

"An Investigation into Some Aspects of the Thin Layer
Chromatographic Assay of Pregnanediol with Emphasis on the
Suitability of this Method as a Clinical Laboratory Routine."

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LIST OF REAGENTS

The Chemicals used in the experiments in this work are as follows :-

Alumina (100 - 150 mesh)
Acetic acid glacial 'Analar'
Acetone (Merck) nach Waldi
Acetyl chloride 'Analar'
Benzene redistilled water-saturated
n-Butanol laboratory grade (British Drug Houses)
Chloroform (Merck) for spectroscopy
Cyclohexane (Merck)
Ether (diethyl) dry M.D.H.
Ethyl acetate 'Analar'
Hexane (boiling range 67° - 70° C) B.D.H.
Hydrochloric acid fuming (SG. 1.19) B.D.H.
Methyl acetate laboratory grade (Merck)
Orthophosphoric acid (89%) extra pure (Merck)
Perchloric acid (70%) extra pure (Merck)
Phosphomolybdic acid (G.R.) Merck
Phenyl mercuric nitrate laboratory grade
Potassium permanganate 'Analar'
Sodium hydroxide pellets laboratory grade
Sodium metabisulphite laboratory grade B.D.H.
Sodium sulphate anhydrous laboratory grade B.D.H.
Sulphuric acid 'Analar' 98% H_2SO_4
Toluene 'Analar'
Vanillin 'Analar'

LIST OF ABBREVIATIONS

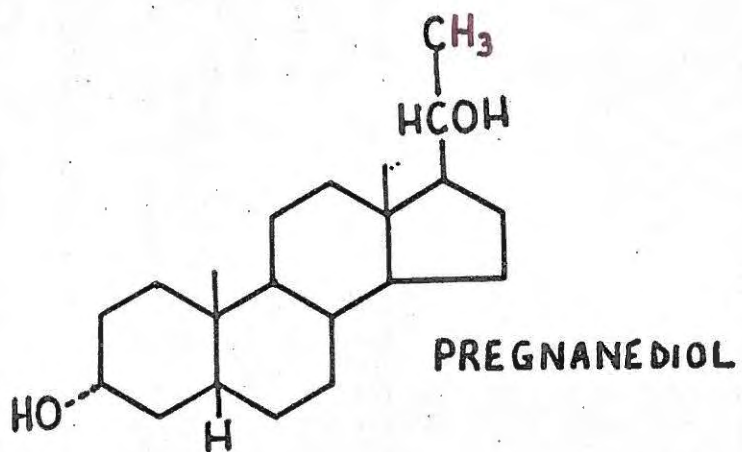
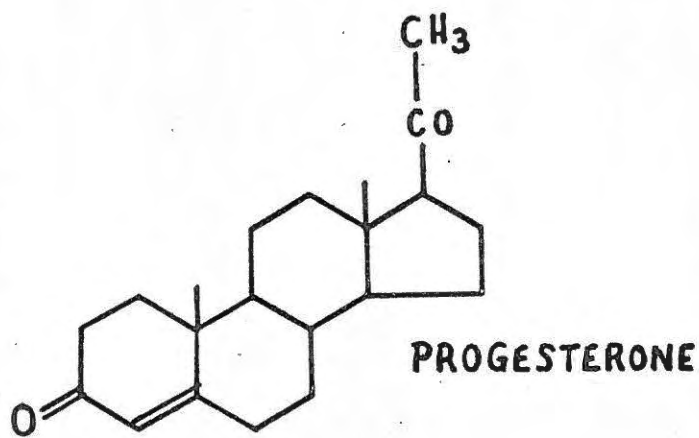
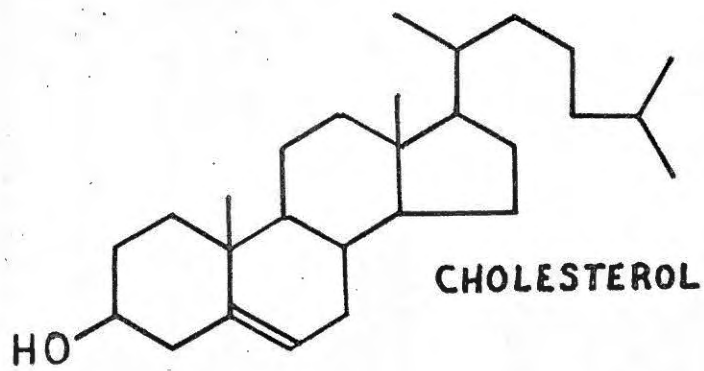
A	absorbance
A.C.T.H.	Adrenocorticotropic hormone
B.B.T.	basal body temperature
E. max	wavelength of maximum extinction
G.L.C.	Gas-Liquid Chromatography
H.C.G.	Human Chorionic Gonadotrophin
H.P.G.	Human Pituitary Gonadotrophin
m.u.	millimicrons
P.G.	Pregnanediol
P.L.G.	Pregnanediol glucuronidate
P.M.N.	Phenyl mercuric nitrate
T.L.C.	Thin Layer Chromatography
u.g.	micrograms
u.l.	microlitres
U.V.	Ultra-violet

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STRUCTURAL FORMULAE



PREAMBLE

Pregnanediol (5 β Pregnane - 3 α - 20 α - diol) is the chief urinary metabolite of progesterone, and as such is important in that variations in its concentration reflect variations in progesterone secretion. Estimations of pregnanediol concentration are therefore of considerable interest to the obstetrician and gynaecologist.

Pregnanediol was first identified in the urine of pregnant women in 1929 by Marrian.⁽¹⁾ Nearly ten years later Venning⁽²⁾ developed a method by which the glucuronic acid ester of pregnanediol could be extracted from the urine and its concentration gravimetrically determined. Numerous variations of the Venning theme were published in the next few years, each being claimed by its authors to be an improvement on the original. Most of these involved the estimation of the conjugated form, and it was a while before the advantage of estimating the hydrolysed aglycone was realized.

Hydrolysis, when it was practised, resolved itself into two methods - namely, hydrolysis by heating the urine with a mineral acid, and enzymic hydrolysis by incubation with beta-glucuronidase. Acid hydrolysis, while producing a less clean hydrolysate, is more rapid and convenient than enzyme hydrolysis, and is used in the Klopper method⁽³⁾ which is presently the most widely used method in clinical studies. Klopper employs a double chromatographic column separation of pregnanediol followed by colorimetric evaluation.

Variations of Klopper's method have also appeared and not a few investigators have published comparisons of the various methods. Klopper himself compared his method to certain other methods and concluded that his was definitely superior. Of the accuracy of the Klopper method there is no doubt. Subsequent methods have proved more sensitive, but in terms of practicability Klopper's is the method of choice. As was pointed out with some complacency, "practicability is most satisfactory, one technician readily performing some twenty determinations in one week."⁽⁴⁾

In contrast to the flood of criticisms, comparisons, variations, claims and counter-claims which accompanied the publication of the above-mentioned methods, the thin layer chromatographic method perfected by Waldi⁽⁵⁾ attracted very little attention. It is very much more rapid than all other existing techniques, is very sensitive, specific and of acceptable

accuracy. In an attempt to ensure its usefulness for clinical and medical research laboratories, the Waldi method has been marketed in 'kit' form. It is intended primarily as a diagnostic aid in establishing pregnancy, and as such it might have enjoyed considerable application had it not been for the advent of the immunological method of pregnancy diagnosis which is very much more rapid. Nevertheless, the Waldi method, used purely as a means of assessing the pregnanediol content of the urine is extremely useful, and it is the purpose of this investigation to establish this usefulness, especially with respect to routine clinical investigations. The validity of some diagnoses which are based on pregnanediol assay results, is also investigated.

As it is impossible to explain the significance or usefulness of a pregnanediol assay without first explaining the functions of progesterone, some time and space must be expended in a brief description, firstly, of the role played by progesterone in the phenomenon of the (female) menstrual cycle, and secondly, of its vital importance in pregnancy.

It must be realized that progesterone is only one of the many hormones involved in these events, but, in order to limit the introduction of extraneous detail, no mention is made of the other hormonal participants except when necessary for the understanding of the whole.

It may be mentioned here that much of the evidence that was used for the elucidation of the functions and origins of progesterone, was derived from studies of its metabolite, pregnanediol.

CHAPTER ONE

The Menstrual Cycle

In the ovaries, the eggs or ova repose in small sacs called follicles. Approximately once a month in the mature human female, a follicle ripens, bursts and expels its ovum. This is termed ovulation.

The ovum travels down the fallopian tube where, if it encounters a spermatozoon, it may be fertilized. The ovum passes into the uterus; if unfertilized it is discharged from the body; if fertilized, it normally lodges in the uterus and develops into an embryo. This lodgement is made possible by the fact that the endometrium or lining of the uterus becomes extremely moist and vascular. It bathes the developing ovum or blastocyst with secretions containing nutriment which maintains the blastocyst and allows rapid cell-division to take place. Should the endometrium regress, as for instance it does just prior to the menstrual cycle, the cellular activity of the developing blastocyst would cease and it would be expelled.

The ruptured follicle from which the ovum is discharged remains attached to the ovary and changes both in shape and colour. Within 24 hours after the discharge of the ovum it develops into a yellow body or corpus luteum. This corpus luteum increases in size until the start of the next cycle, when it ^degenerates. If fertilization has taken place, ^degeneration of the corpus luteum is delayed until parturition.

All this was known at the turn of the century, but the regulative mechanism responsible for the rhythm of the cycle and the maintenance of pregnancy, remained undiscovered. Professor Born, embryologist at Breslau University, suspected that a 'chemical messenger' was involved and recommended a more intensive study of the corpus luteum. One of Born's students, Fraenkel, established that the destruction of the corpus luteum shortly after ovulation, led to the death of the ovum. It was in 1928 that Corner⁽⁶⁾ set out to identify the active substance secreted by the corpus luteum - the substance that was responsible for ensuring the survival of the fertilized ovum. Together with a student (Allen) he extracted a material which proved capable of performing the functions of the corpus luteum when supplied to a pregnant animal in which the organ had been destroyed. This substance

they named Progesterone. Corner, Allen and associates learned that progesterone not only prepared the uterus for implantation and ensured that the embryo was maintained there throughout pregnancy, but it could also prolong pregnancy beyond its normal duration.

The interest stimulated by the above discoveries led many workers to investigate progesterone and the factors governing its release. The sequence of events was finally established: Progesterone is secreted by the corpus luteum. If the ovum is unfertilized the corpus luteum regresses after about 14 days. The absence of progesterone then initiates the degeneration of the endometrium which undergoes autolysis and is expelled. If the ovum is fertilized it undergoes rapid cell division producing among other tissues, primitive placental cells. These cells secrete the hormone Chorionic Gonadotrophin and it is this hormone which acts directly on the corpus luteum, preventing its degeneration and hence preventing the onset of a menstrual cycle. After about three months the placenta also begins secreting progesterone and the supply originating from the corpus luteum is no longer of supreme importance. Indeed, it has been proved that if at this stage the corpus luteum is removed, the foetus will continue to develop to full term.

Experiments with rabbits showed that progesterone was capable of preventing induced abortion, and high hopes prevailed that treatment with progesterone could cut down on the number of miscarriages in humans. G.W. Anderson of the Johns Hopkins Hospital has estimated that 10% of all pregnancies end in spontaneous abortion and 7% in premature delivery. Progesterone treatment was enthusiastically practised in all cases with a history of habitual abortion, but with disappointing results. In few cases did the physicians call for a progesterone assay prior to treatment; had they done so, and had they reserved the progesterone treatment for those cases showing a sub-normal concentration, the percentage success would have been markedly increased.

Investigators sought the method whereby progesterone could prevent induced abortion. That it could exert a blocking action on uterine muscle was soon established. How it exerted this blocking action was not so easily elucidated.

Csapo⁽⁷⁾ starting at the molecular level tried to prove that progesterone inhibited the contractibility of actinomyosin strands, but was unsuccessful. However using electrical stimulation he showed that progesterone reduced the excitability of uterine muscle and inhibited the conduction of impulses. This affect is achieved both through an alteration of the normal concentration gradient of Potassium, Sodium and Calcium at the cell membrane, and through a decrease in the generation of Diphosphopyridine nucleotide (D.P.N.H.)⁽⁸⁾ which would affect the energy produced by oxidative phosphorylation.

In effect then, progesterone limits the likelihood of uterine contractions, even although it does not absolutely preclude them. Similarly an absence of progesterone does not necessarily of itself cause abortion, but the absence does mean that the uterus is in a hyper-excitable state and thus easily stimulated into contraction by posterior pituitary gland secretions, or toxins (either endogenous or foreign e.g. Castor oil) or even by psychic influences. A progesterone or pregnanediol assay will soon indicate such a state of affairs. If the progesterone secretion appears to be normal but abortion seems imminent, the administration of progesterone may alleviate the symptoms, but only rarely. If, on the other hand, an assay indicates subnormal progesterone secretion, the administration of the hormone will usually exert an anti-aborting action. The apparent lack of success attending early progesterone treatments was due to the fact that probably over half the patients had normal progesterone levels anyway and the rest with deficient levels were given too small a dose of injected progesterone. Plasma binds progesterone strongly⁽⁹⁾ and unless a large dose were injected none would be available to the uterine musculature. Csapo recommended a tenfold increase in the dose. The Johns Hopkins Hospital which followed this recommendation, reported numerous successes, but only in cases of innate progesterone deficiency.

DISCUSSION

It has been shown that progesterone is only secreted when there is present a functioning corpus luteum, or a placenta. Pregnanediol excretion would therefore be high at these times, and values lower than normal would

be indicative of malfunction. Progesterone administration in cases of threatened or habitual abortion has proved successful but only in those cases showing evidence of sub-normal endogenous secretion. It has been suggested therefore that a progesterone or pregnanediol assay should precede treatment with progestational agents.

SUMMARY OF THIS CHAPTER

The sequence of events during the menstrual cycle and pregnancy has been briefly outlined with especial reference to progesterone secretion.

Also described was the mode of action of progesterone and its use in treating abnormal pregnancies.

CHAPTER TWO

The biogenesis, metabolism and excretion
of Progesterone and its metabolites

a) The Biogenesis of Progesterone

Jayle⁽¹⁰⁾ describes the methods used to investigate progesterone biogenesis. Labelled precursors (¹⁴C acetate and ³H cholesterol) indicated that the corpus luteum synthesises progesterone from plasma acetate and cholesterol, but that the placenta, which after the third month of pregnancy is responsible for the bulk of the progesterone secreted, synthesises progesterone from cholesterol and pregnenolone. A cholesterol-free diet does not inhibit progesterone biogenesis as the body is perfectly capable of synthesising its own cholesterol. The low pregnanediol level recorded for fasting subjects has been proved to be due to a decreased rate of excretion not a decrease in progesterone secretion. (In cases such as this a pregnanediol assay would tend to give misleading evidence).

b) Metabolism and excretion of progesterone and its metabolites

Whereas it has been established that it takes sixteen hours for an injection of progesterone to exert a therapeutic effect, it has also been reported that the metabolism of progesterone is extremely rapid. Somerville,⁽¹¹⁾ infusing progesterone intravenously into a male patient noted an almost immediate increase in plasma pregnanediol levels. His findings have been confirmed by various works. Pearlman⁽¹³⁾ calculated the half-life of progesterone to be 3.3 minutes. Geigy⁽¹²⁾ quotes 2 minutes. Short and Eton⁽¹⁴⁾ quote 5 minutes. As metabolised progesterone is biologically inactive, it is difficult to understand how an injection of progesterone could have any therapeutic effect that was not immediately produced.

For convenience, progesterone metabolites are usually grouped into four main categories depending on their degree of reduction. These are:-

- i) The pregnanediones
- ii) The pregnanolones (mainly 3 α -OH 5 β pregnan-20-one)
- iii) The pregnanediols (mainly 5 β pregnane-3 α , 20 α -diol)
- iv) More polar compounds.

Quantitatively, pregnanediol is by far the most important metabolic reduction product of progesterone, followed by pregnanolone which is excreted at a quarter the rate of pregnanediol.

A certain amount of confusion exists regarding the percentage of progesterone which is metabolised to pregnanediol. Marrian et al⁽¹⁵⁾ calculated a figure of 20-40% in the presence of a corpus luteum or placenta, or 15% for men and hysterectomised women. Wiest and Fujimoto⁽¹⁶⁾ using labelled progesterone, quote 19%. The lowest figure seen quoted was 6%, and the highest 40%. The progesterone/pregnanediol relationship has been investigated by numerous workers as it is obviously of importance when extrapolating results obtained by a pregnanediol assay, in terms of progesterone secretion. In fact, a precise and concrete relationship does not exist. 15% of the plasma progesterone may appear as urinary pregnanediol on one day, and 20% the next day. Although in one individual the progesterone/pregnanediol relationship is very constant, it may vary. The percentage of progesterone reduced to plasma pregnanediol may alter and/or the percentage of plasma pregnanediol excreted in the urine may alter.

Regarding the excretory pathways of progesterone metabolites, once again many varied results have been quoted. Pearlman⁽¹³⁾ using labelled progesterone found that 55%-75% of the radioactivity subsequently appeared in the urine and faeces. Zander recovered 35% from the urine. Davis and Plotz quote 54% as appearing in the urine and Sandberg found 42% in the bile. In the faeces and (especially) the bile, the principle metabolite is unconjugated pregnanediol. The chief urinary metabolite is conjugated pregnanediol.

Progesterone metabolites are conjugated in the liver. Involved is the reactant uridine diphosphoglucuronic acid and the enzyme glucuronyl transferase. The principle product is pregnanediol glucuronidate. It has been established that the conjugation process is subject to many influences, pathological and otherwise. Holopainen⁽¹⁷⁾ reported that rheumatic diseases decreased the rate of glucuronide formation (and hence the amount of pregnanediol excreted into the urine). Schriefers et al⁽¹⁸⁾ investigated the rate of glucuronide formation in the fasting animal and decided that the ability of the liver to synthesise glucuronides is dependant on "an

undisturbed carbohydrate metabolism." Fasting induces a 20% decrease in the rate of conjugation - a decrease which is reversed by an injection of glucose.

It has been stated that large quantities of pregnanediol are conjugated to the sulphate ester. In 1963 Chang-shen et al found that the percentage of pregnanediol appearing as the sulphate ester was as high as 60%. More recent well-confirmed estimations put the proportion of sulphate ester at less than 1%. If a high proportion of the sulphate ester were excreted it would profoundly influence those pregnanediol assay methods which make use of enzymes as hydrolytic media. Purified β -glucuronidase is often used to hydrolyse the conjugate, and this enzyme would not affect the sulphate conjugate. Of course, those methods using the less specific acid hydrolysis would be unaffected.

DISCUSSION

A number of authorities have investigated the biogenesis, metabolism and excretory pathways of progesterone. While few conditions affect the biogenesis and metabolism of progesterone, the excretion of the metabolite is subject to many influences.

Ideally, the concentration of a metabolite in the urine is directly proportional to the concentration of the precursor in the blood stream. In these cases, providing the ratio is known, it is possible to establish exactly the blood concentration of a substance by assaying the concentration of metabolite in the urine. Unfortunately such is not the case with progesterone and its metabolite pregnanediol. Much work has been done in an attempt to establish a firm progesterone/pregnanediol ratio, but instead this labour has established that such a ratio does not exist. The proportion of progesterone which is metabolised to pregnanediol varies from individual to individual, and possibly shows a week to week variation in the same individual. The position is further complicated by the fact that the pregnanediol once formed has not one but many excretory pathways, and the proportion of the total plasma pregnanediol which appears in the urine shows an individual variation. The rate of pregnanediol excretion in the urine is affected by many physiological and pathological conditions. Fasting or toxins may diminish the efficiency of the liver conjugation mechanism. Rheumatic diseases may

do likewise. Toxins or renal disease may affect the excretion of the conjugated pregnanediol by the kidneys. These factors which may vary the excretion rate are discussed in greater detail in the next chapter.

SUMMARY OF CHAPTER TWO

The biogenesis, metabolism and excretion of progesterone has been described and the methods by which these mechanisms were elucidated, briefly outlined.

CHAPTER THREE

Conditions influencing progesterone production and/or pregnanediol excretion

A progesterone or pregnanediol assay is ~~usually~~ ^{often} used as a pregnancy diagnosis or prognosis, or it may be used when investigating cases of sterility, threatened abortion, amenorrhea etc., etc., or for diagnosing any of the factors causing abnormal secretion or excretion of progesterone and its principal metabolite. Some of these factors are described hereunder.

1) Non-pathological conditions

From the description of the menstrual cycle in Chapter one it will be seen that an increase in pregnanediol levels would follow ovulation and would be maintained until menstruation or parturition, as the case may be. Early reports stated categorically that no pregnanediol was present in the urine until ovulation occurred. The more sensitive present day techniques have disproved this. Pregnanediol is in fact sometimes found prior to ovulation. This preovulatory pregnanediol is a metabolite of adrenal cortex progesterone (19,20) or of other corticoids. (12)

It has been histologically determined that progesterone is secreted shortly before ovulation takes place - (Harrison²¹), but the rate of increase is dependent on a corpus luteum and so pregnanediol only becomes apparent in the urine at the time of ovulation. If this is followed by pregnancy, the increase in urinary pregnanediol levels continues until shortly before parturition. Pregnancies involving twins and triplets have higher pregnanediol titres than those involving single births.

2) Pathological Conditions

A number of diseases have, as one of their ^{features.} symptoms, an abnormal progesterone and/or pregnanediol output.

- a) Adrenal disease - Higher than normal values have been reported.
- b) Gynaecomastia (abnormal mammary development in males). Supra-normal concentrations have been reported.
- c) Sterility - It has been suggested by Venning⁽²²⁾ that one of the causes of sterility in women is a luteal phase of too short a duration. The critical amount of progesterone necessary for the

establishment of a functioning endometrium has not been determined, but if the total excretion of pregnanediol during the luteal phase is less than 20 mgm, it is unlikely that the endometrium would be able to support a foetus.

d) Abnormal pregnancies -

(i) Pre-eclamptic toxæmia : progesterone secretion is unaffected but pregnanediol renal clearance is inhibited.

(ii) Pregnant diabetes: Increased values have been reported.

(iii) Threatened and habitual abortion : Many such cases are due to luteal or placental deficiency and have consequently low values for progesterone and pregnanediol.

(iv) Foetal death : Steadily diminishing levels have been reported.

(v) Chronic nephritis in pregnancy : Low urinary pregnanediol concentrations have been reported.

e) Hepatic Insufficiency : Decreased pregnanediol concentrations are reported.

3) Variations caused by Medical Treatment

a) Gonadotrophin administration : Certain cases of sterility in women are treated with the so-called Anti-sterility hormone which comprises a mixture of human chorionic gonadotrophin (H.C.G.) and human pituitary gland extract (H.P.G.) A frequent response to this treatment is the development of "football" ovaries, or grossly enlarged ovaries containing multiple corpora lutea. This is undesirable as fertilization will result in multiple-births. An assay of pregnanediol will indicate whether ovarian hypertrophy exists and would help the specialist in adjusting dosage schedules. (Buxton²³).

b) Corticoid therapy : Many investigators have reported an increase in pregnanediol output following the administration of some of the adrenal cortex hormones. Injected 11-deoxy corticosterone is apparently metabolised to pregnanediol and appears as such in the urine. Geigy⁽¹²⁾ notes an increased pregnanediol output following cortexone administration, and Gay et al note an increase

following injected dehydroepiandrosterone. Klopper and associates⁽²⁴⁾ suggest that a pregnanediol assay is of value in assessing adrenocortical function in male subjects.

c) Surgery : Urinary pregnanediol levels rise following surgical treatment. This increase is not a reflection of an enhanced progesterone output, but is due to increased deoxycorticoid secretion induced by surgical stress.

d) Stilboestrol administration : Smith et al reported a sharp increase in pregnanediol levels following stilboestrol administration. This report was never confirmed by subsequent work: in fact most investigators reported a decrease in pregnanediol levels following stilboestrol therapy. Smith used the Venning method. Davis and Fugo⁽²⁵⁾ reported that the gravimetric Venning technique is un-specific in that it also extracts stilboestrol metabolites. This probably accounts for the increased values originally obtained by Smith.

e) Administration of progesterone or progestins : Injections of progesterone obviously cause an increase in the urinary pregnanediol levels due to the metabolised injected material. Endogenous progesterone secretion, which functions under pituitary control and is subject to feedback mechanisms, is suppressed.

Progestins or synthetic progestational agents, such as are used in oral birth control tablets suppress the secretion of endogenous progesterone by inhibiting ovulation, and hence the development of a corpus luteum. Fears have been expressed that the frequent use of progestins will cause a permanent endocrine imbalance, so it is reassuring to note that Bell et al⁽²⁶⁾ who investigated a number of long-term users of "the pill", reported that although the hormonal secretions during the first cycle after cessation of therapy were subnormal, the following cycles showed normal values.

It is convenient at this point to include a few more details concerned with progesterone or progestin therapy. As has already been mentioned, these substances are prescribed for the treatment of pregnancy disorders and

contraceptive purposes. They are also administered in cases of menstrual cycle disorders, for some types of carcinoma, and for the treatment of ovarian cysts.

The main physiological effects of progesterone have already been mentioned or implied. A number of associated secondary effects are as follows:

- 1) Inhibition of A.C.T.H. release.
- 2) Diuresis.
- 3) Haematopoiesis.
- 4) Lipaemia and an increase in body weight.
- 5) Thermogenicity.
- 6) Vaginal epithelium desquamation and changes in the nature of cervical mucus.

These latter two effects are of importance and will be discussed later.

DISCUSSION

The many factors, both pathological and non-pathological, which may influence the rate of progesterone secretion and pregnanediol excretion have been examined in some detail. In spite of the fact that urinary pregnanediol concentrations are not directly proportional to blood progesterone levels, a great deal of useful information may be obtained from a pregnanediol assay. It has been stated that pregnanediol concentrations are high in those conditions where progesterone secretion is high, and vice versa. When this rule is not obeyed it is safe to expect the presence of some malfunction or abnormality, and a pregnanediol assay may sometimes be the only practical method of discovering the complaint. Even in non-pathological conditions, an assay is useful in ovulation or pregnancy determinations. Methods of assay are discussed in the following chapter.

SUMMARY OF THIS CHAPTER

The various conditions which affect progesterone or pregnanediol concentrations have been described. Some of the secondary effects induced by progesterone administration have also been listed.

CHAPTER FOUR

Assay methods for progesterone and pregnanediol

1) Progesterone in blood plasma

Unmetabolised progesterone may appear in the urine, but where it does so it is in such minute concentration that its assay is clinically impracticable and is employed merely as a demonstration of technical dexterity. Prior to the 1950's estimations of progesterone secretion depended almost entirely on the measurement of pregnanediol levels in urine. Since then a number of different methods have been devised.

a) Biological methods using rats, rabbits or mice. Although it has been claimed that it is possible to detect as little as 0.002 g of progesterone, the methods are time consuming, laborious and of poor specificity.

b) Colorimetric and Fluorimetric methods. The first usable method is credited to Edgar⁽²⁷⁾ who employed paper chromatography and U.V. spectrophotometry. Subsequent variations of Edgar's method have increased its sensitivity, and have made use of column or multiple paper chromatographic separations. The maximum accuracy claimed for these methods is 80%.

Fluorimetry was used by Heap (1964) who after extraction and paper chromatographic separation, enzymatically converted progesterone to 20 α -hydroxy - preg - 4 - en - 3 - one and measured the fluorescence in H₂SO₄. This method is claimed to be more specific and sensitive than any other hitherto devised.

c) Gas chromatography. Various methods have been reported. Collins and Somerville⁽²⁸⁾ extolled the undoubted advantages of their method but in spite of this it has had little clinical application. The gas chromatograph is not yet inexpensive enough for its adoption by routine clinical laboratories.

The extraction and purification of progesterone is carried out in the usual way and it is only the purified product which is quantitatively assessed by G.L.C. Nowadays it is possible to apply to the

manufacturers themselves for such data as column packings, flow rates and temperatures. (29)

- d) Isotopic methods. Many methods are described in the literature, the one which has seen most employment is that of Woolever and Goldfiens³⁰ It is a method most attractive in its elegance and simplicity. ^{14}C progesterone is added to plasma which is then extracted and chromatographed. Elution is with tritium-labelled Sodium borohydride. Thus the original progesterone in the plasma becomes ^3H labelled and the added progesterone is labelled with both ^3H and ^{14}C . A ^{14}C count enables a correction factor to be calculated. A ^3H count indicates the total progesterone present, and the difference between the two counts gives the amount of progesterone originally present in the sample.

2) Pregnanediol in urine

Methods for assaying pregnanediol in blood have been described, but as such assays have no advantages not shared by urinary estimations, and many disadvantages, they are seldom used.

It has been previously pointed out that owing to variations in the rate of progesterone metabolism and pregnanediol conjugation, it is not possible to correlate pregnanediol urine levels directly with progesterone secretion. This has led some authorities to query the usefulness of a pregnanediol assay. R.U. Short⁽³¹⁾ who has done much work on the assay of progesterone in blood, writes "On the whole pregnanediol assays have proved disappointing as diagnostic aids, and it is doubtful if they can provide much information that is not readily available from a clinical examination of the patient." On the other hand there is the opinion expressed by Loraine and Bell⁽⁴⁾ both men of wide clinical experience and members of the Medical Research unit in Edinburgh ... "It cannot be denied that pregnanediol excretion in urine does to some extent at least reflect the secretion of progesterone. Thus pregnanediol excretion is high in clinical conditions in which it might be reasonable to expect that progesterone secretion is high and conversely its output is low in patients suffering from luteal deficiency or placental failure. Bearing in mind the fact that

urine progesterone assays are of doubtful accuracy, it appears reasonable to conclude that clinically useful information regarding the secretion of the parent hormone can be obtained by urinary pregnanediol assays."

Neither party has pointed out the main advantage of a pregnanediol assay, namely, that as far as ease of collection and of subsequent treatment of the specimen is concerned, urine is definitely the physiological fluid of choice.

The first assay method for pregnanediol in urine was developed by Venning⁽²⁾ It was widely used and contributed much to the early knowledge of the progestational changes which take place during the female menstrual cycle and pregnancy. The Venning method is described in a later chapter. It consists of a butanolic extraction of conjugated pregnanediol (P.L.G.), purification and finally gravimetric evaluation of the purified P.L.G. Specificity and sensitivity are poor, accuracy doubtful, and procedure lengthy and tedious. Yet this method was very extensively used. Venning was the first to report that patients with a tendency to abort had a lower than normal urinary pregnanediol concentration.

Variations of the gravimetric method were developed by many workers. Beall⁽³²⁾ described a benzoic acid absorption method, and not a few papers appeared claiming improvements in accuracy due to minor modifications such as centrifuging during extractions, adjusting the initial pH of urine, etc. etc.

Pregnanediol was a latecomer to the hormone field. Most androgens and oestrogens had been earlier identified and each had a well-established method of assay. Pregnanediol, however, escaped detection for so long because it is biologically inactive. Injections of urine fractions gave no hint of the presence of pregnanediol, and only physico-chemical means could be employed in its determination. Small wonder then that when Venning published her method and it was realized that pregnanediol was a metabolite of progesterone, so many workers turned their attention to its estimation and were responsible for so many modifications.

The spate of publications began to diminish in the early 1940's. From the point of view of technique, the only contributors who introduced new ideas were Maughan⁽³³⁾ who used Tollens reagent (alcoholic naphthoresorcinol), to estimate colorimetrically the glucuronic acid fraction of the

purified conjugate and thus avoided the fallible gravimetry; and Allen et al⁽³⁴⁾ who developed a titrimetric method.

These early methods relied upon the extraction of the conjugated form of pregnanediol. It was Astwood and Jones who in 1941 advocated an initial acid hydrolysis, but their method still involved a final gravimetric evaluation. Talbot used acid hydrolysis and gravimetry.

The next major development occurred in 1954 when Bongiovanni and Clayton of the Johns Hopkins hospital separated hydrolysed pregnanediol from urine by way of an alumina column. Colour was developed with concentrated sulphuric acid and read in a spectrophotometer at 420 m.u. These workers also suggested enzymic hydrolysis with β -glucuronidase in order to avoid producing the coloured breakdown products which result from acid hydrolysis, and which contaminated the column eluate.

Borth, in 1957 used column separation followed by gravimetric evaluation. This rather retrogressive step may have been in response to a publication by Verboom⁽³⁵⁾ who reported that "the colour developed by pregnanediol is not specific and is shared by many steroids." Verboom also noted that pregnanediol E.max. values depended on the temperature, time and rate of heating and cooling, and ambient light. The column separation was still not specific. Pregnanetriol appeared with pregnanediol in the eluate and falsely elevated the spectrophotometrically determined results.

The method of Klopper, Mitchie and Brown avoided this fault. It is the method that is still the most commonly used today and is in fact the standard against which all subsequent methods were evaluated and compared. The method involves an initial hydrolysis and extraction followed by alumina chromatography, acetylation and further alumina column chromatography. The pure pregnanediol diacetate is finally coloured with a modified sulphuric acid reaction. The method illustrates the principle of two chromatographic steps with a modifying step between them, namely acetylation. (Bush³⁶). Any substance accompanying pregnanediol through the first column will be removed by the second column in which the acetylated pregnanediol will have a different polarity and hence elution rate.

Other methods of pregnanediol separation were developed using paper chromatography or electrophoresis. One paper chromatographic method

followed by a spectrophotometric evaluation was claimed by its authors to have an accuracy of 97.8%. The methods have been reviewed by Bush⁽³⁶⁾ and Neher⁽³⁷⁾, Electrophoretic methods have been described (Miyamoto⁽³⁸⁾, Edwards³⁹⁾ but these are mainly qualitative. Gas chromatographic methods of evaluating the purified extract have been utilized for research purposes, but seldom for clinical routines. Accuracies (in terms of recovery values) of 80 - 90% have been quoted.

The most rapid and practical of the methods used for the determination of pregnanediol is undoubtedly the one involving Thin Layer Chromatography (T.L.C.). A number of similar techniques have been described but the most well-known of them is that of Waldi⁽⁴⁰⁾. It is semi-quantitative. Some investigators, notably Bang⁽⁴¹⁾ and Starka⁽⁴²⁾ have made use of a T.L.C. separation followed by elution, colour development with H₂SO₄, and spectrophotometric evaluation. Waldi's method is still the one that has attracted the most attention. Neher⁽⁴³⁾ Stahl⁽⁴⁴⁾ Randerath⁽⁴⁵⁾ and Kirchner⁽⁴⁶⁾ all discuss the method. Alternative developing solvents have been suggested but none of these has any advantage over Waldi's original Chloroform/Acetone system.

Not all of the many different methods developed in the past have been described above. Some of the hybrid techniques seem to have lacked the usual mongrel virility, for their appearance in the literature was sporadic and they did not survive for any length of time. Only those works which have contributed materially in whole or in part to the amassing of data on pregnanediol or progesterone, have been reviewed in the preceding pages.

DISCUSSION

The different methods by which progesterone is assayed have been described. The earlier biological assays have largely given way to instrumental techniques and these have been developed to a high degree of technical perfection.

The methods used to determine the concentration of pregnanediol in urine have also been described. The opinions regarding the usefulness of such determinations as expressed by authorities in the field have been quoted.

The history of the pregnanediol assay is an interesting one. It began with Venning who developed the first practical method, and continued by way of the numerous workers who were each eager to play a part in the history, either as participant or critic. Klopper and his associates have undoubtedly produced a noteworthy performance with their double chromatographic column assay, but it may well be the advocates of Thin Layer Chromatography who will write the final chapter.

SUMMARY OF CHAPTER FOUR

The various methods which have been used to measure the concentration of progesterone in blood, or pregnanediol in urine, have been briefly described.

CHAPTER FIVE

Clinical applications of progesterone/pregnanediol
assay results

The pathological conditions which give rise to abnormal or sub-normal progesterone and pregnanediol concentration have already been described. A progesterone or pregnanediol assay is a valuable diagnostic aid by which suspicions may be confirmed. An assay may also be used to 'individualise' or tailor the dose of gonadotrophins during sterility treatment. However it is likely that the main uses to which the assays will be put, will be connected in some way with human reproduction.

- 1) A pregnanediol assay is an ideal method to use for establishing the day of ovulation. This knowledge may be used in two ways: it may be used when practising the "Rhythm Method of birth control", and, conversely, it may be used by those people desirous of conceiving, or by doctors practising artificial insemination.
- 2) A pregnanediol assay may be used as a means of pregnancy diagnosis. Waldi stressed this use of his method and when it was marketed in 'kit' form by Desaga, pregnancy diagnosis was the main feature in the published list of the tests uses.
- 3) Finally, a pregnanediol assay may be used as a basis for a prognosis in cases of habitual abortion, or it may be used, in cases of threatened abortion, to indicate which cases are likely to benefit from progesterone treatment.

Items 1) and 2) will be discussed in greater detail in the following pages.

Determining the day of ovulation, and the uses of such determinations

Determining the time of ovulation is important to those who desire conception as well as to those who practice natural contraception, that is, who use the so-called 'safe period' or rhythm method of birth control.

When ovulation takes place the ovum travels to the uterus, taking three days for the journey. Long before it reaches the uterus, it ceases to be fertilizable. Hartman⁽⁴⁷⁾ has calculated that the fertilizable life

of the human ovum is only 6 - 24 hours. If, before that period has passed it is not penetrated by a spermatozoon, it will not nidate. The chances of fecundation are further reduced by the fact that the sperm does not retain its fertilizing ability for more than 28-48 hours (Fincus⁵³). Theoretically, therefore, fertilization is only possible within a 3-day period starting two days prior to ovulation. Knowing when those three days occur is necessary before contraception or artificial insemination can be successfully practised.

The various methods by which ovulation may be detected, have been exhaustively reviewed by Hartman. He has listed the individualistic symptoms, or those which occur in only a few subjects, such as midcycle pain, bleeding or excessive cervical secretion : and also the cyclical or regularly occurring changes common to all normal subjects, such as vaginal desquamation, endometrial changes, B.B.T. changes and hormonal variations. Obviously the most determinable of all these is the increase in B.B.T. or Basal Body Temperature. It is claimed that providing the body temperature readings are efficiently and regularly carried out, it is possible to detect the time of ovulation with remarkable accuracy.

This increase in B.B.T. is induced by progesterone (see Chapter three), and was first reported by Buxton and Atkinson⁽⁴⁸⁾ when treating a number of patients with progesterone. This thermogenic response has been frequently confirmed, and attempts were made to correlate the midcycle increase in B.B.T. with other ovulatory phenomena. Hartman used the criteria mentioned earlier and Marshall⁽⁴⁹⁾ and Vollman related the B.B.T. change to the period of maximum fertility as determined in a study of a series of single fertile copulations. The following facts were established:

- i) The midcycle rise in B.B.T. (of $\pm 1^{\circ}\text{F}$) occurs thirteen days before the next menstrual period.
- ii) It precedes the onset of cervical mucorrhoea by one day.
- iii) It occurs two days after the period of maximum fertility.

As an indicator of the fact that ovulation has taken place, the B.B.T. recording is a method offering a great many advantages, especially to the layman - and it is the layman rather than his doctor who is likely

to display the most interest in knowing the exact day of ovulation. It is only on fairly rare occasions that the medical practitioner deems it important to detect the day of ovulation, but there is a great number of the lay-public who for one reason or another, practice the rhythm method of birth control and who base their activities on a calculation of the day of ovulation.

Unfortunately, the B.B.T. graph is easily influenced by environmental, metabolic and even emotional circumstances. Although the body temperature is under excellent homeostatic control, there are many other factors which may cause a transient temperature increase.

A daily vaginal smear offers a routine means of detecting ovulation. Since the method was first described by Papanicolaou many workers have added their improvements and the test is now a rapid and efficient one. The disadvantages of the method detract from its usefulness : Both the collection and examination of the specimens requires the services of trained personnel.

Another simple slide test has been described⁽⁵⁰⁾ which hinges on the fact that cervical mucus dried on a slide forms an arborization or fern-leaf pattern only in the preovulation phase.

Other criteria used as ovulation indicators are

- a) Plasma enzymes: It is reported⁽⁵³⁾ that lactic dehydrogenase undergoes a significant drop in concentration at ovulation. β -glucuronidase concentration also decreases.
- b) Urinary constituents (non-hormonal): Ascorbic acid concentrations diminish at midcycle.
- c) Cervical mucus viscosity: The 'threadability' or 'Spinnbarkeit' test on a microscope slide is claimed as a rapid and reliable test. (The mucus shows maximal 'threadability' at midcycle.
- d) Vaginal pH : this decreases at midcycle.
- e) Glucose concentration of vaginal secretions: There is a mid-cycle increase.

f) Chloride concentration of vaginal secretions: There is a mid-cycle increase.

The initiators of the rhythm method of birth control, Ogino and Kraus, relied on arbitrary timing for sexual abstinence, but it was soon realized that by pinpointing the time of ovulation, the method would be very much more reliable. So it is that a number of patented contrivances have been marketed, all purporting to enable the user to definitely establish the day of ovulation. The methods make use of some of the criteria listed above, especially items d, e and f. Intravaginal applicators containing test paper measure the pH, or glucose or chloride concentration of the vaginal mucus, and although the design and theory behind the design of the contrivances is valid, they have two main handicaps: 1) They are used to measure secondary effects only, effects which are induced by hormonal changes but which could also result from factors not concerned with hormone production, and 2) They are for use on a single biological system subject to numerous influences capable of completely altering the results which could legitimately be expected from the same test performed in vitro.

Any system of ovulation detection which relies upon such secondary effects will be similarly handicapped. Failing the direct observation of ova or corpora lutea there is only one method by which ovulation may indubitably be proved. In the words of Pincus⁽⁵³⁾ "A rise in urinary pregnanediol during the menstrual cycle is an unequivocal demonstration of the establishment of an active corpus luteum."

Unfortunately a simple method of pregnanediol detection for use in the home has not yet been developed. It is possible however, if the demand were great enough, that ovulation determination in clinics could be established in the larger centres, but only if a reliable, rapid and economical routine method were available. The only technique which at present has all these qualifications is the one using T.L.C.

The rhythm method of birth control is practised by a fairly large segment of the population who for religious or health reasons may not use any alternative system. Few of the practitioners of this method have been completely successful in limiting the size of their families, and contemporary literature makes frequent and scathing reference to the apparent

fallibility of the system. This would seem to be unfair because in theory at least, the method is a valid one. It might be wise to re-examine the theory in the light of most recently developed techniques in order to see whether the practical results cannot be brought more in line with theoretical predictions.

The Rhythm Method of Birth Control

Much has been written on the subject, most of the literature being directed at the layman. Facts and findings are quoted which when applied to the individual rather than to the species, are extremely misleading.

The method is based on the following tenets:

- i) The ^{ovum}ovum is only pregnable for a short period after ovulation.
- ii) Spermatazoa have a short life in the female reproductive tract.
- iii) Ovulation always takes place 14-16 days before the onset of the next menstrual cycle. (T.S. Weldon⁽⁵¹⁾).

On this basis sliding scales, coloured calendars and strings of beads have been devised so that the subject knowing the length of her cycle can calculate the time of maximum fertility. For extra safety the system advises a few days margin both before and after ovulation, so that the recommended periods of sexual abstinence range in length from 8 days to 13 days, depending on the length and regularity of the cycle.

Apparently then, the system is perfectly safe, a fact which is pointed out by Weldon who quotes the figure of 97% success. This figure compares rather strangely with the confirmed findings of G.R. Venning⁽⁵²⁾ who quotes a figure of 24 pregnancies per 100 women years for the Rhythm method making it second only to the douche as far as contraceptive ineffectiveness is concerned, and 24 times less reliable than the oral contraceptive.

One is then forced to the conclusion that at least one of the above tenets is not valid. It is beyond the resources of this project to carry out an investigation into all of them, but tenet No. 3, that is, that ovulation occurs at the same stage of the cycle each time, is eminently suitable as an object of research using the T.L.C. method of pregnanediol estimation. ||

Pregnancy Diagnosis

As was mentioned earlier, the T.L.C. pregnanediol assay has been recommended as a suitable means of diagnosing pregnancy and so it joins a long list of procedures - a list which has its origins in ancient times.

For some reason man has long been preoccupied with the art of diagnosing pregnancy. The cabalistic rites and ceremonies persisted until fairly modern times and it was only in 1927 when the first reliable pregnancy test was developed by Aschheim and Zondek. Acceptance was slow. Even six years later a medical dictionary⁽⁵⁴⁾ asserts that the only sure sign of pregnancy is when the medical attendant hears the beating of the foetal heart.

The Aschheim Zondek test makes use of the fact that chorionic gonadotrophic appears in the urine soon after conception. Chorionic gonadotrophin is biologically active and when detoxified ^{pregnancy} gravid urine is injected into an animal it induces certain physiological responses. The A.Z. test uses immature female mice, a positive response being the appearance some days later of haemorrhagic follicles and corpora lutea. The test takes five days. Friedman developed a two-day test using rabbits. The Kupperman test (1943) was the last one making use of mammals, subsequent tests used fishes and amphibia, and could be carried out fairly rapidly (just over two hours). The quoted accuracy of some of these tests was 98-100%.

The above tests all relied upon the excretion of Chorionic Gonadotrophin (H.C.G.) which is only produced during pregnancy and also in some, very rare, diseases. Methods based on the estimation of substances other than hormone have been devised and used but without much general acceptance. The drop in the Basophil count which occurs in pregnancy was suggested as a method, and the increase in urinary histidine, likewise occasioned by pregnancy, was also advocated as a pregnancy test. (81)

A method which still enjoys some popularity involves the administration of an Oestrogen/Progesterone combination to the patient. The failure to induce vaginal bleeding within 15 hours indicates pregnancy (92% certainty). A similar 'endpoint' is used in a test involving an injection of Neostigmine⁽⁵⁵⁾.

That the concentration of urinary hormones (other than gonadotrophin) could be used to diagnose gravidity, was first suggested by Patterson⁽⁵⁶⁾ in 1937. He devised a method for detecting Oestriol in the urine, a positive result indicating pregnancy. Jayle and Crepy suggested basing a diagnosis on an estimation of the total urinary glucuronide concentration, and Soule and Yanow⁽⁵⁷⁾ quoted limits for a pregnancy test involving a pregnanediol glucuronide estimation. (92.2% accuracy). The Waldi test also makes use of urinary pregnanediol concentrations.

The great advantage of the tests devised to detect Gonadotrophin is that if chorionic gonadotrophin is proven to be definitely present, then there is over a 99.9% certainty of gravidity. Other substances including pregnanediol are normal constituents of non-gravid urine and their presence is only diagnostically significant if the concentration is above a certain value. Thus while the chorionic gonadotrophin test need be qualitative only, the other tests must be quantitative or at very least semi-quantitative.

Nevertheless, the quoted accuracy (according to Waldi et al) of the T.L.C. pregnanediol assay pregnancy test is an impressive "greater than 99%". It takes the same time to perform but is better suited to routine work than the biological tests, and no specialized apparatus is necessary. True, the test has been marketed by Desaga in kit form but any laboratory possessing Thin layer apparatus can carry out the test. The Desaga "mini-set" would undoubtedly have commanded a considerable degree of popularity were it not for the development of the new immunological method of pregnancy diagnosis⁽⁵⁸⁾ which was introduced at about the same time. In the immunological test, an anti-serum to chorionic gonadotrophin (obtained by injecting H.C.G. into rabbits or sheep) is mixed with urine and 'Latex' (plastic particles coated with gonadotrophin). If the urine contains gonadotrophin it reacts with the antibody in the serum neutralizing it. The Latex will be unaffected. If the urine contains no gonadotrophin the antibody in the serum will remain active, and attacking the latex, will cause an easily-observed agglutination or clumping of the latex particles. There are a number of variations to the test described above.⁽⁵⁹⁾ Most of them are very reliable and rapid (3-5 minutes). The accuracy of the immunological test has been quoted as 96.6% in positive cases and 98.7% in non-pregnant

cases. (62)

Even with its allegedly superior accuracy, the T.L.C. test would be unable to offer much competition to the immunological test as far as the normal clinical laboratory is concerned where economy of time and labour is a vital factor. One must bear in mind, however, that the immunological test is purely a method of diagnosing pregnancy whereas the T.L.C. pregnanediol assay has many more uses. Medical science uses less and less empiricism and demands more help from the medical research units, with each passing year. Thus the need for new techniques and diagnostic aids becomes increasingly greater with the passage of time.

Before a new technique is incorporated into the repertoire of the clinical laboratories, it must have certain basic attributes. It must be productive of reliable and useful information of a type demanded by the medical fraternity. It should lend itself to routine use and not require excessive time and technical attention. Finally, it should be inexpensive. Expense in these cases is a relative term and almost entirely dependent upon the importance attached to the nature of the results produced by the test. Expense must also be assessed not only in terms of initial expenditure, but also in terms of maintenance, reagents required and manpower needed for the technique.

The factors mentioned in the preceding paragraph are some of the criteria which are employed in assessing the "practicability" of a method (60) and it was the purpose of this research to investigate the practicability as well as other aspects of the Thin Layer Chromatographic method of pregnanediol assay.

DISCUSSION

To use a pregnanediol assay as a means of determining the concentration of progesterone in the blood is, as has already been explained, impossible. A pregnanediol assay can however establish whether the rate of progesterone secretion is higher or lower than normal. It can detect such occasions as are heralded by an increased progesterone secretion, and it can indicate the possibility of a pathological condition. To do this it is not necessary to ~~tie in~~ ^{correlate} urinary pregnanediol levels with blood

progesterone levels. All that is necessary is the availability of empirically determined limits for the condition being investigated. For instance, if a pregnanediol assay result is determined on a specimen taken during the second trimester of pregnancy, and if the result falls within the limits pertaining to the period of pregnancy, then it can be stated with certainty that the placenta of the donor of the specimen is functioning normally as far as progesterone secretion is concerned. Similarly pregnancy can be diagnosed if a pregnanediol determination yields values which are well above the upper limit of pregnanediol excretion during a normal (non-pregnant) cycle. It is of course necessary to know the relevant limits but these can be obtained by a study of the figures quoted by the numerous workers in the field. Providing the limits are correctly chosen, the diagnoses or prognoses based on a pregnanediol assay will have a very high degree of certainty.

To establish the occurrence of ovulation, various criteria have been made use of. None, however, are as certain as the results of a progesterone or pregnanediol assay. A sustained increase in pregnanediol output is a sure indication that ovulation has taken place. What is more, it is an immediate indication, unlike the rise in Basal metabolic temperature which occurs two days after ovulation. Such an assay can therefore produce results which would be extremely useful to those who wish to limit or increase their family, or to the physician investigating menstrual disorders.

The various methods used to detect pregnancy have been described, and this application of the T.L.C. pregnanediol assay is mentioned. The validity of the T.L.C. pregnancy diagnosis will be reviewed at greater length in subsequent chapters.

SUMMARY

The clinical applications of the pregnanediol assay have been described with especial reference to ovulation determination and pregnancy diagnosis. A brief description of the theory behind the "Rhythm Method" of birth control, has been included in this chapter, and also a history of the methods used to detect pregnancy.

CHAPTER SIX

The T.L.C. pregnanediol method described hereunder is not the original as published by Waldi, but is the modified form devised for use with the Desaga Miniset. The method has been registered as a patent by Merck A.G., Darmstadt. It is virtually identical to the originally published method: the few modifications being introduced in order to make the method more suitable for routine use.

The method is divided into stages for convenience of reference.

STAGE ONE. HYDROLYSIS.

20 ml of urine (filtered) are acidified by the addition of 2 ml of Hydrochloric Acid B.P. (SG 1.19). The urine is either part of a normal daily specimen or, if the most quantitative results are desired, is an aliquot of the total 24 hour production of urine. The acidified urine is heated on a steam or water bath to 90°C and is left at that temperature for exactly 10 minutes, after which it is rapidly cooled under running water to room temperature.

STAGE TWO. EXTRACTION.

The hydrolysate is extracted three times in a separating funnel, each time using a fresh 25 ml portion of cyclohexane. Some urines have a tendency to form emulsions. This tendency can be minimised by running the urine (prior to hydrolysis) through a strongly acid cation exchange column. The emulsion-forming tendency can be further reduced by the addition of 2-3 ml I.N. Sodium Hydroxide to the urine-cyclohexane mixture prior to the first extraction.

After extraction, the combined cyclohexane is washed twice, each time with a 20 ml portion of I.N. Sodium Hydroxide. The washed cyclohexane is then dried in a 100 ml flask containing 5-7 g. of anhydrous sodium sulphate.

After 10 minutes drying, the cyclohexane is filtered through dry filter paper, the sodium sulphate being washed with a few ml of fresh cyclohexane and the washings added to the cyclohexane extract.

STAGE THREE - EVAPORATING TO DRYNESS

The extract is evaporated to dryness on a water bath, preferably using reduced pressure. The dry residue is quantitatively transferred to a 10 ml tube using four portions of chloroform. The chloroform is removed by evaporation after which exactly 0.5 ml chloroform is used to dissolve the residue thus producing the final test solution which is to be applied to the thin layer plates.

STAGE FOUR - SPOTTING THE PLATES

The Desaga miniset employs ridged glass plates (150 x 100 mm) as described by J.M. Bobbit.⁽⁶¹⁾ They are coated with Silica Gel G (Merck), 250 mu thick, and activated by heating at 110°C for half an hour. When testing for pregnancy the following spotting scheme is recommended:

Lanes	1	2	3	4	5	6	7	8	9	10	11	12	13
ul spotted	5a	25b	1c	25b	3c	25b	5c	26b	7c	25b	10c	25b	5a

where a = test mixture of dyes, b = urine extract, c = Standard P.G.soln. (0.1% P.G. in alcohol i.e. 1 ul = 1 ug P.G.).

The spotting may be carried out using a micrometer pipette or by using a capillary, depending on the degree of accuracy required. A dosage scheme for a capillary is included in the Desaga instruction sheet.

If a cycle rather than a pregnancy test is to be carried out, 50 ul of urine extract are used instead of the 25 ul specified above.

The plate is developed in a saturated atmosphere using as solvent a chloroform : acetone (85 : 15) mixture. The proportions can be slightly varied in order to ensure that the P.G. spot has an Rf of about 0.2. Developing distance is 10 cm, which usually takes half an hour.

STAGE FIVE - DETECTING THE STEROID

The plate is sprayed with a 30% v/v orthophosphoric acid solution until just transparent, and is then heated at 110°C until the layer colour is once again matt white. (10 - 20 minutes of heating). The pregnanediol is then visible under ultraviolet light (long-wave) as a greenish-grey fluorescence. Its position may be confirmed by over-spraying the plate with 1.5% phosphomolybdic acid in ethanol which causes

pregnanediol to appear as deep blue spots.

STAGE SIX - QUANTITATIVE EVALUATION

By comparing the spot sizes of standard and unknown it is possible to calculate the concentration of pregnanediol in the urine to within fairly narrow limits. Randerath⁽⁶²⁾ discusses the validity of the method and points out that it is generally sufficient to derive mean values (of spot sizes) for each concentration, from determination under standardized conditions on several identically treated plates of equal thickness. The most accurate results, however, are obtained by running known amounts on the same chromatogram and comparing the relative spot areas on this one plate. This is the method chosen by Waldi.

The urine extract is 40x more concentrated than the original urine specimen. 25 u.l. of urine extract are used to spot the plate, so this is equivalent to 1 ml of the original urine specimen. If the spot size of the extract is found to be the same size as, for instance, the spot of the standard solution on lane 11, the concentration of pregnanediol in the original urine specimen is 10 u.g. per ml or 10 mg per litre.*

The following claims have been made for the cycle and pregnancy test patented by Merck:

- 1) The reliability of the tests is 99 - 100%.
- 2) The test is positive 5 - 8 days after conception.
- 3) Disturbances in hormone changes during pregnancy may be established with this test.
- 4) The material cost for a single pregnancy test is only half that for the immunological test.
- 5) The complete test may be carried out in only two hours.

PREGNANCY DIAGNOSES BASED ON T.L.C. RESULTS

When the urine extracts have been evaluated, the results are interpreted as follows:** "The maximum pregnanediol concentrations found during

* In the dosing scheme printed on the Desaga Miniset instruction sheet (Ref. No. 12 04 00) there are a number of errors. The list of microgram concentrations is incorrect and the final assessment is quoted as "mgm of pregnanediol per 24 hours urine instead of per litre of urine. As few people produce exactly 1 litre of urine per 24 hours, this error could in some cases be a very large one.

** Paraphrased from the instruction sheet (12 04 00).

the secretion-phase (post-ovulatory phase) will usually lie between 3 - 5 mgm/litre urine. About 10 days after conception, the pregnanediol content lies about 2mgm higher than the maximum normally found during the secretion phase. This has been observed in all cases which have so far been investigated (over 800 cases). Urine for pregnancy tests is most usually taken after the subject has failed to menstruate, that is to say, in the proliferation-phase (pre-ovulatory phase), so that a content greater than 3 mgm pregnanediol/litre urine must be taken as positive. If pregnancy is on hand (sic) the pregnanediol concentration would be from 5 - 10 mgm/litre urine during this time. If the urine sample is obtained during the secretion phase and shows less than 6 mgm pregnanediol/litre urine, a repeat test must be made after 5 days. If in that time there has been no decrease, then the test may be taken to be positive."

As can readily be observed, the conclusions to be drawn from the above instructions are not particularly clear. Assuming the validity of the figures quoted for maximum and minimum pregnanediol concentrations during the various phases of the cycle, it appears that a positive diagnosis can only be made on the basis of a concentration of 7 mgm or more per litre urine. Lower concentrations however do not necessarily indicate a definite negative, and the test must be repeated in such cases, which is not very satisfactory. It appears as if the method of Waldi is at once too quantitative and not quantitative enough, depending on the object of the test. As a pregnancy test, it would be far more suitable for routine use if it were converted to a limit test, that is, if instead of attempting a semi-quantitative evaluation by spotting the plate with increasing volumes of the standard, one volume only were used. As the positive diagnosis depends upon a concentration of 7 or more mgm pregnanediol/litre urine, the plate need only be spotted with 7 u.l. of standard solution, or if a more detailed assessment is desired, three standard spots could be used, for instance 3, 7 and 10 u.l. This method would make more lanes available for the use of urine extracts.

If, on the other hand, variations in abnormal pregnanediol production during the cycle or during pregnancy are being studied, it is desirable to use a method which is as quantitative as possible. Evaluation

by means of spot size comparisons is subject to errors of between 5 and 10%. An elution technique followed by spectrophotometric or colorimetric evaluation is capable of reducing these errors to between 2 and 5%.

It was decided to introduce modifications as described above, to the Waldi method. During the preliminary work carried out, some anomalies were noted; viz. results which did not accord well with figures quoted by Waldi or even with some of the current text-book theory. This led to the decision to carry out a thorough investigation into as many aspects as possible of the Thin layer chromatographic assay of pregnanediol. This investigation was aimed at establishing the following points:

- 1) The validity of T.L.C. pregnanediol estimations when used as a basis for pregnancy diagnosis.
- 2) The use of pregnanediol estimation in establishing the date of ovulation, and other cyclical phenomena.
- 3) The feasibility of using a more quantitative method of assaying pregnanediol in urine, for routine use in clinical laboratories.
- 4) The accuracy of the above method (see No. 3) compared to other methods which have had extensive clinical employment.
- 5) The usefulness of the above method in following the pregnanediol excretion in some cases of:
 - a) Atypical menstrual cycles.
 - b) Pregnancy.
 - c) Drug induced variations in excretory patterns.

In carrying out the investigation it was desirable to devise a preparative method of extraction in order to ensure a supply of pregnanediol for making up standard solutions. This preparative method is described on page 61.

At all times especial attention was paid to the problem of making the various procedures more suited to routine use. Modifications were introduced wherever it was possible to effect a saving in time, labour, apparatus, or cost of materials. These modifications are discussed in the relevant sections.

DISCUSSION

The Waldi T.L.C. pregnanediol assay marketed by Desaga and patented by Merck makes two claims which appear in the light of subsequent research to be extravagant. These claims are:

- i) 99 - 100% reliability.
- ii) Positive results 5 - 8 days after conception.

The terminology is not precise. In its literal sense the claim of 99 - 100% reliability for the test is no doubt correct as the method gives very reproductive trustworthy results. However the implication is that the diagnosis based on the results of the test is correct in 99 - 100% of cases. Randerath⁽⁴⁵⁾ who discusses the Waldi method chooses this latter interpretation. He writes: "This diagnosis (of pregnancy) can be made with 99 - 100% certainty."

The second claim, that positive results are obtained 5 to 8 days after conception seems, in the light of modern theory, to be somewhat astonishing. Chorionic gonadotrophin which is responsible for maintaining the corpus luteum in a functioning state, is produced a few days after the implantation of the ovum.⁽⁶³⁾ It seems unlikely that the luteotropic activity of this hormone is such that it can, within a matter of hours, stimulate the secretion of progesterone to such an extent that it exceeds (in over 99% of cases) the highest limit of production of which the unstimulated corpus luteum is capable. That there is an early increase in progesterone secretion is not queried, but that this increase is invariably higher than the uppermost level found in non-pregnant cases is doubted, bearing in mind the widely differing pregnanediol concentrations found in the urine of different individuals at the same stage of the menstrual cycle. Jayle and Henry state that "a pregnanediol estimation has no practical value in the early diagnosis of pregnancy."⁽⁶³⁾

It is apparent that the validity of an early pregnancy test using as basis for a diagnosis the urinary pregnanediol concentration, is not definitely established and that further investigations in this field are necessary.

SUMMARY

The thin layer chromatographic method of pregnanediol assay according to Waldi has been described in detail. The validity of this method as a means of pregnancy diagnosis has been queried.

CHAPTER SEVEN

The validity of the Waldi method of pregnancy diagnosis

Most of the investigations carried out elsewhere (e.g. at the Biochemical Department of the Research Laboratories of E. Merck A.G., Darmstadt) were aimed at obtaining positive diagnosis in as many established cases of early pregnancy as possible. This method requires the cooperation of a large hospital or clinic, the services of a large number of technicians for carrying out the tests and the availability of a large number of volunteers, both male and female. Such resources are beyond the reach of this project.

It is possible, however, to employ another method: Instead of, as above, calculating the percentage of positive results obtained from testing assuredly ^{pregnancy} gravid urine, it is possible to calculate the number of false positives obtained from tests on definitely non-gravid urine, which will indicate the inaccuracy of the method. (It must be stressed that this research was not undertaken for the purposes of invalidating the Waldi pregnancy tests, but when, in the course of the investigation, results were obtained which did not ^{tally} ~~tie in~~ with claims made for the test, it seemed only logical to pay some attention to this aspect of the Waldi pregnancy test).

The method chosen to assess the validity (or otherwise) of the pregnancy test, was the alternative mentioned above, that is, ascertaining the number of false positives obtained from tests on non-gravid urine. Thus, the second aim of the investigation would be carried out at the same time and only one series of experiments would be necessary.

The second aim, which involved testing for variations in pregnanediol output during normal menstrual cycles, was carried out using urine specimens donated mainly by female student volunteers. As the primary object was to establish the date of ovulation which is indicated by a sudden and very obvious increase in pregnanediol excretion, a limit test rather than a quantitative assay was used. Only in those cases where the excretion rate was abnormal, were quantitative methods used on the urine extract.

In the initial experiments, the Waldi method was used unmodified excepting that only two concentrations of standard solution were run with

the urine extracts on the thin-layer plate. As the tempo of the trial increased, and as no technical assistance was available, it became necessary to alter the various procedures in order to cut down on the time taken by each test. When any such modification was introduced, duplicate tests were performed using both modified and unmodified methods in order to ensure that no loss of accuracy or efficiency was involved in the alteration. In its final form it was possible to carry out twenty estimations in one day. It must be pointed out that although it is claimed that the cycle and pregnancy test of Dr. Waldi takes only two hours to perform, this is only true under ideal conditions. It does not take into account the recommended initial column filtration through cationic exchange resin. Nor does it take into account the formation of emulsions - (which can be a frequent occurrence) - and the time necessary to 'break' them. The solitary worker has, in addition, to perform such mundane tasks as bringing water-baths to the boil, weighing out materials (e.g. Sodium sulphate), folding filter paper, arranging the apparatus etc., all of which, additively, considerably increases the time taken for a single estimation, though admittedly certain of these factors do not apply to a well-organised clinical laboratory.

The procedure followed during this series of trials was as follows:

Collection of Specimens

The volunteers were supplied with a funnel and a supply of sterile, amber, labelled bottles with a water-tight screw cap. They were asked to supply the following information:

- 1) The date of the first day of the previous menstrual period.
- 2) The normal duration of a menstrual cycle. (The answer was required in a form which indicated both the length and the regularity of the cycle. Thus a typical regular cycle would be 28 ± 1 day, and an irregular cycle could be for instance 32 ± 5 days).
- 3) The occurrence or otherwise of any regular mid-cycle phenomena, such as abdominal pain, bleeding, nausea, etc.
- 4) Other details for reference purposes e.g. age, place of residence etc.

Each volunteer was assigned a reference number which was printed on the label of the bottle together with the date on which the specimen was

supplied. The specimens were of morning urine taken at intervals of 1, 2, or 3 days depending on the phase of the cycle, and were placed as soon after voiding as possible, in a receptacle outside the laboratory door. If for any reason it was impossible to extract the urines on the day of delivery, they were refrigerated at about 5°C until immediately prior to extraction.

A number of the volunteers were supplied with clinical thermometers and were asked to record their morning temperatures (measured immediately after awakening) on the label of the bottle containing that days urine specimen.

Hydrolysis and Extraction

Acid hydrolysis was carried out as already described. The urines were not first passed through a cation exchange column as it was impossible to predict at this stage which of the specimens would form an emulsion while being extracted. It was considered that carrying out a column filtration on all the urine specimens in order to avoid the possibility of a few of them forming emulsions, would have been in most cases an unnecessary waste of time.

The method of extraction described earlier was altered as follows:

a) Two separating funnels were used per specimen. The heavier aqueous portion could thus be poured directly from funnel 1 to funnel 2 and could be extracted with a fresh portion of extractant while the contents of funnel 1 were draining into a 100 ml flask.

b) Only two extractions were carried out on each urine specimen. Duplicate experiments involving both two and three extractions were carried out and showed that provided the urine and extractant were very thoroughly shaken up, the final result was the same.

c) When emulsions formed, they were centrifuged until broken.

d) Alternative extractants to cyclohexane were sought; and hexane was eventually used for all extractions not involving a final fully quantitative analysis. Hexane has the following advantages :

i) It seldom forms an emulsion.

ii) It extracts fewer interfering substances from acid hydrolysed urine and so produces a 'cleaner' final extract.

iii) It may be evaporated to dryness much more rapidly and at a lower temperature than cyclohexane. This is important with respect to time saved and to the validity of final result. (It has been reported⁽⁴⁶⁾ that excessive heating may cause pregnanediol breakdown).

iv) As hexane is more hydrophobic than cyclohexane it entrains less aqueous phase during extraction. Drying may therefore be carried out using only 2 - 3 g. of anhydrous sodium sulphate.

Hexane has one major disadvantage - it is a less efficient extractant than cyclohexane, and larger quantities must be used for each extraction (30 ml instead of 20 ml). Its advantages outweigh this disadvantage.

When more than two urine specimens were required for assay, a simultaneous extraction procedure using a battery of units was devised. Each unit comprised two 250 ml separating funnels, six 100 ml conical flasks, funnels, filter paper, measures etc., and reagent bottles containing the materials required for the extraction process. The extraction was divided into stages, each stage being carried out at each unit in turn before beginning the following stage.

Using these techniques it was possible to carry out the extractions in considerably less than the time required to complete the same number of extractions using the method originally described by Waldi.

Evaporating to Dryness

One only distillation unit was used. It comprised a vacuum pump, water-cooled condenser, one 1-litre receiving flask and two 50 ml distilling flasks. The quick-fit adaptor between distilling flask and condenser was fitted with a stop-cocked funnel and air-bleeder tube. These were fitted in such a way that it was unnecessary to move them when changing distilling flasks. The heat required for distillation was provided by a water bath.

Using the above arrangement it was possible to carry out almost continuous evaporation of the hexane extracts. As each extract was dried to a gummy residue, the distilling flask was removed and replaced with a fresh flask. While the contents of the fresh flask were being evaporated, the gummy residue was quantitatively transferred from the used flask which

was then cleaned ready for attachment to the distilling unit.

Chloroform was used to transfer quantitatively the dried extracts to 5 ml tubes or micro beakers. These were placed in front of an ordinary hot air heater fitted with a deflection vane which directed the warm air on to the chloroformic extract, causing rapid evaporation. The extracts were left in the dry state until shortly before spotting on the T.L.C. plate at which time they were dissolved in 0.5 ml Chloroform.

Spotting and Evaluating the T.L.C. Plates

20 x 20 cm. plates were coated with Silica gel (250 u.m. thick) and activated in the usual way. Sixteen half-inch lanes were ruled on each plate with a sharp stylus - a procedure which minimises edge effects and ensures a horizontal solvent front.

Spotting was carried out according to the following scheme, using a 10 u.l. micropipette.

Lanes 1 and 11 : 3 u.l. of Std. P.G. solution (0.1%)

Lanes 6 and 16 : 7 u.l. of Std. P.G. solution (0.1%)

The remaining lanes were spotted with 25 u.l. of each urine extract.

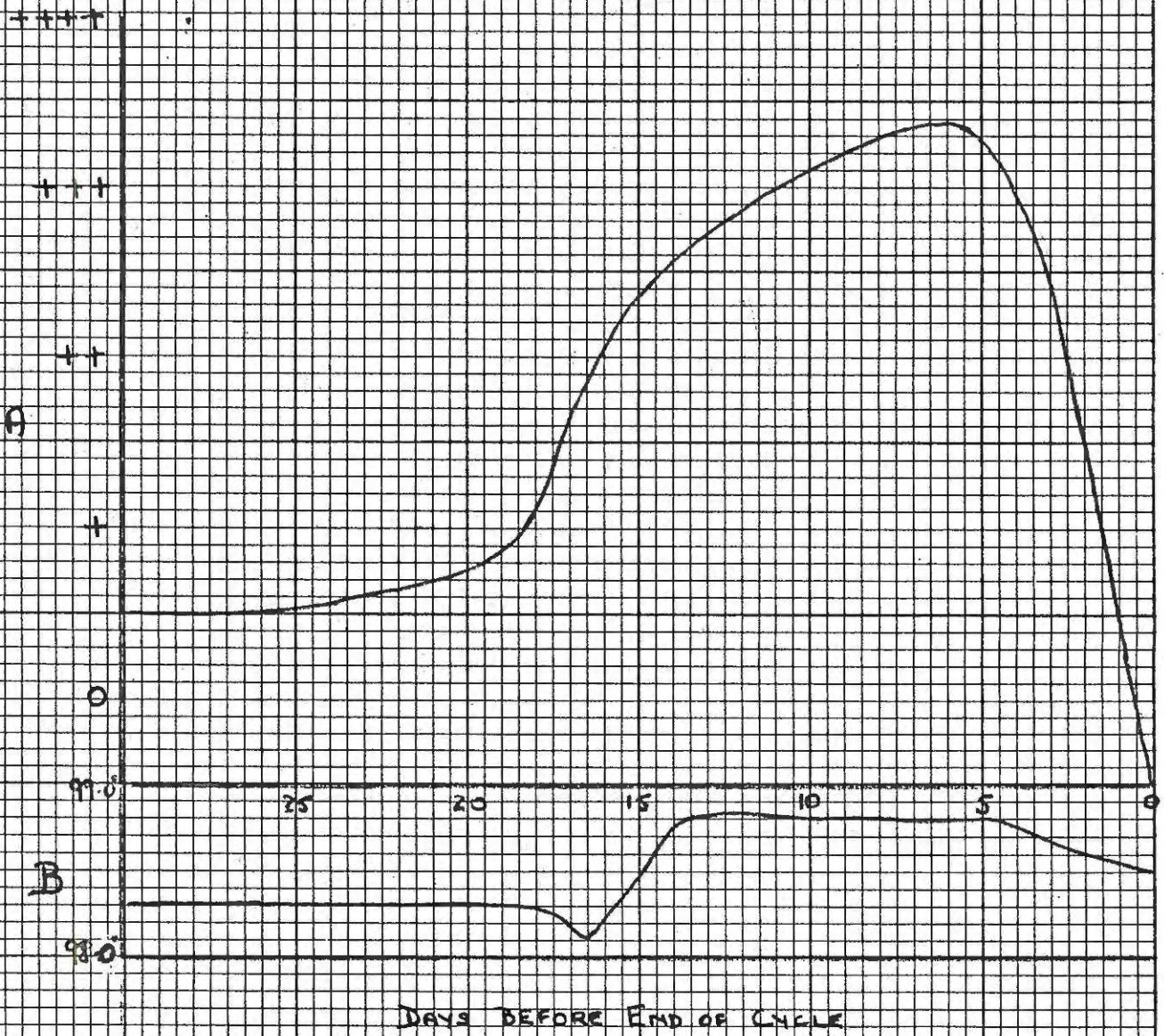
This scheme was adopted for most of the assays carried out in this project but the method of spotting was altered slightly in an effort to ensure maximum accuracy of results. For instance, the concentration of both the standard solution and urine extracts was altered in order that they could be conveniently applied by a Hamilton microsyringe (for quantitative analysis) and by capillary (for semi-quantitative analysis and limit tests). It was found to be more accurate to use standard solutions of different concentration rather than to use one standard solution and different spotting volumes.

The thin layer plates were 'visualized' after development by spraying with the phosphoric acid/phosphormolybdic acid reagent. Spot sizes were compared and the urines were evaluated according to the following scheme :

No pregnanediol (P.G.) present	—
P.G. present but less than 3 mgm/litre urine	+
P.G. present at a concentration equal or close to 3 mgm/litre urine	++

FIG 1.A

TYPICAL PG EXCRETION PATTERN
DURING NORMAL CYCLE.



A = PG assay value
(Mean of 12 typical cycles)

B = Basal Body Temperature
(Mean of 4 cases)

P.G. present at a concentration greater than 3 mgm but less than 7 mgm/litre urine	+++
P.G. present at a concentration greater than 7 mgm per litre of urine	++++

Those extracts having a response equal or greater than ++ during the pre-ovulatory phase of the cycle were earmarked for quantitative analysis. Extracts with a ++++ grading at any time of the cycle were likewise earmarked.

Results

26 volunteers supplied urine for this series of assays. They were followed over two and in some cases three cycles, but owing to the intervention of university vacation or illness, it was possible to obtain a complete record of only 49 cycles altogether.

For the most part the pattern of pregnanediol excretion was remarkably similar in the different donors. A graph of twelve typical excretion patterns is included opposite (fig. I).

In most cases the rise in pregnanediol occurred between 12 and 14 days prior to the first day of the next cycle. The results are tabulated in table No. I.

In nearly all cases in which daily temperatures were recorded, the temperative increase occurred two days after an increase in pregnanediol. Only in one case did the temperature rise on the same day as the pregnanediol output increased. (The initial temperature increases ranged from 0.3 to 0.7°F).

In the few cases who reported midcycle pain, this pain was associated with an increase in pregnanediol output on the day following the report. The pains were variously described as "sharp stabbing pain on the side of stomach", "mild but noticeable pain in lower abdomen" and "a pain like appendicitis." Only the fact that the symptoms were associated with a P.G. increase led to their being accepted as genuine and not discarded as being psychosomatically induced.

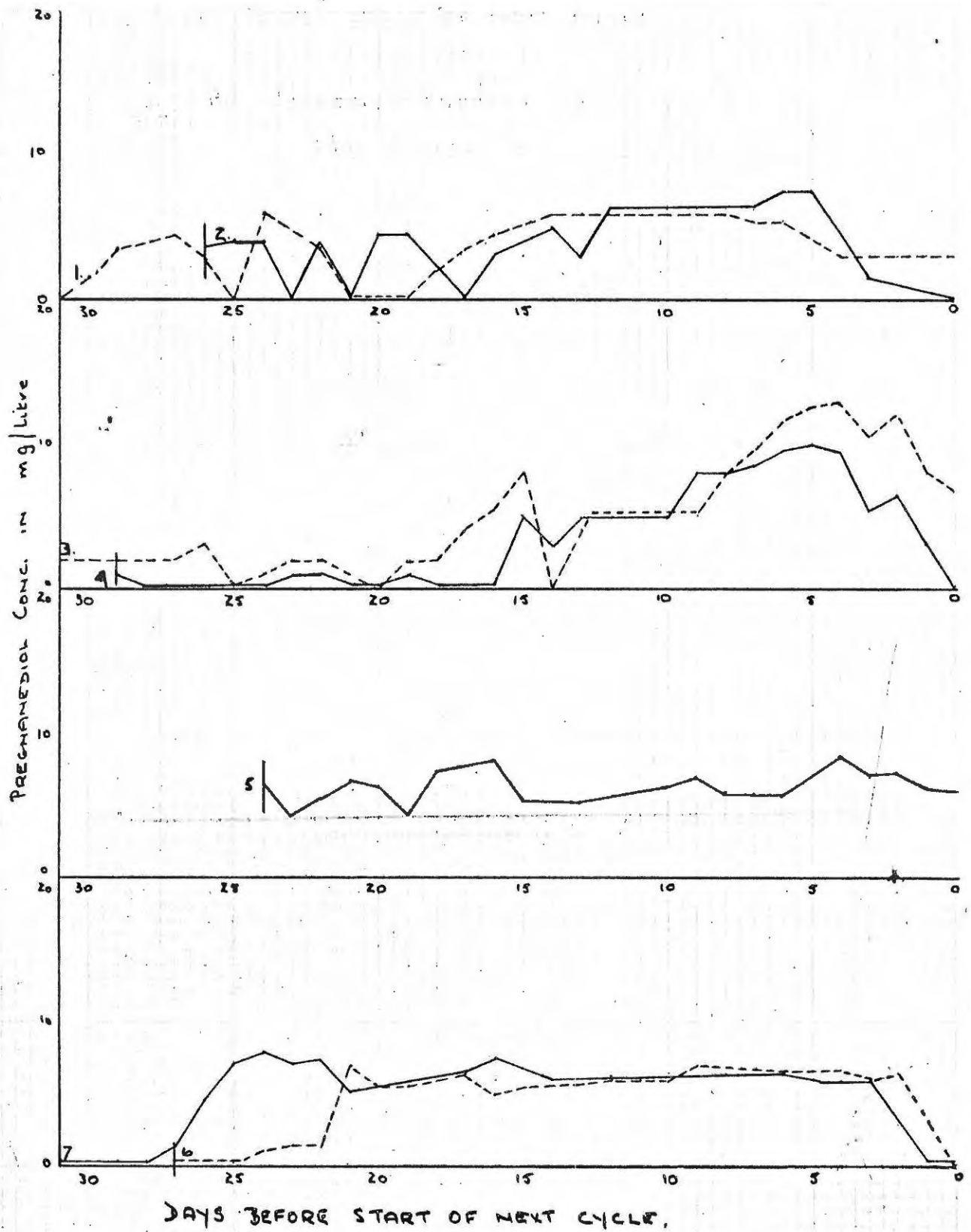


FIG 1. B. PG EXCRETION PATTERN IN SEVEN ATYPICAL MENSTRUAL CYCLES.

TABLE I - 42 cycles

	Days prior to start of next cycle:					
	15	14	13	12	11	10
P.G. increase	3 cases	5 cases	17 cases	12 cases	5 cases	
Temp. increase	-	1 case	-	1 case	4 cases	3 cases
Pain in abdomen	-	-	2 cases	2 cases	-	-

The graph showing the abnormal excretion rates is a composite of seven cases.

Cases 1 and 2 show a number of 'false starts' before finally maintaining a steady P.G. increase.

Cases 3 and 4 show a normal excretion pattern for the major portion of the cycle but towards the end of the cycle supranormal concentrations of P.G. appear in the urine.

Case 5 shows a high P.G. concentration throughout the cycle with a slight midcycle increase presumably due to ovulation. (This case was followed through a whole cycle and most of the next one. At no time were low P.G. levels recorded).

Cases 6 and 7 show an unusually early increase in P.G. output. Unfortunately a temperature record of these two cases was not available, but seeing that the increase of P.G. was maintained throughout the cycle, it must have been due to ovulation.

These seven cases are discussed more fully in the following chapters. Urine supplied by them was re-extracted and quantitatively assayed in order to provide a more accurate basis from which to draw conclusions.

The table and graphs included in this chapter are constructed from data obtained from just under 500 urinary pregnanediol assays.

DISCUSSION

The original testing (by Waldi et al) of the T.L.C. pregnanediol assay pregnancy test, was carried out on a large number of pregnant volunteers. It is possible however to assess the accuracy of the method by

using non-pregnant volunteers as described in this chapter.

For the most part it was morning urine only which was assayed. This is the usual practice when it is known that the concentration of a substance in morning urine and combined 24 hour urine is very similar (Jayle and Henry⁶³). This similarity regarding urinary concentrations of morning urine and 24 hour urine was established by a series of tests described later. (See Table No. 10).

The method was altered to make it more conformable to routine use and the urinary pregnanediol excretion was followed through a total of 49 complete menstrual cycles, not merely in order to establish the average rate of excretion of pregnanediol, but to determine the proportion of abnormal levels in a random sample of volunteers. That a number of such abnormalities were noted even in such a comparatively small trial as the one described, is an indication that as an early pregnancy test, a pregnanediol assay cannot be as reliable as reported by Waldi. The full implications are discussed later.

With regard to the average pregnanediol excretory patterns, there is nothing which contradicts contemporary theory. Ovulation as indicated by a pregnanediol increase, occurs on an average 13 days prior to the start of the next cycle, and is followed within two days by a rise in the B.B.T.

SUMMARY

The pregnanediol excretory patterns were followed through a number of menstrual cycles using a modified method. The results have been described in tabular and graphic form.

%T = Percentage light transmitted by solution.

λ = Wavelength of incident radiation

Ultra-violet Absorption Spectrum of Pregnenediol
(5% w/v PG in ethanol)

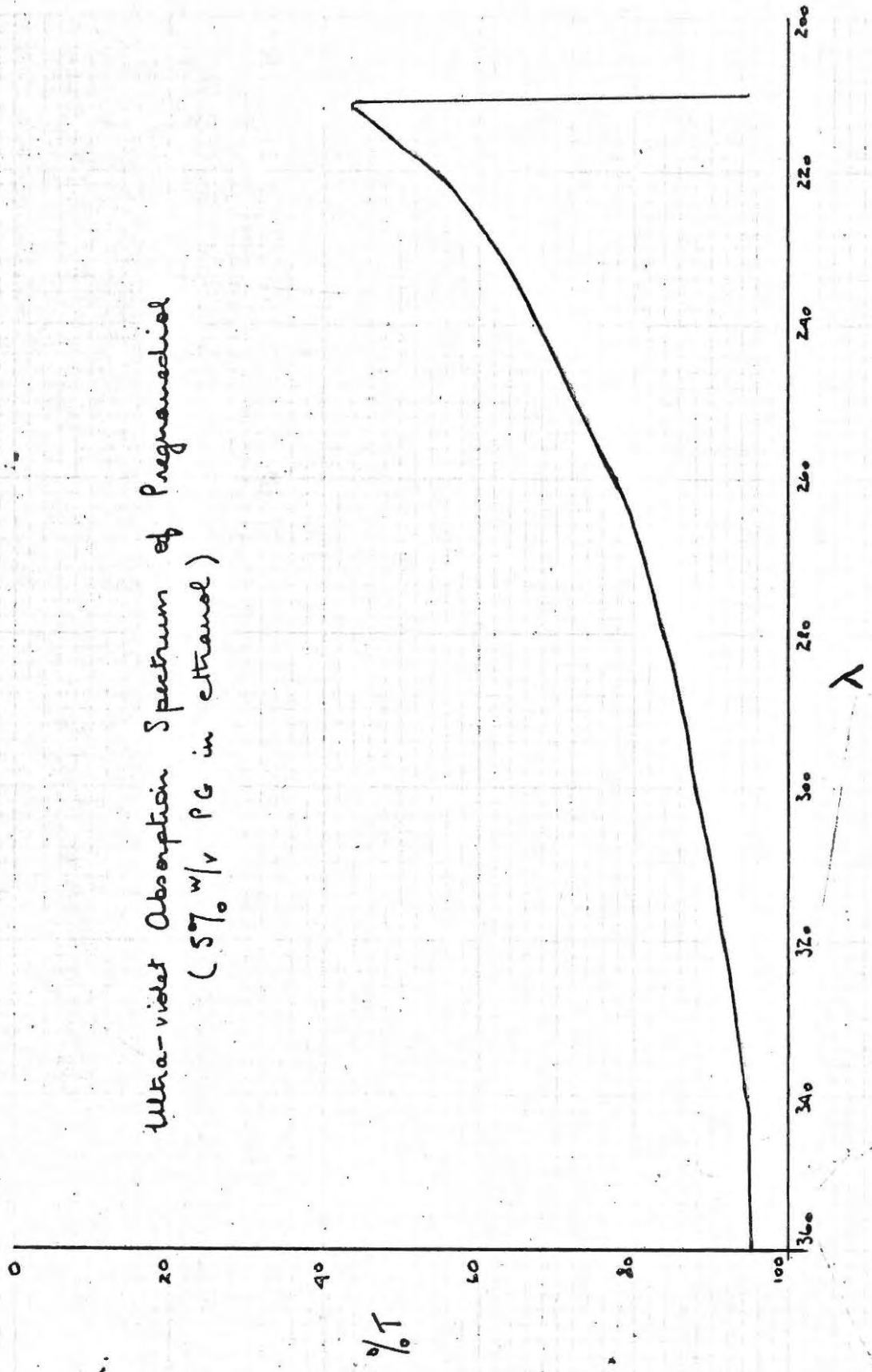


FIG. II

CHAPTER EIGHT

Quantitative Pregnenediol Assays

As can be seen from the ultra-violet absorption spectrum of pure pregnenediol in alcohol (fig. 2.) the profile offers no sharp peak which could be of use in a spectrophotometric assay. It was necessary therefore to use a colour-producing reagent, and a number of these were investigated.

- 1) Concentrated Sulphuric Acid A.R. (Bang,⁴¹).
- 2) Sulphuric Acid/Sodium bisulphite reagent (Starka et al⁴²).
- 3) 10% Vanillin in Glacial Acetic Acid/Perchloric acid reagent.⁶⁵
- 4) Phosphomolybdic acid.

Methods used

1) Colour produced by Sulphuric Acid A.R.

The developed P.G. spots were scraped from the thin layer plate with a spatula made of a celluloid blade clamped in a light wooden handle. This was found to be more efficient than using a metal blade or spatula. Locating the spots prior to scraping can present difficulties. Three methods were used

a) Spraying the two outer lanes of the plate (spotted with P.G. standard) with phosphomolybdic acid reagent, heating, and using the visible spots thus produced as a guide for scraping the untreated lanes. This method may only be used if it is certain that the solvent front has run straight, otherwise it is easy inadvertently to scrape off some of the neighbouring allopregnenediol with resultant inaccuracies in the final reading.

b) Spraying with distilled water (Bang's method). The P.G. appears as a greasy white spot on a darker background and can either be scraped directly into the sulphuric acid, or can be outlined with a stylus and scraped off when the plate is dried.

c) Spraying lightly with 30% phosphoric acid solution (v/v), heating at 110°C for five minutes and scraping the spots off under ultraviolet light. The phosphoric acid does not appear to interfere with the colour produced by the sulphuric acid.

The pregnanediol was scraped directly from the plate into centrifuge tubes containing 5 ml of concentrated sulphuric acid. After standing at room temperature for fifteen minutes the tubes were centrifuged and the contents transferred to spectrophotometer cells to be read at 430 m μ in a Beckman D.B. spectrophotometer. A standard calibration curve was prepared, the values for the unknowns being read from it.

The method is simple and rapid. Reproducibility was not ideal (table No. 2a) but by running triplicates acceptable results were obtained.

TABLE No. 2(a)

Standard Pregnanediol solution assayed by Bang's Method. A calibration curve was prepared and exactly measured volumes of standard solution were chromatographed, the results being read from the curve.

Actual concentration on plate	Concentration according to Bang's Method	%
24 ug	22.0 ug	91.7%
18 ug	17.5 ug	97.2%
12 ug	11.5 ug	95.7%
6 ug	5.5 ug	91.7%
3 ug	3.5 ug	111.65%

The standard volumes were then spotted on the plate in triplicate and the test was repeated.

TABLE No. 2(b)

Actual Concentration	Mean Value	%
20 ug	19.7	98.4
18	17.8	98.9
16	15.5	97.0
14	13.8	98.5
12	11.8	98.3

Mean accuracy : 98.2%

Pregnanediol glucuronidate was added to P.G.-free urine. The urine was hydrolysed, extracted, chromatographed and the P.G. spots treated with sulphuric acid as previously described. Two estimations were carried out

in triplicate.

TABLE No. 2(c)

Pregnanediol glucuronidate added to urine	Conc. of P.G. in urine	Mean Conc. as per Bang's method	% accuracy
60 mgm/litre	37 mgm/litre	35.5 mgm/litre	96.0%
80 mgm/litre	49.3 mgm/litre	47.5 mgm/litre	96.3%

2) Using the Sulphuric acid/Sodium bisulphite reagent

The method is very similar to that described above. The spots are scraped directly into centrifuge tubes containing 5 ml of reagent (made by adding 10.g. of sodium bisulphite to 40 ml of sulphuric acid and decanting off 30 ml). The P.G. was mixed well with the reagent and allowed to stand for 2 hours before centrifuging. Optical density was measured at 425 μ ⁶⁴

The necessity of waiting for the colour to stabilize itself, makes this method rather longer than the one previously described. In addition, the readings obtained were rather high. (Table 3). The addition of the sodium bisulphite to the sulphuric acid makes the reagent more sensitive, and lower concentrations of P.G. standard may be used. In spite of this advantage, method No. 1 appears to be of greater practical use.

TABLE No. 3(a)

(Using the sulphuric acid/sodium bisulphite method)

A calibration curve was prepared using pure pregnanediol. Known volumes of standard P.G. solution were chromatographed, coloured and read at 425 μ . Concentrations as per calibration curve are listed below.

Actual concentrations T.L.C. plates	Mean concentrations ex calibration curve	%
3 ug	3.3 ug	110
9 ug	9.2 ug	102
27 ug	27.8 ug	103

TABLE No. 3(b)

Urine containing known quantities of conjugated pregnanediol was assayed with the following results.

Conc. of Conjugate	Conc. of P.G.	Concentrations as per calibration curve	%
60 mgm/litre	37.0 mgm/litre	38.0 mgm/litre	102.5
80 mgm/litre	49.3 mgm/litre	48.5 mgm/litre	98.4

3) Using the Vanillin/Perchloric Acid reagent

This reagent was first used by Godin in 1954⁽⁶⁵⁾ and later reviewed by J.D. Few.⁽⁶⁶⁾ The method was adapted as follows.

The P.G. was scraped directly into empty centrifuge tubes. To each tube was pipetted 0.1 ml of a 10% W/V solution of vanillin in glacial acetic acid (freshly prepared). After a thorough mixing, 0.2 ml of 72% perchloric acid was added to each tube, and well mixed in by shaking. The tubes were then clamped in a frame and suspended in a water bath at 70°C for exactly 15 minutes. After cooling, 10 ml of glacial acetic acid was pipetted into the tubes, which were then centrifuged.

The peak of maximum absorbance was found to be at 580 mμ and the solutions were read at this wavelength in a spectrophotometer.

This method was found to have little to recommend it. The colour produced is influenced by reaction temperature, presence of moisture, age of vanillin reagent and the presence of any organic matter. It is also rather insensitive, relatively high concentrations of P.G. being necessary to produce a quantitative colour curve.

4) Using phosphomolybdic acid reagent

It was noticed when washing thin layer plates used for pregnanediol determinations and treated with phosphomolybdic acid, that the colour which was rinsed from the plates was persistent enough to turn a sink-full of washing water blue. Accordingly it was decided to investigate the possibility of using this reagent to impart colour to P.G. on a quantitative basis.

At first the P.G. was scraped from the plate into a tube of the reagent. However the results obtained from a reaction on the plate were found to be as good or better than a reaction performed on the eluate in tubes.

The Lambert-Beer law is obeyed over a fair range of concentrations (0.01 - 0.5 mg), and although the sensitivity is not as great as with the sulphuric acid/sodium bisulphite reagent, it is adequate.

The method finally employed was as follows : The developed plate was carefully sprayed with a 1.5% solution of phosphomolybdic acid in ethanol (^w/_v). It was heated at 110°C to maximum colour density (7 - 10 minutes). The plate was then allowed to cool to about 60°C and resprayed with 30% orthophosphoric acid in water, after which it was again heated to 110°C for five minutes.

The coloured spots were carefully scraped off into centrifuge tubes containing 5 ml of distilled water, and a similar area of thin layer which had undergone the spraying process but which contained no P.G. spot, was also scraped into 5 ml of water and treated like the rest.

The tubes were centrifuged and read in the spectrophotometer at 690 mμ which was the wavelength of maximum absorption. The solution obtained from the eluate containing no P.G. was used as the blank.

Plate to plate reproducibility was poor and it was found that the best method was to run on each plate, 5 standard spots of increasing concentration with which to construct a calibration curve for determining the concentration of the extracts run on the same individual plates.

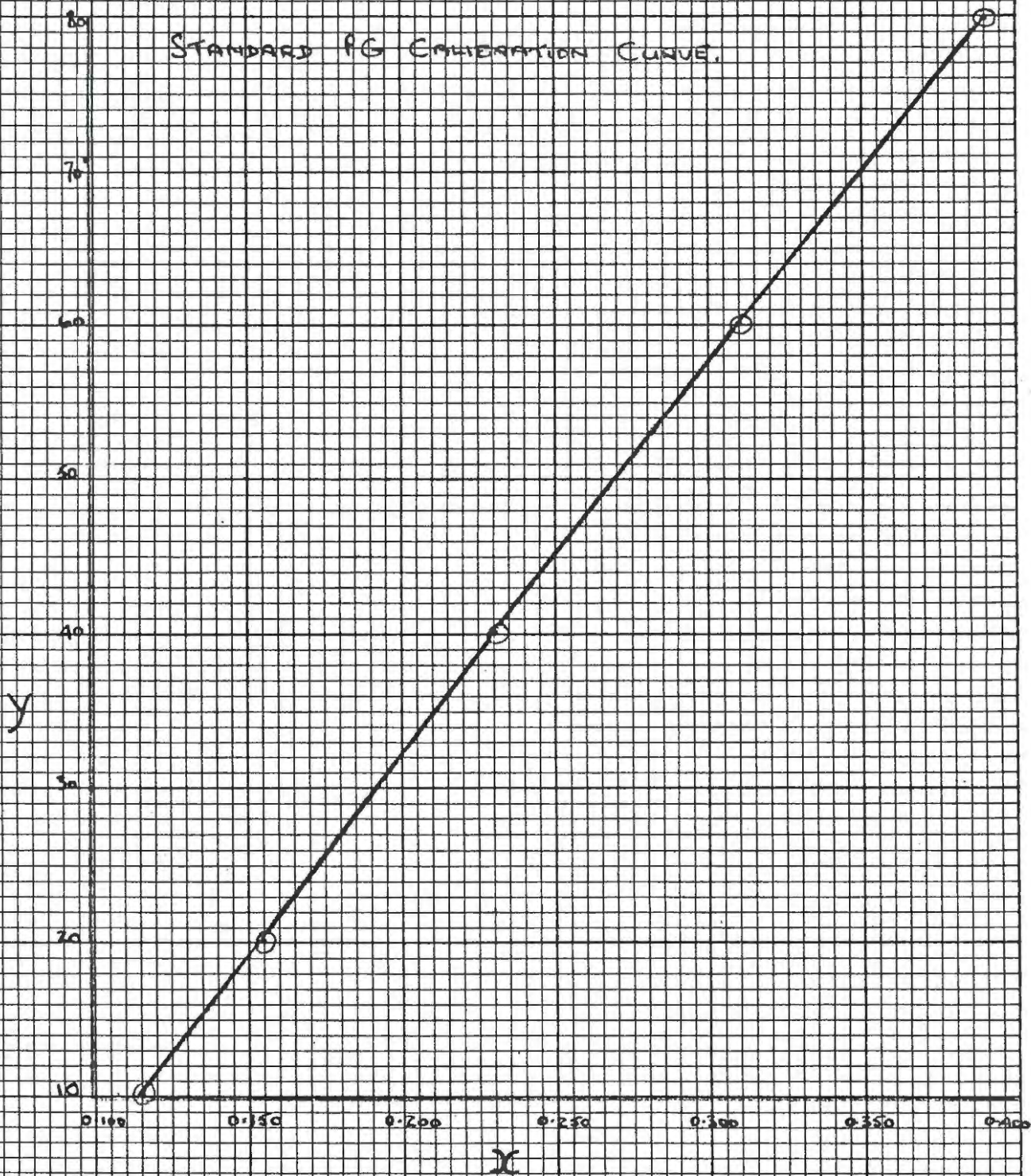
The following precautions were necessary :-

a) Spraying was carefully performed. The phosphomolybdic acid solution was sprayed on to the horizontal plate until a persistent sheen remained. The phosphoric acid solution was sprayed until the plate was evenly transparent.

b) The plate was placed in the oven in such a way that the whole plate was subjected to the same amount of heat. (An uncluttered oven should be used). Overheating during the second heating (i.e. after the phosphoric acid spray) causes the spots to become hard and difficult to remove, but a light spraying with water softens the spots again.

FIG. 3

STANDARD PG EMULSION CURVE.



y = Concentration of PG in micrograms
x = Absorbance at $\lambda = 690 \text{ m}\mu$
(See TABLE A.)

The main disadvantage of the method is the fact that 5 standard spots must be run on each plate for constructing a calibration curve. However this still leaves up to eleven lanes free for the use of urine extracts. The advantages of this method are in its simplicity, rapidity and adaptability to routine use. A quantitative and semi-quantitative assessment can be made using the same T.L.C. plate, i.e. the spots can be made visible for a normal evaluation by spot size comparison, and only those spots which require it need be scraped off for a fully quantitative assay.

This was the method finally chosen for all the quantitative work required by the project. Experiments were performed to prove its reliability in terms of accuracy and reproducibility which were tested for by triplicate assays of solutions of known P.G. concentration. (Table 4). Satisfactory results being obtained it was decided to see if improvements could be made to the earlier stages of the Waldi test.

TABLE No. 4

Chromatographed plates spotted with standard P.G. solution were sprayed with phosphomolybdic acid reagent and the resultant colour was read at 690 mu. Results are tabulated below:

4(a)

A	10	10	10	20	20	20	40	40	40	60	60	60	80	80	80
B	76.2	76.5	76.8	70.2	70.1	69.7	58.4	58.4	58.7	40.2	40.5	40.7	49.5	50.5	50
C	.115			.155			.23			.31			.39		

A = plates spotted in triplicate with following concentrations of P.G. in ug

B = % transmission readings at 690 mu

C = Mean Absorbance readings at 690 mu

A calibration curve was constructed from the above readings (A/conc.) and the concentration of spots on the same plate were determined.

4(b)

Actual concentration of spots (ug of P.G.)	25	30	50	70
Concentration ex curve (mean of 3 readings)	24.4	29.9	49.7	
Concentration ex curve (mean of 2 readings)				68.4
%	97.6	99.8	99.3	97.8

4c. Urine containing known concentrations of conjugated pregnanediol was assayed. Recovery values are listed below:

Actual concentration in mgm/litre	37	49.3	74
Mean assay conc. in mgm/litre	35.5	48.0	71.5
%	96%	97.3%	96.8%

4d. Pure pregnanediol in alcoholic solution was added to pregnanediol-free urine. Recovery values are listed below:

Actual concentration in mgm/litre	40	50	60
Mean assay conc. in mgm/litre	39	49	58.5
%	97.5%	98%	97.5%

It may be noted at this point that the % accuracy figures in table 4d are higher than those in table 4c. As the assay methods involved in both these sets of data are identical excepting for the hydrolysis stage which was not carried out in 4d, it seems likely that this technique (hydrolysis) is responsible for the lower recovery values.

Stage 1 - Hydrolysis

(a) Enzymatic Hydrolysis - The hydrolysis of conjugated glucuronides by means of glucuronidase extracted from ox spleen was first reported in 1939 by W.H. Fishman.⁽⁶⁷⁾ Various workers investigated the method, extracting the enzyme from various sources. Levvy⁽⁶⁸⁾ used calf spleen as did Cohen.⁽⁶⁹⁾ Buehler⁽⁷⁰⁾ extracted the enzyme from *Escherichia coli*. Henry and Thevenet used snails.

It was Cohen et al who first suggested using the enzyme for pregnanediol assays. Henry and Thevenet suggested that its use would avoid the production of the many chromogens caused by acid hydrolysis.

Numerous reports appeared in the 1950's regarding this method of hydrolysis. Inhibiting substances were listed, and substances which potentiated the reaction (e.g. Propylene glycol) were also noted.



Beta-glucuronidase became commercially available and gradually standardised enzymatic hydrolytic procedures became accepted alternatives to acid hydrolysis.

It was decided to carry out a few hydrolyses using beta-glucuronidase (B.D.H. Batch 0200010 - ex Mollusc) to see whether the method had any advantages not shared by the acid method.

Method

25 ml of ^{pregnancy} gravid urine were buffered to p.H. 4.6 with 0.1N Acetate buffer. 5,000 units of beta-glucuronidase were dissolved in the mixture and finally 1 ml of chloroform was added. Incubation was carried out at 37°C for 24 hours. The urine was then extracted in the usual way. Acid hydrolysis was carried out on more of the same urine specimen and it too was extracted. The two extracts were quantitatively compared. (Table 5a).

The chief disadvantage of the method is its need for a long incubation period. This would be offset by results proving a higher P.G. yield, but in fact the increase is too slight to make the method suitable for routine use. Its main advantage, that of producing a very much cleaner hydrolysate, would be of some account in column chromatographic techniques where interfering chromogens are difficult to get rid of, but as T.L.C. separation is complete, this does not apply.

TABLE 5a

Gravid urine was hydrolysed a) enzymatically
b) with H Cl

It was extracted and quantitatively assayed.

Method of Hydrolysis	Urine 1	Urine 2	Urine 3	Urine 4
Acid hydrolysis (H Cl) concentration mgm/litre	54.0	50.5	21.5	48.0
Enzymatic (beta-glucuronidase) concentration mgm/litre	54.0	51.0	23.0	48.5
% increase of Enzyme vs Acid	0	0.99	7.0	1.04

Excluding urine No. 3 results, the mean percentage increase in yield is $\pm 0.7\%$.

(b) Acid Hydrolysis - In 1965 Waldi published an alternative method of acid hydrolysis.⁽⁷¹⁾ This involved heating urine on a steam bath for 20 minutes with a mixture comprising 25 ml cyclohexane, 2g Trichloroacetic acid, 5 ml of 10% (V/v) acetic acid and 5 ml of 25% (W/v) sulphuric acid, per 20 ml of urine.

This method was carried out using ^{pregnancy} gravid urine. After hydrolysis the cyclohexane fraction was purified with I.N. sodium hydroxide and evaporated in the usual way. Normal hydrochloric acid hydrolysis was also carried out on urine from the same specimens and the results were compared. (Table No. 5b). Although this method is superior it was not put to use in this project because most of the experimental work had been completed before the reference to the method was discovered.

TABLE 5b

Gravid urine was hydrolysed in the normal way with HCl and by the method described above. Assay results are listed below:

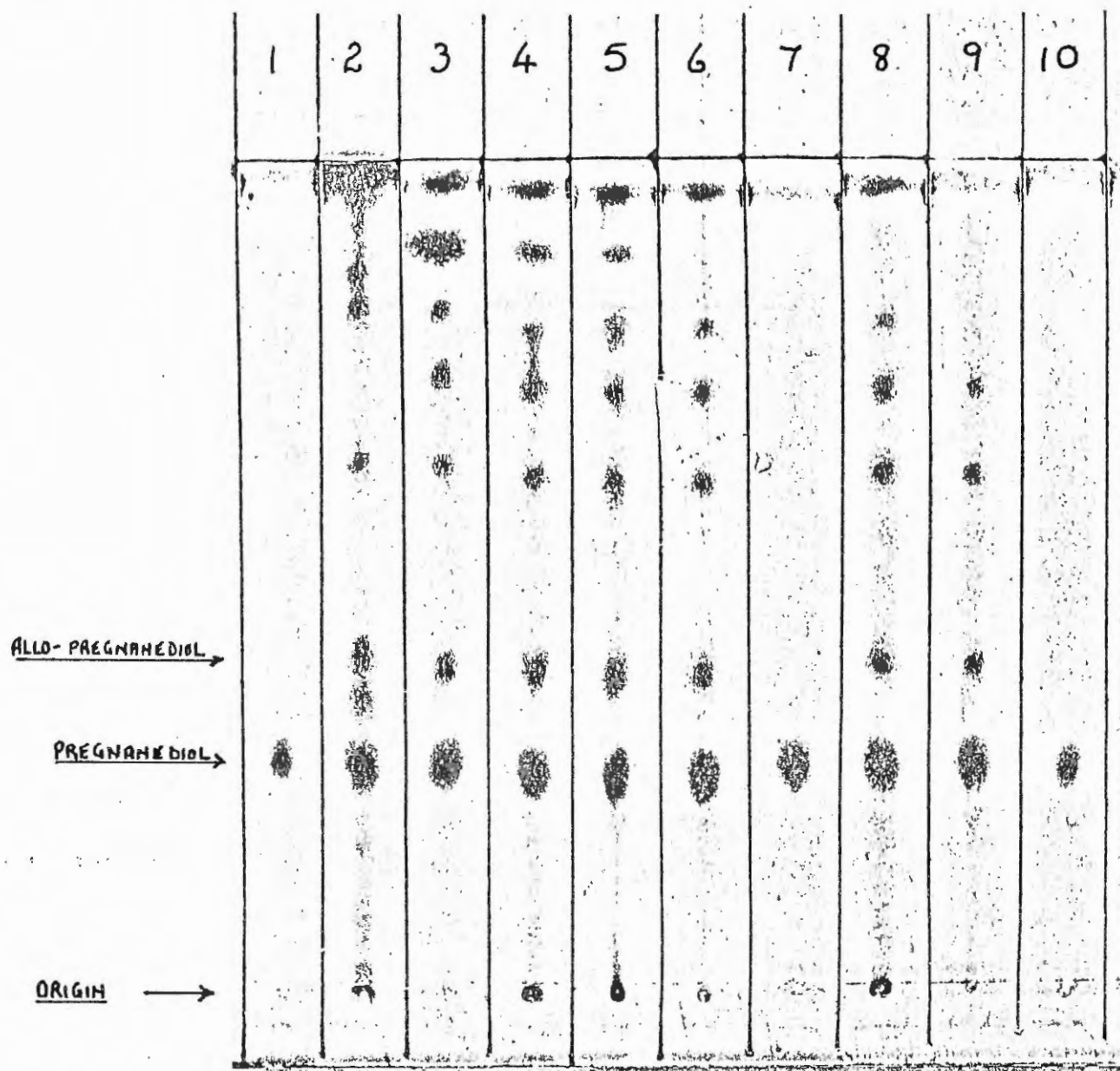
Method of Hydrolysis	Urine 1	Urine 2	Urine 3	Urine 4	Urine containing 49.3 mg P.G./l.
HCl hydrolysis concentration mgm/litre	53.0	55.3	55.0	55.7	47.8
Mixed acid hydrolysis concentration mgm/litre	53.5	55.5	55.4	55.8	48.1
% increase	0.95	0.38	0.73	0.10	0.63

Stage 2 - Extraction

The Waldi method of extraction was altered as previously described. The difference in yield following one, two and three cyclohexane extractions was calculated and the advantages of using cyclohexane instead of other solvents was queried by comparing results obtained with a cyclohexane extraction to those obtained using ethyl acetate, chloroform, toluene, hexane and ether (Table No. 6). Emulsion-forming tendencies were also noted, as was selectivity.

FIGURE N° 4

XEROX OF THIN LAYER CHROMATOGRAM OF URINES EXTRACTED
WITH DIFFERENT SOLVENTS



- LANES 1 & 10: 3mg STANDARD PREGNANEDIOL
- LANE 7: 7mg STANDARD PREGNANEDIOL
- LANE 2: CHLOROFORM EXTRACT OF GRAVID URINE (25ml)
- LANE 3: TOLUENE EXTRACT OF GRAVID URINE "
- LANE 4: ETHYL ACETATE EXTRACT OF GRAVID URINE "
- LANE 5: CYCLOHEXANE EXTRACT OF GRAVID URINE "
- LANE 6: HEXANE EXTRACT OF GRAVID URINE "
- LANE 8: HEXANE EXTRACTED ACID HYDROLYSED URINE
- LANE 9: HEXANE EXTRACTED ENZYME HYDROLYSED URINE

TABLE 6

Progynon

Gravid urine was extracted with a range of different solvents and the results were evaluated using a number of different criteria.

Solvent	1	2	3	4	5	6	7	8	9
Assay result in mg/litre	49.00	52.10	52.20	51.15	52.20	52.10	51.80	49.60	52.15
A Emulsion forming tendencies	++	++	++	++	+++	++	+	+	+
B Non-selectivity	++	++	++	++	+++	+++	+	+	+
C Water-carrying capacity	++	++	++	+++	+++	++	+	+	+

Code Solvent 1 = Cyclohexane (1 extraction)
 Solvent 2 = " (2 extractions)
 Solvent 3 = " (3 extractions)
 Solvent 4 = Ethyl Acetate
 Solvent 5 = Chloroform
 Solvent 6 = Toluene
 Solvent 7 = Ether
 Solvent 8 = Hexane (20 ml)
 Solvent 9 = Hexane (30 ml)

- = negative + = slight ++ = reasonable +++ = considerable

A was evaluated by shaking up the solvent with 10 different urines.

+ indicates emulsion forming in not more than 2 cases.

++ indicates emulsion formation in more than 2 and less than 6 cases.

+++ indicates emulsion formation in 6 or more cases.

B Lack of selectivity was judged only after the extracts had been chromatographed. In all cases separation of PG was adequate, but in some cases (especially with chloroform) faint streaking indicated the presence of impurities.

C Water holding capacity was assessed by slowly adding anhydrous sodium sulphate to the combined solvent after extraction and washing. The presence of moisture is indicated by the behaviour of the salt. If it remains loose and amorphous and is easily swirled in the flask, no water is present, but if it forms hard lumps water is present.

It must be appreciated that the solvents were assessed purely in terms of extractant usefulness, and no attempt has been made to correlate the above factors with any physico-chemical properties such as eluotropicism, hydrophobicity, specific gravity, etc. Using the criteria mentioned in A, B and C, Hexane was the solvent of choice.

Stage 3 - Evaporating to dryness

The initial evaporation under reduced pressure technique was left unchanged, but owing to the report that pregnanediol is broken down by excessive or prolonged heating, a number of duplicate extracts were evaporated without using reduced pressure, in order to see whether the values thus obtained were lower than those for reduced pressure evaporation. No significant differences were noted. Similarly, no difference was noted when duplicate quantitatively transferred chloroform extracts were dried in a stream of hot air and by a jet of hot nitrogen gas (bottled nitrogen flowing through a heated glass tube and directed onto the extracts).

Dried extracts left in an open vessel at room temperature began to show reduced pregnanediol values (5%) after 9 - 11 weeks. Dried extracts left in an oven at 110°C showed reduced values (5 - 10%) after one hour. (c.f. Section 5, below). The rate and degree of degradation was not established, as the conditions causing the reduced values do not normally pertain during the usual evaporation procedures.

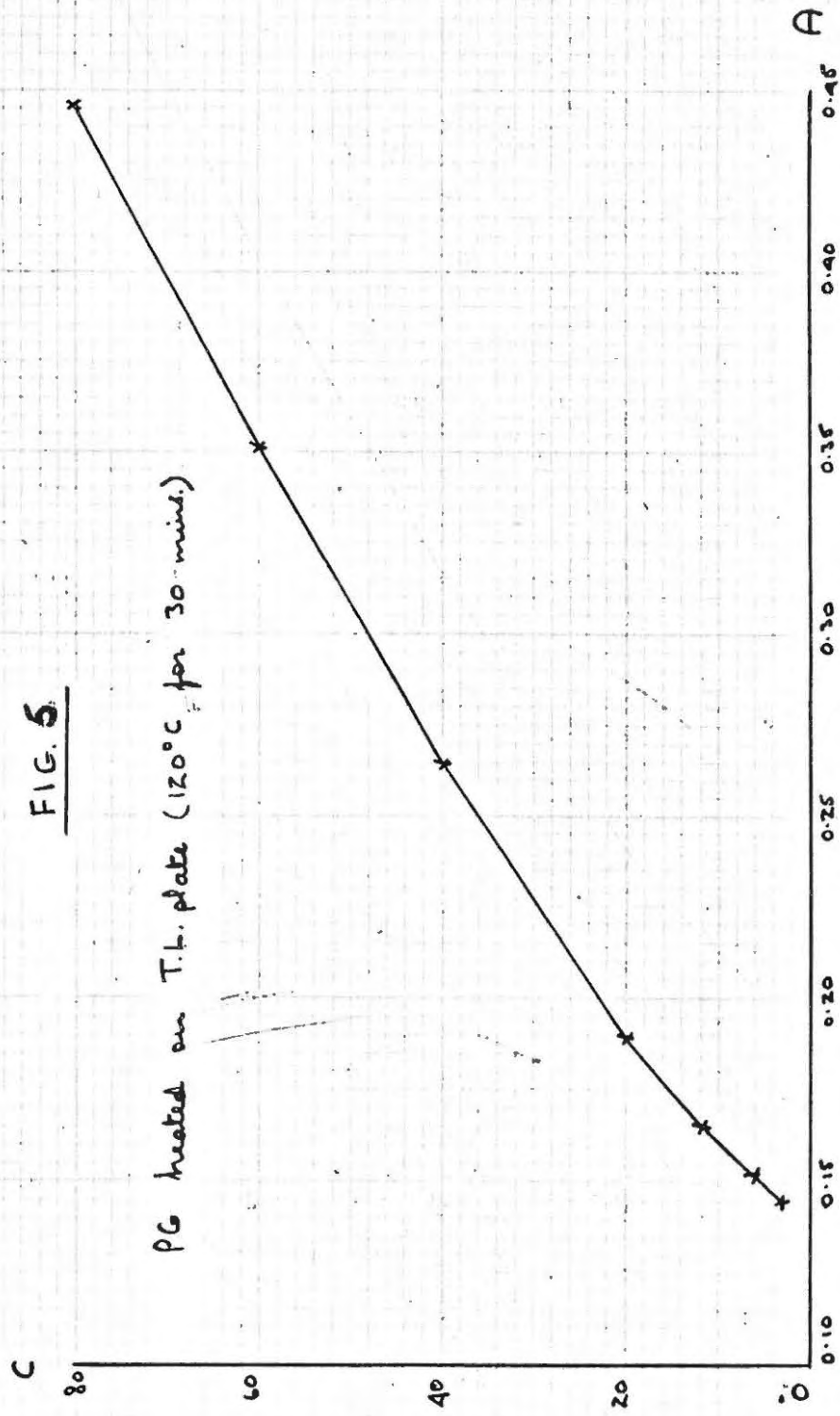
Stage 4 - Spotting the plates

A number of variations to the published method were introduced to the spotting procedure and the results were quantitatively assessed to check the accuracy. The method finally chosen has already been described. (page 41).

Stage 5 - Development

The solvent mixtures Ethyl Acetate/Toluene 4:1 and Chloroform/Methanol 12½:1 as devised by Chang-Shen et al⁽⁷²⁾ were tried but were not an improvement on the Chloroform/Acetone solvent previously described.

After development a number of plates were placed in an oven at 110°C for 10, 20, 30, 60, and 120 minutes. The plates left in for 20 minutes or more showed depressed PG values when compared to a series of



C = Conc. of PG in μg .

A = Absorbance
($\lambda = 690 \text{ m}\mu$)

standards which had not been heated. This agrees with the findings of Iau and Jones as reported by Kirchner.⁽⁴⁶⁾ It should be noted however that if the standards for the calibration curve are also heated for the same length of time as the P.G. being tested the final results are still quantitative.

A number of plates were similarly tested at 120°C. A loss of P.G. was evident after 10 minutes. As some of the cheaper laboratory ovens have poor thermostats, they sometimes reach 120°C when set at 110°C, this loss could significantly alter the final results. It thus becomes apparent how important it is to include standards for calibration on each plate run. The loss, if it occurs, is then proportional and the final result is still acceptably accurate. Even with this precaution it is desirable to use no unnecessary heat during any of the procedures of the assay. (See Table 7).

TABLE 7

A number of Thin layer plates (5 x 20 cm) were spotted with three concentrations of pregnanediol standard and after development were left in an oven at 120°C. A plate was withdrawn every 10 minutes. Concentrations were calculated from a calibration curve prepared in the usual way.

	0 mins	10mins	20mins	30mins	40mins	50mins
Concentration in mg/litre	19.4	19.1	18.6	18.3	18.2	18.1
% decrease		1.55	4.20	5.75	6.30	6.80
Concentration in mg/litre	39.3	39.1	38.7	38.5	38.4	38.2
% decrease		0.51	1.53	2.05	2.30	2.80
Concentration in mg/litre	59.7	59.5	59.1	59.0	59.0	58.8
% decrease		0.35	1.00	1.18	1.18	1.51

The error is more noticeable at lower concentrations of standard.

A calibration curve prepared from a plate left in an oven at 120°C for 30 minutes has a slope increase at lower concentrations. (See fig. 5). Concentrations calculated from the curve are still acceptably accurate provided that the spots were taken from the same plate as was used to construct the calibration curve.

DISCUSSION

In an attempt to limit any inaccuracies inherent in the Waldi method of pregnanediol assay, a number of colorimetric evaluations were tested.

The Bang method was found to be rapid and accurate, more so than the method of Starka, but the most convenient and rapid method is the one involving phosphomolybdic acid reagent. Consequently this was the technique used for all cases which required quantitative evaluation. It was used to compare the relative efficiencies of acid hydrolysis, enzyme hydrolysis and mixed-acid hydrolysis.

Although beta-glucuronidase hydrolysis offers an increased yield and a cleaner hydrolysate, it is much more lengthy than acid hydrolysis and cannot be recommended for those assays where the time factor is of importance. Under these circumstances the mixed-acid technique would be the method of choice.

Quantitative determinations were also employed to ascertain the most useful solvent for the extraction process (see Table 6). Probably the most suitable all round solvent would be ether (diethyl) but only in a laboratory which did not rely upon gas or open flame for its sources of heat. The less explosive hexane was the solvent decided upon for the extractions performed during this project. The hexane used in extracting one urine specimen may be evaporated to dryness in three minutes, under reduced pressure. Reducing the time to less than three minutes does not offer any advantages because the quantitative transfer of the dried residue to the final container which is carried out during the evaporation process, cannot be accurately performed in less than just under three minutes.

It has been reported that excessive heating will vitiate the extracted pregnanediol, and the quantitative assay of extracts heated as described in Table 7, tend to confirm these reports. The advantages of treating extracts and calibrationary standards exactly alike (i.e. running them on one T.L.C. plate) is therefore apparent.

By making use of the quantitative evaluation procedure it was possible to establish the optimum method of carrying out the various stages of the T.L.C. pregnanediol assay. Thus wherever a modification was responsible for an increase in efficiency or accuracy of the assay, it was incorporated, and the assay emerged in the final form as described on pages 38 to 42.

SUMMARY

A number of colorimetric techniques were tested, and one of them was used for a series of quantitative evaluations involving the different stages of the T.L.C. pregnanediol assay. The results have been described in graphic and tabular form.

CHAPTER NINE

One of the aims of the project was to compare the T.L.C. assay of urinary pregnanediol with other methods which were or are used by clinical laboratories. The methods chosen for comparison were those of Venning, and Klopper, Mitchie and Brown.

Although the Venning method has been replaced by more recent techniques, it had in its day fairly wide employment. It is a cumbersome method and it is interesting to observe to what lengths clinical laboratories were prepared to go if they considered the objective of sufficient importance. Another reason for choosing the Venning method for comparison purposes was that the end-product of the method is the conjugated glucuronide of pregnanediol which same could be put to use in subsequent experiments.

Pregnanediol Assay using the Venning Method

Urine from pregnant donors was collected, bulked and assayed by the T.L.C. method at 36 mg of P.G./litre. 2 litres of this was extracted as follows. The urine was adjusted to p.H. 11.0 and was well shaken up with four successive portions of n-Butyl alcohol, centrifuging each time to separate the two phases. The organic portions were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 0.1 normal sodium hydroxide which was then extracted with four portions of butanol. The combined butanol portions were washed with water, centrifuged, and the alcohol phase was evaporated to dryness under reduced pressure. The residue was dissolved in warm methyl acetate and allowed to digest overnight in a refrigerator at 5-10°C. The mixture was then centrifuged and the separated precipitate dissolved in hot ethanol. This was filtered with suction into a tared beaker, evaporated to dryness and weighed.

In order to assess its purity, the melting point of the conjugate was measured. Pure pregnanediol glucuronidate melts at 270°C. The first extract was impure and it was redigested in methyl acetate as described above until the melting point was that of the pure substance.

The white amorphous conjugate was then tested for purity by paper electrophoresis using an acetic acid/formic acid/water (15 : 5 :80) buffer

in a Shandon electrophoresis apparatus (5 hours). From the number of spots obtained it was obvious that the extract was still impure - a fact which was confirmed by T.L.C. using the method of Kay and Warren.⁽⁷³⁾ Further purification was carried out by precipitating the extract from methyl acetate/water (95 : 5) and acetone/water (90 : 10), until a (chromatographically) pure product was obtained.

A number of Venning extractions were carried out as described above. The actual yield compared to the theoretical yield was on no occasion satisfactory. (See Table 8).

TABLE 8.

Three extractions by Venning's method were carried out. A technical error resulted in the loss of all the pregnanediol glucuronidate (PLG) in the first extraction. Relevant details of the next two extractions are tabulated hereunder:

Vol. of Urine Extracted	Concentration of P.G. by assay	Total P.L.G. Conc.	Weight of Impure Extraction	Weight of Purified Extraction	Time taken for initial extraction	Total Time Taken
2.0 litres	36 mgm/litre	118mgm	71 mgm	17 mgm	32 hours	37 hr.
1.6 litres	47.3mgm/litre	119mgm	104.5mgm	68 mgm	27 hours	30 hr.

A supply of pregnanediol glucuronidate was now available. Some of it was used to test the efficiency of the various hydrolytic processes as described earlier. The rest of it was employed in establishing the relative accuracy of the T.L.C. and Klopper's assay method. Known amounts were added to urines containing no pregnanediol so that the final concentration of P.G. in the urine was accurately known.

At one stage of the project pure pregnanediol for use as a standard became rather scarce. It was needed for the various tests being carried out in duplicate and triplicate and no local supplies were available. In due course a large supply was donated by Organon Laboratories, but just prior to this gift it was decided to devise a method for the large-scale extraction of pregnanediol from urine and subsequent purification by

preparative T.L.C. This method is described hereunder :-

A large supply (9.3 litres) of pregnancy urine was collected and acidified with hydrochloric acid of S.G. 1.19 (0.4 litres). The rotating flask of a Rotovap evaporator was adjusted over a water-bath, the vacuum was applied and the acidified urine drawn in at a rate that balanced the amount evaporating off.

The mixture was evaporated to a viscous dark-brown mass, only just fluid. The water-bath was removed and two solid glass cylinders (10 x 20 mm) were dropped into the flask. 100 mls of cyclohexane was drawn in and the flask was allowed to rotate rapidly for 15 minutes. The glass cylinders prevented the thick evaporated urine from plastering itself over the walls of the vessel and caused enough agitation to ensure a thorough mixing of cyclohexane with the urine.

The cyclohexane was drawn off and replaced with a fresh 100 ml portion and the process was repeated until four 100 ml portions had been collected. These were combined and washed with three 100 ml portions of I.N. sodium hydroxide in a 1 litre separating funnel, after which the cyclohexane was dried with anhydrous sodium sulphate. The rotovap was then used again to evaporate the cyclohexane extract to a gummy residue, which was carefully washed from the flask with five 100 ml portions of chloroform. This was evaporated in a stream of warm air and further chloroform was used to adjust to a final volume of 30 ml.

Preparative T.L.C. plates were coated with Silica Gel H. in a layer 2 mm thick. These plates were activated at 110°C overnight (14 hours) and then charged with the extract. Applying the extract to the plate required a high degree of technical expertise. It was found to be advisable to practice on a test-plate using pure solvent before attempting to use the extract. An uncoated plate was used as a guide or template to ensure a straight base-line. Spotting the extract was found to be very tedious and unsatisfactory. Eventually a record syringe (5 ml) was fitted with a cotton wick instead of a hypodermic needle and using this, straight fairly thin base-lines were applied. When practice had developed the required degree of manipulative skill, the five plates were charged with the extract

and were run in the usual chloroform/acetone solvent.

Locating the required zone was achieved by lightly spraying the plate with distilled water, which causes the pregnanediol to appear as a greasy streak. The pregnanediol zone was scraped from the plate and eluted with chloroform. After centrifuging off the silica gel the chloroform was evaporated to dryness. The purity of the pregnanediol^d was established by T.L.C. and by melting-point determinations. The theoretical yield was 307 mg and the weight of the extracted pregnanediol was 185 mg; that is, just over 60% of the available pregnanediol was extracted.

The method was used once only. The arrival of the Organon donation eliminated the need for further preparative extractions.

Pregnanediol Assay using the Klopper Method

This method was employed on

- a) ^{Pregnant} Gravid urine
- b) Pregnanediol-free urine containing a precisely measured amount of added P.G. standard.
- c) Pregnanediol-free urine containing a precisely measured amount of added pregnanediol glucuronidate.

The results were compared with those of a T.L.C. pregnanediol assay on duplicate specimens of urine.

The method is widely used and consequently descriptions of the procedure appear in most biochemistry practical text-books. Only a brief description appears hereunder; it is according to Varley.⁽⁷⁴⁾

Method

50 ml of toluene was added to 150 ml diluted urine in a round-bottomed flask. After acidifying with 15 ml of hydrochloric acid (S.G. 1.19) the mixture was boiled under reflux for 10 minutes. After rapid cooling the urine layer was removed in a separating funnel and then re-extracted with a further 50 ml of toluene. The combined extracts were shaken up with a solution of 25% w/v sodium chloride in I.N. sodium hydroxide. The toluene layer was then separated and shaken with 50 ml of freshly prepared 4% w/v of potassium permanganate in I.N. sodium hydroxide. The separated toluene layer was then washed four times with distilled water.

After filtering, the toluene was distilled down to a final volume of ca 10 ml.

A column was then prepared using benzene and deactivated alumina. The toluene extract was added to the column and firstly eluted with 25 ml of 0.8% ^v/v ethyl alcohol in benzene (the eluate being discarded) and then with 12 ml of 3% ethyl acetate in benzene. This final eluate was collected and evaporated under reduced pressure. The still-warm residue was dissolved in 2 ml of benzene, and 2 ml of acetyl chloride was added to the mixture which was then left to stand for one hour at room temperature. After adding 25 ml of light petroleum to the mixture, it was transferred to a separating funnel and washed with water, 8% ^w/v sodium bicarbonate solution and then again with water. The non-polar phase was then separated and run through a column of deactivated alumina prepared with light petroleum. The acetylated pregnanediol was eluted with benzene and evaporated to dryness under reduced pressure, after which it was stored for an hour in a calcium chloride desiccator.

To the tube containing the residue was added 10 mg of sodium sulphate followed by 10 ml of concentrated sulphuric acid. After a thorough mixing the tube was allowed to stand for 17 hours at room temperature (25°C). The solution was then read in a spectrophotometer at 430 mu using sulphuric acid as a blank. A standard had been prepared by dissolving 0.2 mg of pregnanediol in benzene and acetylating it with 2 ml of acetyl chloride as described above. It was then taken up in light petroleum (25 ml) and washed with water, bicarbonate and water. It was not chromatographed but was evaporated and treated with sodium sulphite and concentrated sulphuric acid as already described.

The amount of pregnanediol present in the urine was calculated from the formula:-

$$\frac{\text{Absorbance reading of unknown}}{\text{Absorbance reading of standard}} \times \text{urine dilution factor} \times 0.2 \\ = \text{mg of P.G./24 hours urine}$$

The method takes two days to complete and requires experience before reproducible results are obtained.

TABLE 9.

Relative values for P.G. concentration of urines assayed by T.L.C. and Kloppe's method:

Method of Assay	Gravid urine		Urine containing 10 mg PG/litre		Mean % Accuracy	Urine containing 60mg P.L.G. per litre. i.e. 37mg PG/litre		Mean % Accuracy
	mg/l	mg/l	mg/l	mg/l		mg/l	mg/l	
Kloppe's	16.05 mg/l	16.20 mg/l	9.88 mg/l	9.90 mg/l	98.90	36.2 mg/l	36.15 mg/l	97.70
T.L.C.	16.00 mg/l	15.9 mg/l	9.80 mg/l	9.75 mg/l	97.75	35.8 mg/l	35.6 mg/l	96.50

DISCUSSION

The Venning method of pregnanediol glucuronidate (P.L.G.) estimation was carried out on a supply of gravid urine. The yields, though disappointing from an assay point of view, were useful in that the purified pregnanediol glucuronidate could be added to the urine when testing the efficiency of hydrolytic techniques as described in the previous chapter.

Unconjugated pregnanediol for use as a standard was also extracted from pregnancy urine by a simple and comparatively rapid method involving simultaneous hydrolysis and extraction in a rotary evaporator followed by preparative T.L.C. separation.

The Kloppe, Mitchie and Brown pregnanediol assay was performed on a number of urines and the results compared to those of a T.L.C. assay. The accuracy of the Kloppe assay is superior to the T.L.C. assay but the method is tedious and far less suited to routine use. Performed simultaneously, it is possible to carry out 20 pregnanediol assays, (using Kloppe's method) in one week. Using the T.L.C. method the same number of assays can be easily completed in less than two days.

SUMMARY

Described in this chapter is the Venning method of estimating pregnanediol glucuronidate concentrations. Also described is a preparative pregnanediol extraction.

The assay results obtained from Kloppe and T.L.C. methods are compared and discussed.

CHAPTER TEN

The T.L.C. pregnanediol assay gives results which are only slightly lower than those of the method of Klopper. On the other hand it is very much more rapid than the Klopper method. It was certainly the most convenient method for studying variations in pregnanediol excretion during pregnancy, abnormal menstrual cycles and drug therapy, as follows.

Pregnanediol Excretion during Pregnancy

During the period that this research was undertaken six volunteers were used as a source of gravid urine. In two cases assays were made on the urine at a very early stage i.e. less than three weeks after conception and before an official diagnosis had been made. In the other cases urine was supplied on or before the seventh week of pregnancy and in all but one case assays were continued until full term.

The volunteers were supplied with sterile bottles on which they were asked to label their reference number and the date of the donation. Morning urine was asked for although for a few assays a 24 hour sample was used. Bachman et al⁽⁷⁵⁾ studied the variation in 24 hour excretion rates of 13 pregnant women and reported that there is very little variation in output during a 24 hour cycle. He also stated that the amount excreted is not related to the volume output of urine.

To confirm this, two donors were asked for 24 hour samples, each urination to be contained in a separate bottle. The contents of each bottle were assayed separately, the urine was combined and an aliquot of the combined sample was assayed. It was found that the pregnanediol concentration (expressed as ug/ml) for the morning urine sample and the combined sample showed very little difference (Table 10). It was thus deemed permissible to use morning urine only for the assays, especially as this would cause the donors the least amount of inconvenience. The results were expressed as mg/litre, but provided the average 24 hour urine volume was known, the results could be calculated as mg/24 hours by proportional adjustment.

TABLE 10

Diurnal variations in pregnanediol secretion.

Voiding Order		Donor A	Donor B
1	Volume of urine specimen in ml.	530	318
	Concentration of P.G. in mg per litre	40.0	32.5
	Total quantity of P.G. in specimen (mg)	21.20	10.32
2	Volume of urine specimen	226	120
	Concentration of P.G.	37.5	29.0
	Total quantity of P.G. in specimen	8.47	3.48
3	Volume of urine specimen	135	78
	Concentration in P.G.	37.0	32.2
	Total quantity of P.G. in specimen	4.99	2.50
4	Volume of urine specimen	264	245
	Concentration of P.G.	39.0	33.0
	Total quantity of P.G. in specimen	10.30	8.10
5	Volume of urine specimen	145	165
	Concentration of P.G.	38.0	33.0
	Total quantity of P.G. in specimen	5.52	5.44
6	Volume of urine specimen	150	140
	Concentration of P.G.	37.5	31.0
	Total quantity of P.G. in specimen	5.62	4.34
7	Volume of urine specimen	170	-
	Concentration of P.G.	38.5	-
	Total quantity of P.G. in specimen	6.55	-
	Total 24 hour volume of urine	1620	1066
	Concentration of P.G. in mg/litre	38.6	32.0
	Total 24 hour quantity of urine	62.65	34.18
	Assay concentration of bulked 24 hour urine specimens in mg per litre	39.85	33.50

As can be seen in table 10 the morning urine of Donor A assays at 40 mg/litre and the bulked 24 hour sample assays at 39.85 mg/litre. Donor B has a morning urine value of 32.5 mg/litre and a 24 hour urine concentration of 33.5 mg/litre (by assay).

The assay of the pregnant urine gave results which conformed to the expected values excepting in two instances. (Whenever abnormal values were obtained the assay was repeated using a further aliquot of the same urine or, if this was impossible, another sample of urine was collected from the donor as soon as convenient).

The individual pregnanediol concentration was assayed at weekly intervals excepting during the 10, 11, 12 and 13th weeks of pregnancy when assays were carried out twice or three times a week.

Quantitative Pregnanediol Assays of Excretion Rates during atypical Menstrual Cycles.

In the series of semi-quantitative assays of pregnanediol excretion during menstrual cycles, a number of atypical cases were observed. These showed abnormal peaks of pregnanediol output in either the pre- or post-ovulatory phases, and it was decided to examine them more closely, i.e. by quantitative assay. The results are depicted graphically in Fig. 1B.

Pregnanediol excretion variations caused by drugs.

(a) Administration of glucuronic acid

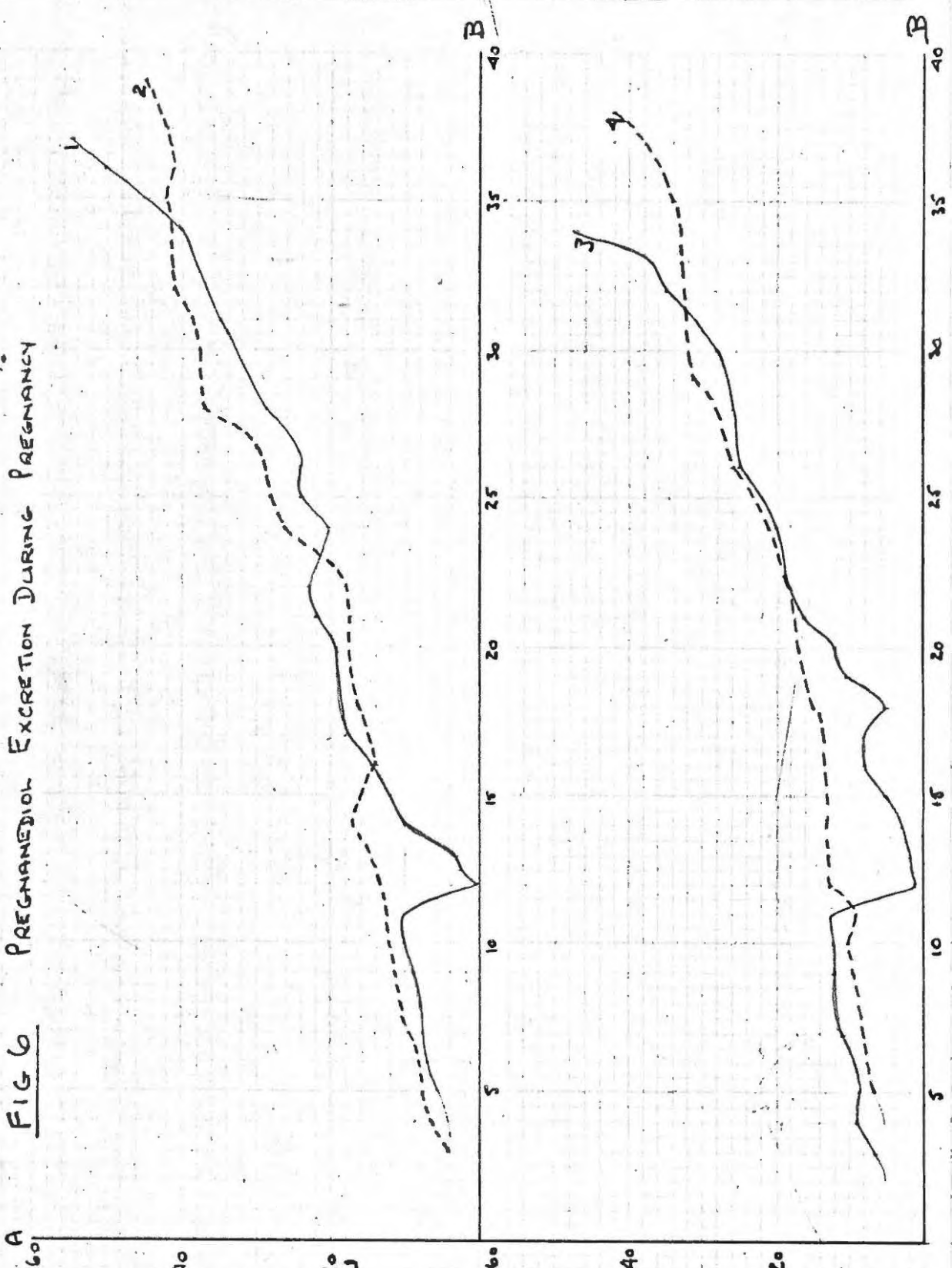
Glucuronic acid in tablet form is available as a commercial preparation called "Guronsan". These tablets are prescribed in order to aid and hasten the elimination of undesirable waste-product from the body; the reasoning being that since most waste-products are excreted as a conjugate of glucuronic acid, the administration of exogenous glucuronic acid is bound to facilitate excretory processes.

It was thus decided to see whether the administration of glucuronic acid would have any effects on the rate of pregnanediol excretion. Two donors who showed a very steady rate of pregnanediol excretion were requested to take 5 Guronsan tablets each. The pregnanediol content of their urine was assayed on the two days prior to taking the tablets and on the day following. Results are listed in table 11.

TABLE 11

	Day 1	Day 2	Day 3
P.G. concentration of urine of Donor A	44.30 mg/litre	44.80 mg/litre	45.42 mg/litre
P.G. concentration of urine of Donor B	46.15 mg/litre	45.65 mg/litre	46.30 mg/litre

FIG 6 PREGNAMEDIOL EXCRETION DURING PREGNANCY



A = Conc. of PG in mg/litre
 B = Weeks of pregnancy.

1 and 3 = Graph of abnormal PG secretion.
 2 and 4 = Graph of typical secretion.

The Guronsan tablets were taken in the evening of day 2. Although the pregnanediol concentration of the urine is higher on day 3 than on the two preceding days of the test, the increase is not significant. It is no more than might be expected in normal day to day variations of output.

(b) Administration of a diuretic

Urine was collected from a pregnant donor who, showing signs of incipient odoema, was directed by her doctor to begin a course of diuretics. (Navidrex K). The drug induced an increase of just over 35% in the 24 hour urine volume. The total pregnanediol excretion per 24 hours appeared to be unaffected by the increased urine output. As it has never been suggested that the kidneys were actively involved in the rate of excretion of pregnanediol, this result was not surprising.

DISCUSSION

The pregnanediol excreted during pregnancy was followed in six volunteers. In two cases the excretion curves showed a decided drop near the end of the third month (fig. ~~10~~ 6).

In the first case, the initial increase in pregnanediol output following conception was maintained until the eleventh week after the last menstrual period. The value of the assay on day 75 showed an expected increase over the previous assay figure. The assay on day 81 showed no pregnanediol at all. Experimental error was suspected but another assay performed the following day also showed a very much depressed value. Thereafter the urinary pregnanediol concentration increased gradually to a high level just prior to parturition. The birth was normal, a $8\frac{1}{2}$ lb. male child being delivered.

In the second case, the initially high pregnanediol values dropped dramatically in the twelfth week and stayed at a very low level for nine days after which the concentration increased. However, until the sixth month the pregnanediol level was lower than the normal value. This case had a history of abortion, but the pregnancy on this occasion terminated successfully. A female child was delivered a month prematurely but is otherwise healthy and normal.

Jayle et al report falls in pregnanediol to very low levels between the sixth and twelfth weeks and suggest that it is due to luteal insufficiency.

However in the second case mentioned above, the pregnanediol decrease occurred just at the time when placental progesterone secretion should have dominated the luteal secretion, and the low levels persisted long after the corpus luteum secretion held any significance. It appears likely, therefore, that the placental secretion was at fault.

Mention was made in an earlier chapter of abnormalities noticed during a series of pregnanediol assays to determine cyclic variations. These urines were reassayed quantitatively and concentration curves were constructed. (fig. 1B).

Cases one and two show sporadic pre-ovulatory pregnanediol levels in excess of 3 mg/litre. These isolated peaks are quite different from the post-ovulatory pattern which shows a steady increase until just prior to the menses. According to Waldi, a proliferative phase pregnanediol content greater than 3 mg/litre must be taken as a positive indication of pregnancy. The unusual pre-ovulatory peaks described above probably reflect a transient cortical secretion increase. Close questioning of the donors concerned did not elicit any detail (e.g. of abnormal stress or strain) which could account for the phenomenon, nor had the donors taken any kind of medication.

Cases three and four are normal in every way except with regard to the pregnanediol levels reached a few days before the start of the new cycle. The concentrations reached (9.8 mg and 12.7 mg/litre) fall well within the range defined by Waldi as indicating pregnancy.

Case five shows persistently high pregnanediol levels throughout the cycle. At times the concentrations exceed the lower pregnancy limits. The slight midcycle increase is probably indicative of ovulation. It is possible that the continuous high pregnanediol level is due to a persistent corpus luteum. This is a rare condition and is usually associated with amenorrhoea. In the case mentioned above, a normal menses occurred, and also apparently, ovulation, both of which phenomena are usually suppressed by high progesterone levels in the blood. The alternative to a persistent corpus luteum is that the high level of pregnanediol is due to a corticoid breakdown and is therefore indicative of adrenal hyperplasia. The volunteer is unfortunately not available for further study.

Cases six and seven are abnormal in that ovulation (as indicated by a sustained rise in pregnanediol levels) occurred very early in the cycle. In case six the sustained increase began on day 7 of a 27 day cycle i.e. ovulation occurred 20 days before the end of the cycle. Case seven ovulated on day 8 and had a 32 day cycle. Unfortunately it was possible to follow these two volunteers for one complete cycle only, and so it is not known for certain whether this early ovulation is a regular cyclic occurrence. From that portion of the following cycle which was tested (case 7 only) it seems likely that such an early ovulation was not a regular feature of the subject's normal cycle. If either of these cases were ever to practice the Rhythm method of birth control it is likely that they would not be very successful.

SUMMARY.

The results obtained in pregnanediol assays of pregnancy urine and from urine of atypical menstrual cycles, have been described and discussed. An attempt to induce variations in pregnanediol excretion rates by the administration of drugs has also been described.

CHAPTER ELEVEN

The T.L.C. method of pregnanediol assay as a standard procedure in the Clinical Laboratory

The times required to perform the various stages of the assay in its original form (according to Waldi) and its modified form is shown in table 12.

TABLE 12

Ten specimens were assayed by each of the two methods and the times required by the stages are listed :-

Stage	Procedure	Waldi method		Modified T.L.C. method	
		Time (minutes)	Details	Time (minutes)	Details
1	Hydrolysis	25	-	25	-
2	Extraction (inc. breaking emulsions).	502	4 emulsions formed	410	1 emulsion formed. Battery system used.
3	Evaporation and drying	70	-	30	-
4	Spotting (with capillary)	36	10 plates used (multiple spotting)	11	1 plate used
5	Development	33	5 tanks used	30	1 tank used
6	Spraying and evaluation	32	Semi-quantitative (6 standards/plate)	20	Semi-quantitative (5 standards)
7	Quantitative evaluation		-	65	Spectro-photometric
<u>Total:</u>					
	Semi quantitative assay	698		526	
	Quantitative assay	-		593	

In the tabular form it is easy to see the advantages and disadvantages of the different methods, but there are some aspects which cannot be expressed in tables and these are expressed below:

1) The Collection and Storage of Urine Samples

In the research laboratory it is possible to ensure that the urine is collected and then assayed with a minimum of delay. If delay there must be, then the specimens can be refrigerated until used. The routine

clinical laboratory on the other hand, is forced to use samples which may have spent many days in transit; samples for instance, sent from another town by unrefrigerated carrier. Even if the doctor responsible for ordering the collection and dispatch of the urine provided the patient with sterile equipment and containers, it is still highly unlikely that the urine would have remained uncontaminated with bacteria during micturition.

In order to test the effect of bacterial contamination, a number of samples of urine were inoculated with a range of different bacteria and were left standing at room temperature for three days. They were then hydrolysed and extracted in the usual way while a set of duplicates was extracted without prior hydrolysis. The results indicated that the bacteria caused hydrolysis to a lesser or greater degree and that total pregnanediol values were depressed probably due to the breakdown of the steroid by the bacteria. (Table 13).

Another set of urine specimens were placed unopened and uninoculated in the incubator at 35°C for two days. Six of the urine specimens (i.e. 66.7%) showed evidence of bacterial growth as indicated by extreme cloudiness and unwholesome odour. Sterile bottles and funnels had been used for the collection of these specimens, so the contamination probably occurred during micturition. (Catheterisation would avoid this source of contamination, but this would be a most disagreeable counter-measure). The contaminated specimens were extracted unhydrolysed and all showed the presence of free pregnanediol. No attempt was made to identify the bacteria involved.

Obviously the most convenient method of avoiding the vitiation induced by bacteria, is by preventing the growth rather than the presence of bacteria. This is simply performed by adding a bactericide to the specimen bottles before they are filled. Thymol crystals were used but were unsatisfactory and Phenyl Mercuric Nitrate (P.M.N.) was then used with apparent success. Tests were carried out to prove the reliability of this substance as follows:

Eight bottles were each filled with 20 ml of urine of known pregnanediol concentration. Four of these bottles had been treated with 1 ml

of 0.05% P.M.N. (to give a final concentration of 0.0025%). All eight bottles were then inoculated with a loopful of bacterial suspension (from a mixed culture), and were incubated for two days at 35°C. Growth occurred only in the specimens containing no P.M.N. The remaining four bottles were assayed and it was found that the pregnanediol content had not been affected nor had the bactericide caused any alteration or displacement of the pregnanediol spot on the chromatogram.

TABLE 13

Effects of bacterial contamination

A number of urine specimens of known concentration (P.G.) were inoculated with a range of bacteria. After incubation they were assayed.

	Bacterial Inoculants				
	A.a.	B.s.	E.c.	P.v.	S.a.
Assay values of inoculated urine following acid hydrolysis (in mg/litre)	31.5	30.5	31.6	34.3	29.5
Assay values of inoculated urine extracted without prior hydrolysis (in mg/litre)	22.5	7.6	19.6	2.5	16.0
Assay values of the uncontaminated urine (mg/litre)	32.0	32.0	32.0	34.5	34.5
Percent hydrolysis induced by bacteria	71.50	23.80	61.25	7.25	46.30
Percent destruction of P.G. due to bacteria	1.6	4.7	1.3	0.58	14.5

A.a. = *Aerobacter aerogenes*

B.s. = *Bacillus subtilis*

E.c. = *Escherichia coli*

P.v. = *Proteus vulgaris*

S.a. = *Staphylococcus albus*

It should be noted that while *A.aerogenes* brings about a high degree of hydrolysis, *S.albus* causes actual destruction of the pregnanediol.

Even without bacterial contamination it is possible for pregnanediol to be deposited from the urine. It was noted that a quantity of gravid

urine which had been stored in the refrigerator for 9 weeks gave lower pregnanediol assay values than was expected. It was decided to trace this loss. As the urine had been stored in the dark at a temperature of 3-5°C, it seemed unlikely that the loss was due to chemical breakdown. The following experiment was carried out:

Six flasks containing 20 mls each of urine were stored in the refrigerator for three weeks. They were then removed, opened and placed upside down on a draining board. After 10 minutes each flask was washed out three times with a small quantity of warm cyclohexane. The washings were combined, dried over anhydrous sodium sulphate and evaporated. The residue was assayed in the usual way.

The quantity of pregnanediol washed from the flasks assayed at 0.6 mgm per total volume of urine (120 mls). This is equivalent to 5 mgm/litre - an appreciable amount.

2) The Evaluation of the Thin Layer Chromatogram

With a fully quantitative assay, the pregnanediol is colorimetrically assessed and the results filed. With a semi-quantitative evaluation where spot sizes are compared on the Thin layer plate, it is possible that difficulties may arise if the following precautions are not observed :-

a) The plates must be evaluated as soon as possible after development, or certainly not later than 24 hours after development. Spots caused by the pregnanediol - phosphomolybdic acid reaction, fade fairly rapidly especially in the presence of light, warmth and air. The spots of smaller pregnanediol concentrations appear to fade more rapidly than the higher pregnanediol concentration, and after 24 hours it is no longer possible to arrive at an accurate evaluation.

b) Burette-grease containing silicones must not be used on any of the apparatus used in the T.L.C. assay. Even in trace quantities it reacts strongly with the phosphomolybdic acid reagent to give a dark-blue stain which can completely obscure the pregnanediol spot on the Thin layer plate.

3) Storage of the dried extracts

It may be necessary for reference purposes to store the dried extract for a period of time. The degradation of the extract may be

minimised if it is stored in a completely dry state in a closed container (amber glass) under nitrogen, preferably in a refrigerator. Under these conditions there will be no significant loss of pregnanediol even after a period of years. For periods of less than three months, it is sufficient to store the well-dried extract in a stoppered container placed in a cool dark cupboard.

DISCUSSION

In the previous chapters, the accuracy, reproducibility and reliability of the T.L.C. pregnanediol assay has been assessed and compared with other methods. The time factor, also of considerable interest to the clinical laboratory, is analysed in this chapter.

A breakdown of the method with respect to time is presented in table 12. A comparison of the modified T.L.C. method with Waldi's method indicates clearly that the modified method provides a saving of time and material as well as increased accuracy.

There is one difficulty experienced by the routine clinical laboratory which is not shared by the research laboratory: that is, the difficulty of preserving the specimens in a fairly fresh or uncontaminated state. A study of table 13 will show how bacterial contamination can affect the pregnanediol concentration and cause false results. It is necessary therefore to include a bactericide in all specimens which have to spend some time in transit from the source to the testing laboratory. Even in the absence of bacteria, it is possible for pregnanediol concentrations to alter on storage. Refrigerated urine often develops a finely divided precipitate after standing for a while. It is probable that the less soluble components in the urine (including P.G.) are absorbed onto the precipitate which cakes on the glass of the container, and is left behind when the urine is poured into the hydrolysis flask. This loss could be avoided by washing the original container with the solvent or the hot hydrolysing mixture - the washings to be included with the urine in all subsequent processes.

SUMMARY OF THIS CHAPTER.

The times taken to perform the various stages of the T.L.C. pregnanediol assay have been tabulated. Losses of P.G. due to bacterial

contamination of the urine specimen, have been described and the loss of pregnanediol by deposition onto the glass walls of the specimen bottle have been discussed.

Some precautions necessary to produce accurate results are also listed.

CHAPTER TWELVE.

Miscellaneous qualitative and quantitative methods of
pregnanediol evaluation

In the course of this research a number of different assay methods not hitherto described were investigated in order to see if they offered any advantages not shared by the Klopfer or T.L.C. method. These methods are briefly discussed hereunder.

1) The colorimetric method of pregnanediol assay as
described in Martindale. ⁽⁷⁶⁾

This method involved acid hydrolysis, toluene extraction precipitation and absorption on 'Celite' (B.D.H.). The pregnanediol was purified with activated charcoal and after colouring with sulphuric acid as described earlier, was read on an absorptiometer (E.E.L., filter 601). Evaluation was by a calibration curve constructed from pure pregnanediol standard solution.

Comment

The method was lengthy (2 days) and of doubtful accuracy. (89% of the T.L.C. assay value).

2) The gravimetric determination of pregnanediol as
devised by Huber. ⁽⁷⁷⁾

Acid hydrolysis and toluene extraction were followed by evaporation and column chromatography using deactivated alumina. The pregnanediol was recrystallised from methyl alcohol until it melted at 234 - 236°C. It was then weighed.

Comment

Although of the correct melting-point, the final product contained impurities (as assessed by T.L.C.). In spite of this the weight of the final product was lower than the calculated value. The method takes under four hours.

3) Paper chromatography of pregnanediol glucuronidate, as
described by Lewbart et al. ⁽⁷⁸⁾

This method was used on freeze-dried urine and on some of the product of a Venning assay. It was hoped that a simple assay method could be devised which would involve a minimum of experimental techniques i.e.:

freeze-drying, paper chromatography and colorimetry of the eluate.

Comment

Suitable separation of the numerous urinary constituents was not achieved. It appeared that a preliminary purification of the freeze-dried urine was necessary for the pregnanediol ester to be freed from interfering constituents, and the introduction of such preliminary steps automatically nullified the method's main advantage (i.e. its simplicity).

(Note: Electrophoresis was substituted for the paper chromatography stage but no better separation was achieved).

DISCUSSION

The pregnanediol assays described above were found to possess no features which could justify their inclusion in this project. The Martindale method is as lengthy as the Klopper method and is less accurate. The Huber gravimetric method does not yield a pure product and the paper chromatographic method is only feasible where initial purification of the urine is carried out in order to limit the possibility of "mimics" causing false positives.

SUMMARY

Some pregnanediol assay methods not previously mentioned are described and discussed in this chapter.

FINAL SUMMARY AND CONCLUSION

Progesterone and its metabolite pregnanediol have been the subject of much research. When the primary functions of the hormone began to be appreciated intense activity was directed toward the full elucidation of the role played by progesterone in the female sexual cycle and pregnancy. The discovery by Venning of a method to assay pregnanediol in the urine provided a further stimulus to this field of research and the development of new techniques for estimating both progesterone and pregnanediol has continued to the present day. There has been a renewed upsurge of interest in recent times primarily due to the use of progestins in ovulation control.

An outline of the significance of pregnanediol, and a history of its assay methods has been included in this work. As a diagnostic aid, the pregnanediol assay is of widespread importance, but it is not the more esoteric uses of the assay which have been stressed : rather it is the more mundane but topical applications which have been investigated; namely pregnancy diagnoses and menstrual cycle determinations. The research has been especially directed toward devising and assessing the most practical form of P.G. assay, and toward establishing the validity or otherwise of diagnoses made on the basis of such an assay.

Experimental Work

The original method described by Waldi was adapted so as to include a final colorimetric evaluation of the pregnanediol extract. A number of techniques were investigated and assessed and in the light of these findings, one method was chosen for all quantitative work.

Being thus able to detect small changes in the concentration of the final extract, the initial hydrolytic, extractive and evaporative stages of the method were altered to an optimum form. This final form of the assay was then compared to the assay method of Klopper, Mitchie and Brown in terms of accuracy, reproducibility and rapidity. Though rather less accurate than the Klopper method, there is no doubt at all that the Thin Layer chromatographic method is far more suitable for routine use. That it is an ideal routine system is proved by the fact that over 500 assays were carried out singlehanded on urines supplied by female volunteers

in the course of $2\frac{1}{2}$ months and nearly 50 complete menstrual cycles were followed. This was not only an attempt to discover the variation in secretion during a normal cycle, for such values are known, having been published by a number of workers. Instead, this trial was performed to discover abnormal pregnanediol levels.

The modified T.L.C. method was used excepting that instead of a quantitative evaluation, a limit-test type of assessment was employed. Only the extracts showing abnormal values were referred for the full quantitative treatment.

In further tests to evaluate the T.L.C. pregnanediol assay in terms of routine usefulness, the effects of excessive heating, bacterial contamination, and deterioration on storage were investigated. The precautions necessary to avoid arriving at false P.G. values have been described in the text.

The reliability of the T.L.C. pregnanediol assay has been established, but the validity of diagnoses based on the assay results, remains in question.

With regard to ovulation detection, the assay would seem to be adequate. Certainly there is an easily observed pregnanediol increase at midcycle, this increase usually preceding the B.B.T. increase by two days. Whether in fact the P.G. increase actually occurs on the day of ovulation is impossible to determine without an actual visual observation of the ovaries or ovum, but if the increase is sustained it does indicate the presence of a functioning corpus luteum. Most authorities agree that there is at most a difference of one day between ovulation and the increase of urinary pregnanediol.

Planned parenthood by means of a pregnanediol estimation was first suggested in 1961.⁽⁷⁹⁾ A concentration of over 2.5 mgm P.G./litre urine was presumed to indicate the conception-free period of the menstrual cycle. To rely upon a single estimation is to take an unwarrantable risk. Pre-ovulatory concentrations of over 2.5 mgm P.G./litre are not uncommon, but are never sustained for more than one, or at most two days, and so it would be safer to base the resumption of sexual activity on three consecutive P.G. assay values of over 2.5 mgm P.G./litre.

Those who practice the Rhythm method as described by Weldon,⁽⁵¹⁾ that is, sexual abstinence during a midcycle period the time and duration of which is calculated according to the usual length and regularity of the menstrual cycle, are likely to end up with numerous progeny. There is no immutable natural law which dictates on which day of the cycle ovulation shall occur. It is accurate to say that ovulation usually occurs on the 13th - 15th day before the start of the next cycle : it is incorrect to say that it always occurs at that time. A glance at fig.1B will show that ovulation may occur so early as to nullify completely the efforts of those who observed the usual midcycle period of abstinence even when this period had been lengthened in the manner recommended for 'perfect safety.' Unfortunately it was possible to follow only one of the early ovulating cases through part of a second cycle. Ovulation appeared to occur at the normal time, in this case day 16 of a 31 day cycle. It seems likely then that persons with a perfectly normal cycle are subject to occasional phases in which ovulation may occur at unpredictable times. This may be very occasional indeed, but after all it has to happen only in one cycle every year or two for the person concerned to conceive with reasonable frequency.*

In cases such as these, where ovulation occurs at different times of the cycle, a pregnanediol assay would be useful, not in ascertaining a pre- and post-ovulatory 'safe period' but only in establishing the post-ovulatory conception-free period. For perfect safety it would be necessary to regard as potentially fertile the whole cycle up to the time until the assay concentrations were above 2.5 - 3.0 mgm P.G./litre on a number of consecutive days.

For detecting ovulation a P.G. assay has a legitimate use, but for early pregnancy testing the system which Waldi uses as a basis for diagnosis is of doubtful validity. The assay itself is perfectly satisfactory, as has been demonstrated in the foregoing chapters. It is the criteria which he uses as bases for positive diagnoses which are doubtful. If these criteria are applied to the cases depicted in fig. 1B it will

* Two more cases of early ovulation have been noted, but as the cycles concerned were not fully covered, these instances have not been quoted.

be seen that 5 of them are by Waldi's definition, pregnant. This, out of a series of 49 cycles is somewhat over 10%. The claim of 99 - 100% accuracy for the Waldi pregnancy test cannot therefore be correct.

That pregnanediol levels rise during pregnancy is irrefragable. It follows therefore that a P.G. assay can be used as a means of establishing pregnancy. This much was admitted in an earlier chapter; but it was stressed that the level of P.G. present for a positive diagnosis must be above the highest levels found during a normal non-gravid cycle. Thus the claim that an assay value of over 7 mgm P.G./litre is an indisputable indication of pregnancy is not substantiated by the findings quoted in this project, nor by figures quoted by various authorities. (Martindale Vol.II for instance quotes post ovulatory P.G. values of 1 - 10 mgm/24 hours for non-pregnant cases).

Nor can it be claimed that values of less than 7 mgm P.G./litre definitely preclude non-pregnancy. Jayle et al⁽⁸⁰⁾ quote values of 4.0 mgm/24 hours assayed during the 5th and 6th week of pregnancy. de Water-ville in the same work quotes a figure of 2.0 mgm/24 hours assayed in 7, 9, & 15 week pregnancies. Herve et al quote a value of 3.0 mgm P.G./24 hours for an 18 week pregnancy. Admittedly these are extreme cases, but the fact that they do occur must indicate that the claim of 99 - 100% accuracy for the pregnanediol assay pregnancy test is somewhat optimistic. It is only after the twelfth week that average P.G. values are higher than the upper limits found during a non-pregnant cycle (Jayle⁽⁸⁰⁾) and thus it is only after the twelfth week that a pregnanediol assay can establish pregnancy with any certainty.

The fact that the pregnancy diagnosis based on a pregnanediol assay is refutable must not be allowed to detract from the merits of the actual assay itself. There are many conditions which influence the rate of excretion of pregnanediol and which may be diagnosed on the basis of a pregnanediol assay, and it was in anticipation of the possibility that a pregnanediol assay may one day be incorporated in the services offered by the clinical laboratories, that this project was carried out.

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