0	0	0	
EB	.046	.040	.035	000	100% Reference.
25 ESP	.110	.200	.120	.085)	25 gamma = .085
50 ESP	.213	.397	.241	.170)	
Tube 11	.047	.039	.024	-	
12	.073	.102	.061	.035	10.29
13	.091	.138	.087	.049	14.4
14	.048	.051	.034	.010	2.94

Spectrometry a) Column Eluate. (*XI)

Sample: 0.5 ml. from tube plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
0.2 ml. ES	143780	30	4793
MH3S	442201	30	14740
MB	861	30	29
SB	857	30	29
0.2 ml. EIS	1021364	10	102136

Spectrometry a) Column Eluate. (*XI)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	908	30	30				
Tube 5	29	1	29	–			
6	5901	30	197	167	2.74		
7	7481	30	249	219	2.77		
8	48	1	48	18			
9	59	1	59	29			
10	72	1	72	42			
11	2231	30	74	44			
12	54194	30	1806	1776	7.8	15.85	1,120,505) ± 2.84%
13	50985	30	1700	1670	7.6	14.08	1,186,079)
14	4221	30	141	111			
15	29	1	29	–			
Remnant	347	1	347	318			

Spectrometry a) Column Eluate. (*XI)

Sample.	Counts.	Time.		cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	905	30		30				
Tube 5	30	1		30	-			
6	2119	30	30	71	41	1.85		
7	1112	30	30	37	7	1.49		
8	29	1	71	29	-			
9	34	1	37	34	4			
10	41	1	29	41	11			
11	2113	30	34	70	40			
12	8723	30	41	291	261	3.27	24.1	134,814) ± 3.82%
13	4846	30	70	162	132	2.53	15.9	124,882)
14	1397	30	91	47	17			
15	36	1	62	36	6			
Remnant	81	1	47	81	52			
			36					
			81					

Spectrometry a) Column Eluate. (*XI)

Sample.	Counts.	Time.	cpm.	BG.	SD±	Pd.g.	S.A.
Day 3 EB							
Tube 1	966	30	32				
5	29	1	29	-			
6	1188	30	40	8			
7	2104	30	70	38	1.55		
8	1067	30	36	4	1.51		
9	25	1	25	-			
10	27	1	27	-			
11	1266	30	42	10			
12	1450	30	48	16			
13	7911	30	264	232	3.1	35.3	65,722) ± .11%
14	3349	30	112	80	2.2	12.2	65,574)
15	1500	30	50	18			
Remnant	80	1	80	51			

Spectrometry a) Column Eluate. (*XI)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB							
Tube 1	959	30	32				
5	29	1	29				
6	1387	30	46	14	1.61		
7	1041	30	35	3	1.48		
8	31	1	31	-			
9	32	1	32	-			
10	33	1	33	1			
11	1037	30	35	3			
12	2704	30	90	58	2.02	17.1	33,914)
13	3361	30	112	80	1.95	24.0	33,333) ± 1.9%
14	1449	30	48	16	1.64	4.9	32,653)
15	36	1	36	4			
Remnant	72	1	72	43			

Spectrometry b) Urine. (*XI)

Sample: 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.6 ml. tritium free urine as above.

Internal Standard: 0.2 ml. standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine. (*XI).

Samples.	Counts.	Time.	cpm.	Mean.	BC.
MB	837	30	28		
MH^{3}S	450913	30	15030		
SB	1787	60	30		
0.6 ml. UB	1898	60	31.6)	31.5	
0.6 ml. UB	1891	60	31.5)		
0.2 ml. H^{3}IS	514003	30	17133)	17226	17200
0.2 ml. H^{3}IS	519586	30	17320)		

Spectrometry b) Urine. (*XI).

Sample.		Count.	Time.	cpm.	BC.	Mean.	SD±%
0.6 ml.	Day 1	3931	60	65.5	34		
	Day 1	3832	60	64	33	33.5	2.7
	Day 2	2261	60	38	6.5		
	Day 2	2266	60	38	6.5	6.5	11.7
	Day 3	2139	60	35.6	4		
	Day 3	2165	60	36	4.5	4.25	17.4
	Day 4	2105	60	35	3.5		
	Day 4	2080	60	35	3.5	3.5	21.1

Spectrometry c) Urine plus internal standard. (*XI)

Sample.		Counts.	Time.	cpm.	TBC.	QF.
0.6 ml.	Day 1	137375	30	4579	4513	
	Day 1	137474	30	4582	4518	3.8
	Day 2	144790	30	4826	4788	
	Day 2	144383	30	4813	4775	3.6
	Day 3	142913	30	4764	4728	
	Day 3	140847	30	4695	4659	3.65
	Day 4	141840	30	4728	4693	
	Day 4	139843	30	4661	4626	3.7

Total Counts Recovered from Column as Pregnanediol. (*XI)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	111995	21300	15288	6853	155436
Per cent	72.1	13.7	9.8	4.4	100
Per cent injection dose.					16.7

Total Counts Recovered from Column as Peak X. (*XI)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	12545	2602	2254	756	18157
Per cent	69.1	14.3	12.4	4.2	100
Per cent injection dose.					1.96

Total Counts Recovered from Urine. (*XI)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	344771	105690	63363	48023	561847
Per cent	61.2	18.8	11.3	8.6	100
Per cent injection dose.					60.5

Total Pregnanediol Recovered per day. (*XI)

	Day 1	Day 2	Day 3	Day 4	Mean.
Gamma	97.5	163.2	232.75	204.7	174.5

Ratio of $\frac{\text{\% Injected Dose Recovered as Pregnanediol}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{8.5}{1}$

Ratio of $\frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Pregnanediol}} = \frac{3.62}{1}$

Ratio of $\frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{30.9}{1}$

Experimental Injection Solution. (*XI)

0.2 ml. = 1021364 cpm.

Total Radioactivity Injected =

$\frac{10}{11}$ of 1021364 = 928500 cpm.

$= \frac{\text{Total Radioactivity Injected (cpm)}}{\text{Specific Activity of Pregnanediol}}$.

Secretion Rate of Progesterone. (*XI) =

$928500 \div \frac{155436}{174.5}$ gamma

$= \frac{928500 \times 174.5}{155436}$

= 1.042 mgm. per day.

Subject *XIII Results.

Patient: L.M. Height : 5 ft. 6 ins.

Weight : 125 lbs.

Age : 48 years.

34 days after panhysterectomy.

Urine: Day 1 : 1310 ml.

Day 2 : 1100 ml.

Day 3 : 1865 ml.

Day 4 : 1815 ml.

Extraction: 500 ml. aliquots of daily urine extracted by method 3 (b).

Columns: Day 1 : Column II : Front; tube 6

Day 2 : Column IV : Front; tube 5

Day 3 : Column II : Front; tube 5

Day 4 : Column IV : Front; tube 5

Spectrophotometry: Column Eluate.

Sample: 3.0 ml. from tubes.

Experimental blank: 3.0 ml. from tube 1.

Experimental standard: 25 and 50 gamma of standard pregnanediol plus 3.0 ml. from tubes 1 and 2.

Spectrophotometry - Column Eluate. (*XIII)

Day 1 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.110	.112	.108	.003	100% Reference.
25 ESP	.120	.186	.106	.073	
50 ESP	.194	.340	.180	.153	25 gamma = .075
Tube 11	.146	.105	.091	-	
12	.265	.323	.205	.088	29.3
13	.271	.305	.180	.080	26.7
14	.151	.113	.087	-	
Day 2					
AB	0	0	0		
EB	.124	.107	.084	.003	100% Reference.
25 ESP	.071	.141	.071	.070	
50 ESP	.154	.304	.162	.146	25 gamma = .072
Tube 11	.182	.268	.194	.180	27.8
12	.185	.265	.177	.084	29.2

Spectrophotometry - Column Eluate. (*XIII)

Day 3 Sample.	Wavelength Microns. 390	425	460	Allen Correction.	Pregnanediol Gamma.
AB	0	0	0		
EB	.146	.170	.135	.029	100% Reference.
25 ESP	.094	.169	.094	.075	25 gamma = .071
50 ESP	.226	.371	.238	.139	
Tube 11	.170	.163	.160	-	
12	.129	.169	.077	.066	23.3
Day 4					
AB	0	0	0		
EB	.141	.166	.129	.031	100% Reference.
25 ESP	.101	.177	.097	.078	25 gamma = .080
50 ESP	.232	.397	.238	.162	36.875
Tube 11	.245	.365	.249	.118	
12	.144	.134	.093	-	

Spectrometry a) Column Eluate. (*XIII)

Sample: 0.5 ml. from tubes plus 10 ml. Scintillation fluid.

Experimental blank: from tube 1.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Sample.	Counts.	Time.	cpm.
MB	940	30	31
MH^{3}S	447392	30	14913
SB	1065	30	36
0.2 ml. ES	143774	30	4792
0.2 ml. EIS	6875529	10	687553

Spectrometry a) Column Eluate. (*XIII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 1 EB	1079	30	36				
Tube 6	66	1	66	30			
7	7226	30	241	205	3.03		
8	1626	30	54	18			
9	101	1	101	65			
10	109	1	109	73			
11	3435	30	115	79			
12	51097	30	1703	1667	7.6	48.8	339,549) ± 4.5%
13	50658	30	1689	1653	7.6	44.5	371,461)
14	8524	30	284	248			
15	115	1	115	79			
Remnant	438	1	436	406			

Spectrometry a) Column Eluate. (*XIII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 2 EB	1015	30	34				
Tube 5	33	1	33	-			
6	2407	30	80	46	1.9		
7	1263	30	42	8			
8	34	1	34	-			
9	39	1	39	5			
10	1768	30	59	25			
11	9518	30	317	283	3.42	46.3	61,123) ± 4.5%
12	10767	30	359	325	3.62	48.6	66,872)
13	1714	30	57	23			
14	41	1	41	7			
15	36	1	36	2			
Remnant	264	1	264	228			

Spectrometry a) Column Eluate. (*XIII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 3 EB	1261	30	42				
Tube 5	39	1	39	-			
6	1781	30	59	17	1.82		
7	1265	30	42	-			
8	37	1	37	-			
9	39	1	39	-			
10	1412	30	47	5			
11	2404	30	80	38			
12	8727	30	291	249	3.33	38.6	64508
13	1718	30	57	15			
14	50	1	50	8			
15	50	1	50	8			
16	41	1	41	-			
Remnant	72	1	72	36			

Spectrometry a) Column Eluate. (*XIII)

Sample.	Counts.	Time.	cpm.	BC.	SD±	Pd.g.	S.A.
Day 4 EB	1199	30	40				
Tube 5	40	1	40	-			
6	1404	30	47	7	1.7		
7	1136	30	38	-			
8	39	1	39	-			
9	40	1	40	-			
10	1369	30	46	6			
11	4129	30	138	98	2.43	61.4	15961
12	1654	30	55	15			
13	1072	30	36	-			
14	39	1	39	-			
15	36	1	36	-			
Remnant	77	1	77	41			

Spectrometry b) Urine. (*XIII)

Sample: 0.6 ml. urine (after thoroughly shaking up specimen) plus 10 ml. Scintillation fluid and 6 ml. absolute ethanol.

Experimental Blank: 0.6 ml. tritium free urine as above.

Internal Standard: 0.2 ml. Standard H^3 pregnanediol.

Counting: High Voltage tap 7 (Automatic).

Time: in minutes.

Spectrometry b) Urine. (*XIII)

Sample.	Counts.	Time.	cpm.	Mean.	BC.
MB	1756	60	29		
MH^3S	898309	60	14972		
SB	2140	60	36		
0.6 ml. UB	2860	60	47.7)	48	
0.6 ml. UB	2934	60	48.9)		
0.2 ml. H^3IS	514003	30	17133)	17226	17200
0.2 ml. H^3IS	519586	30	17320)		

Spectrometry b) Urine. (*XIII)

Sample.		Count.	Time.	cpm.	BC.	Mean.	SD±%
0.6 ml.	Day 1	4996	60	83	35		
	Day 1	4986	60	83	35	35	3.0
	Day 2	3858	60	64	16		
	Day 2	4062	60	68	20	18	5.4
	Day 3	3257	60	54.3	6.3		
	Day 3	3272	60	54.5	6.3	6.3	14.6
	Day 4	3230	60	53.8	5.8		
	Day 4	3206	60	53.4	5.4	5.6	16.4

Spectrophotometry c) Urine plus Internal Standard. (*XIII)

Sample.		Counts.	Time.	cpm.	TBC.	QF.
0.6 ml.	Day 1	138829	30	4628	4545	
	Day 1	136016	30	4534	4451	3.8
	Day 2	144876	30	4829	4765	
	Day 2	139828	30	4561	4593	3.65
	Day 3	139345	30	4645	4591	
	Day 3	140156	30	4672	4618	3.7
	Day 4	142770	30	4759	4705	
	Day 4	139234	30	4641	4597	3.67

Total Counts Recovered from Column as Pregnanediol. (*XIII)

	Day 1	Day 2	Day 3	Day 4	Total
cpm	86984	13376	9288	3557	113205
Per cent	76.8	11.8	8.3	3.1	100
Per cent Injection Dose.					18

Total Counts Recovered from Column as Peak X.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	5371	1188	634	254	7447
Per cent	72.1	16.0	8.5	3.4	100
Per cent Injection Dose.					1.19

Total Counts Recovered from Urine.

	Day 1	Day 2	Day 3	Day 4	Total
cpm	290383	120450	72455	62069	545357
Per cent	53.3	22.1	13.1	11.4	100
Per cent Injection Dose.					87.2

Total Pregnanediol Recovered per Day. (*XIII)

	Day 1	Day 2	Day 3	Day 4	Mean
Gamma	244.4	208.8	144	223	205

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered as Pregnanediol}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{15.1}{1}$$

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Pregnanediol}} = \frac{4.84}{1}$$

$$\text{Ratio of } \frac{\text{\% Injected Dose Recovered from Urine}}{\text{\% Injected Dose Recovered as Peak X}} = \frac{73.3}{1}$$

Experimental Injection Solution (*XIII) 0.2 ml. = 687553 cpm.

Total Radioactivity Injected = $\frac{10}{11}$ of 687553 = 625000 cpm.

Secretion Rate of Progesterone (*XIII) =

$$\frac{\text{Total Radioactivity Injected (cpm).}}{\text{Specific Activity of Pregnanediol}}$$

$$= 625000 \div \frac{113205}{205} \text{ gamma}$$

$$= \frac{625000 \times 205}{113205}$$

= 1.132 mgm. per day.