RELATIONSHIP OF OVARIAN FUNCTION TO HISTOCHEMICAL REACTIONS.

Summary of Thesis
Submitted for the Degree of
Doctor of Philosophy at the
University of Glasgow.

by

F. HAMILTON LECKIE.

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Histochemistry is accepted as a method for investigating body tissues and organ function, and although much research has been made into many human tissues, the ovary alone would appear to have been neglected: there do exist a few reports which deal with the histochemical findings in ovarian tumours, and also with the vasculature, but the present writer has not found any reports of any organised or formal study of the histochemistry of the human ovary. Such a study was undertaken by the writer, and it is this work and the results obtained which form the Thesis.

In the first instance the mature human ovary was studied, sections being treated by a variety of staining techniques, the main ones being the Schiff reaction and various qualitative modifications of it, and the Sudan reaction. As a result of this a lipo-mucoprotein substance was discovered in the ovary, this substance being particularly associated with the follicular system, being found in the granulosa layer of the developing follicle, to a greater extent in

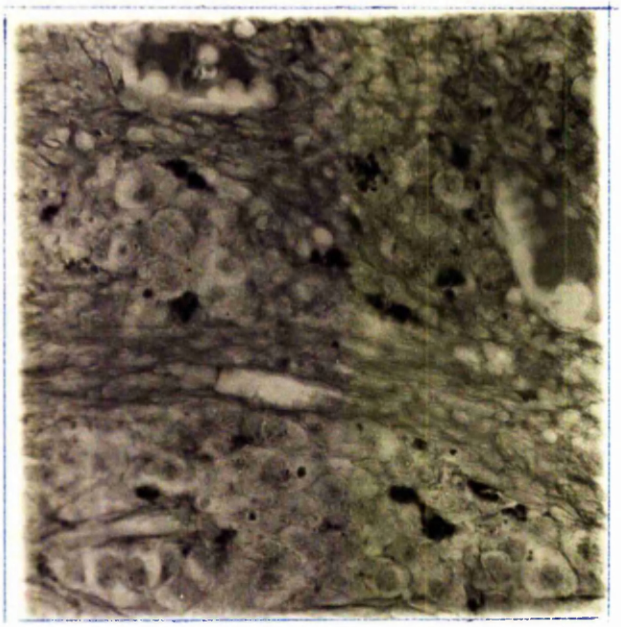

Fig. 1. Section showing Schiff positive cells in a corpus luteum. P.A.S. ×500.

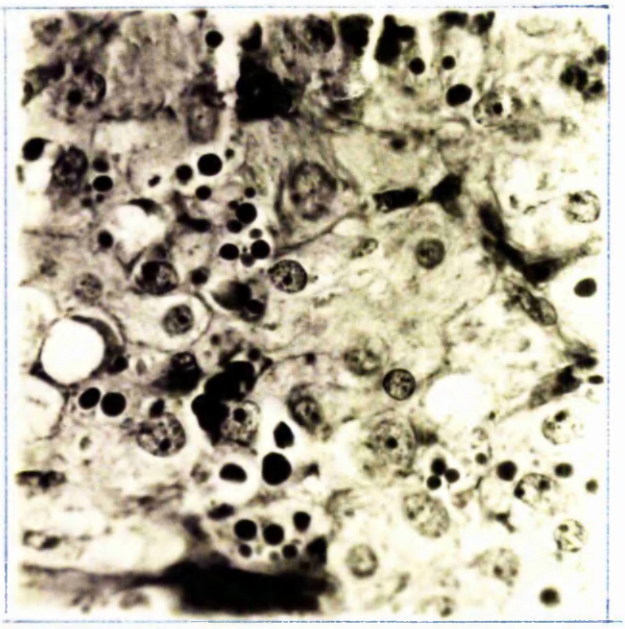

Fig. 2. Section to show globules of Schiff positive material in a corpus luteum. P.A.S. × 750.

the corpus luteum, and to a slight degree in association with the corpus albicans. Closer examination revealed that this substance was visualised in two forms - first, as granules within cells, and second, as globules apparently not contained within cells. (Figs. 1 and 2). It has been noted that there is greater evidence of this Schiff positive material in the corpus luteum associated with the pregnancy state than at any other time. Apparently not the result of break-down products, it seemed that this substance was intimately associated with activity of the ovary.

Study of foetal/baby ovaries and of post-menopausal ovaries revealed that activity in these organs was not an infrequent occurrence, and particularly with regard to the foetal/baby ovaries, this activity was especially associated with a maternal pregnancy toxaemia. As in the mature ovaries, Schiff positive material was found related to the follicular bodies within these foetal/baby and post-menopausal ovaries.

It seemed that the lipo-mucoprotein found

in these ovaries might possibly be related to gonadotrophins, probably of pituitary origin, or perhaps with the ovarian hormones themselves; but whether the Schiff positive material revealed the presence of the actual hormones or merely a carrier substance, cannot be stated.

In an attempt to resolve the question of the true nature and significance of this Schiff positive material, certain animal experiments were carried out. Animals - rabbits, rats, mice - were injected with urine from both pregnant and non-pregnant women, and also with various hormonal preparations. As a result it was shown that urine from pregnant women and gonadotrophic hormones produced activity in the experimental animals, and further, that Schiff positive material, similar to that found in the human ovary, was associated with this activity, and in similar sites. The true nature of the material, however, remains unresolved.

Discussion of the results of the investigation leads to the following conclusions:

1) The human ovary, when active, exhibits

the presence of some substance which is demonstrable by histochemical procedures, in particular the Schiff reaction and its various modifications.

2) The Schiff positive substance is complex and would appear to be a mixture of lipoid and mucoprotein, the protein moiety probably containing tryptophan.

3) This Schiff positive substance is especially associated with the follicular system of the ovary.

4) It seems unlikely that this substance is either an artefact or due to simple break-down products, but is in fact representative of some positive active phase in the ovarian cycle.

5) It seems more than likely that what in the past have been referred to as ovarian interstitial cells are in fact a terminal stage of the Schiff positive substance which is now reported.

6) Animal experiments show that the ovaries of rabbits, rats, and mice respond to stimulation in a way similar to that of the human ovary, and

exhibit Schiff positive material under similar conditions.

7) Further investigation will be required in order to establish identification of the Schiff positive material.

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Thesis submitted for the Degree of Doctor of Philosophy at the University of Glasgow.

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Supervisors -

1. Professor R.A. Lennie. (1952-1954).
2. Professor Ian Donald. (1954-1955).

PREFACE

This study was undertaken in the Midwifery Department of the University of Glasgow, and in the Research Department, Royal Maternity and Women's Hospital, Glasgow. The work was performed over a period of 3 years whilst the candidate was a Research Student at the University of Glasgow. During the final year of the study assistance was received by the award of a Leverhulme Scholarship.

Practically all the tissues for examination were obtained from post-mortem specimens at the Royal Maternity and Women's Hospital, and from patients under treatment in the Obstetric and Gynaecological Department, Eastern District Hospital, Glasgow. Animal experiments and studies were carried out, under licence, in the Research Department, Royal Maternity and Women's Hospital.

Two papers - "A Histochemical Study of the Human Ovary, Preliminary Report", and "A Study of the Histochemistry of the Human

Foetal Ovary" - have been published in the Journal of Obstetrics and Gynaecology of the British Empire, 1954, 61, 772, and 1955, 62, 542, respectively. These papers are incorporated in this thesis.

I wish to express my thanks to Dr. Hugh Stirling, for his co-operation and permission to use tissues obtained at operation on patients in his Unit at Eastern District Hospital, and also to Professor R.A. Lennie, and latterly Professor Ian Donald, of the Midwifery Department, the University, as my supervisors, for their kindly interest, encouragement and advice. I also wish to express my indebtedness to Dr. A.D. Telford Govan, Director of Research, Royal Maternity and Women's Hospital, for his constant encouragement and helpful criticism throughout the course of this work.

Acknowledgment is also made for technical advice received from the Staff of the Research Department, Royal Maternity and Women's Hospital, Glasgow, and for the photographs which are the work of Mr. Andrew Fraser, F.I.M.L.T., Senior

Technician in the laboratory.

TABLE OF CONTENTS.

INTRODUCTION

For some time it has been felt that although much routine histology dealing with human ovarian tissue has been reported from departments of pathology, there has been virtually a complete lack of any organised study of this organ. Pflüger (1863) was probably the first to differentiate particular types of cell in the ovary apart from those associated purely with the development of the Graafian follicle. He described in detail the interstitial cell of the mammalian ovary, which Bouin (1902) later called the interstitial gland because of the lipoid content of these cells. Since that time reports dealing with the human ovary are few, and deal mainly with the vasculature (Collins et al., 1952; Reynolds, 1950, 1951), or with clinico-pathological conditions (Buxton and Engle, 1950; Laffargue and Luscan, 1951). Quite an extensive literature, however, has appeared dealing primarily with the histochemistry of the interstitial tissue of animal ovaries (Dawson and McCabe, 1951;

Hökfelt, 1951; Rennels, 1951; Vincent and Dornfeldt, 1948). These authors describe a cell in the ovarian interstitial tissue which appears to contain a large amount of lipoid or lipo-protein, the main feature being a positive reaction to Schiff's reagent.

As far as can be ascertained from a perusal of the literature no communication has been made or evidence produced to indicate that any other worker has undertaken an organised and controlled study of the histochemistry of the human ovary. It seemed, therefore, that here was a fertile field for investigation. As a result of the study of the histochemistry of the human ovary it was felt that new light would be thrown on the physiology of ovulation and ovarian function, and that a contribution might be made towards the understanding of the pathology of the ovary under varying conditions. Accordingly, such a study was undertaken.

In the first instance it was decided to investigate the histology and histochemistry of the human ovary during what is considered to be

its most active period, that is, from patients in the child-bearing age-group, both in the pregnant and non-pregnant states. Thereafter, it was considered necessary to study, by similar methods, ovarian tissue from the extremes of life, that is, foetal/baby ovaries, and ovaries from post-menopausal patients. Subsequently, animal experiments were carried out in order to corroborate, if possible, the results obtained in the human study, and to endeavour to establish the modus operandi of the histochemical substance which was found in the ovary to be Schiff positive, and resembling to some extent the cells described in animal ovaries by such workers as Dawson and McCabe (1951), and Rennels (1951).

For the sake of clarity the work is reported under five main headings:

I. Histochemical Techniques.

II. Histochemistry of Mature, Active, Human Ovaries.

III. Histochemistry of Foetal/Baby Ovaries.

IV. Histochemistry of Post-Menopausal Ovaries.

V. Animal Experiments.

Thereafter the results are discussed and conclusions drawn.

SECTION 1.

Histochemical Techniques.

General Historical Introduction.

Most authorities appear to agree that histochemistry as a science is as old as histology itself. That modern histochemistry differs markedly from that of about 100 years ago there can be little doubt; with progress it could hardly be otherwise; alterations, improvements and discovery of new techniques have taken place. In its earliest stages the application of histochemistry was mainly botanical, and several publications appeared, such as Raspail's 'New System of Organic Chemistry' in 1834. About the middle of the 19th. century there was a wider application of histochemistry, although in the case of animal tissues the methods involved tissue destruction and was mainly biological chemistry. During the period between 1899 and 1929 the use of aniline dyes in histology became widespread, and this period also saw the rapid expansion of descriptive histopathology.

Mann (1902) contributed a classical work on the subject in his 'Physiological Histology'. Other works published in this period showed that histochemistry still flourished, and includes 'Review of Recent Developments in Histochemistry' by Parat (1927). Between 1930 and 1944 histochemistry came to the fore again, and virtually returned to the domain of histology, by the publication of Lison's 'Histochimie Animale' in 1936. This author set out the new science of histochemistry without tissue destruction. Since that time the main published works have been Glick's 'Techniques of Histo- and Cyto-chemistry' (1949), and 'Histochemistry, Theoretical and Applied' by Pearse (1953).

Baker (1945) has drawn attention to the claims of the French microscopist F.V. Raspail to be considered as the founder of histochemistry. Although earlier references to histochemical practice can be found, such as the use of solutions of iodine for the staining of starch, the first clear appreciation of the science of

microscopic tissue chemistry came from Raspail (1825 a, b; 1829). This author, besides using solutions of iodine as a means of indicating starch in vegetable tissues, produced an aldehyde method for determining protein in the tissues which, modified by many workers since that time, is still applied in histochemistry as the Vaisenet-Füath reaction for tryptophan. Raspail also discovered and applied the xanthoproteic and Liebermann reactions for protein, and tested the reaction of protoplasm with a blue dye which turned pink in acid solution.

Vogel (1845, 1847) was able to detect the presence of iron in the tissues by its conversion to black ferrous sulphide with yellow sulphide of ammonia, and this was the origin of many techniques for demonstrating iron which are among the oldest in histochemistry. Perls (1867) introduced his Prussian-blue method for demonstrating iron, and this remains the method of choice up to the present day. The first report of the

use of enzymes for tissue digestion came in 1861, and by the end of the century the technique had developed into the well-recognised one of enzymal analysis for cytochemical purposes (Kassel and Matthews, 1898). Klebs (1868) and Struve (1872) were the first to record the presence of peroxidases by showing that tincture of guaiac gave a blue colour with pus, and peroxidases are well known to occur in the granules of leucocytes. Nuclear chromatin was isolated by Miescher (1873) who made use of its selective affinity for methyl green. Two years later, Cornil (1875), using methyl violet, noted the pink metachromasia given by amyloid when stained with this dye. From then on much work was carried out on the nature of protoplasm, and in the closing years of the 19th century histochemistry was submerged in the mass of work which arose from the discovery and use of new dyes, as well as the subsequent evolution of new staining techniques. When the 19th. century came to a close, most histologists were occupied in making full use

of these new developments in the art of staining, and histochemistry was ignored until its revival in the 1930s.

Specific Techniques, as used in this study.

Tissue fixation. Although there is a tendency, especially with physical chemists, to assume that freeze-drying of tissues is a necessity for all histochemical work, most authorities do not consider that it is a sine qua non. Two factors which tend to preclude the use of freeze-drying are, first, the troublesome routine which is involved, and second, the fact that much of the material when received is already in the fixed state. It follows that most histochemical research must be, and indeed is carried out on material subjected to preparation by the standard methods of fixation. According to Pearse (1953) the most important tissue component is protein, and further, that formalin is the best protein fixative: he states "only where strong objections to the use of formalin can

be raised will there be any necessity to replace it (formalin) by fixatives of other types". In the present study, therefore, all tissues for examination were fixed in formol-corrosive, embedded and blocked in paraffin, and serial sections cut at 6μ. Duplicate specimens were fixed in formol-saline and serial sections cut on the freezing microtome at 12μ. In practice it was found that the staining reactions differed little between tissues fixed in formol corrosive, blocked in paraffin, and cut on the ordinary microtome, as opposed to tissues fixed in formol-saline and cut on the freezing microtome; indeed, if anything, it was felt that greater contrast was noted in the former.

Staining techniques. As a routine for general histological purposes, examination, and initially for comparisons, all sections were stained by the haematoxylin and eosin (H.& E.) method. For histochemical purposes various tissue constituents may be shown by different staining techniques.

Proteins. The proteins, which are present in all tissues, can be divided into (a) simple proteins, defined by the biochemists as yielding mainly α-amino acids and their derivatives on hydrolysis, and (b) conjugated proteins. Of the various classical methods employed for the identification of proteins in vitro, only two are inherently suitable: these are the Millon and the diazonium reactions.

Since Millon (1849) first described his reaction, which is due to the presence of the hydroxyl-phenyl group in the protein molecule, and which is given by any phenolic compound which is unsubstituted in the position meta to the hydroxyl group, numerous modifications have been made, that introduced by Bensley and Gersh (1933) being the greatest advance.

The diazonium compounds are prepared by the action of nitrous acid in the cold on the salts of primary aromatic amines. The resulting compounds, which act as diazonium hydroxides in alkaline aqueous solutions, combine with the phenol, the indole, and the

heterocyclic imidazole groups respectively of tyrosine, tryptophan, and histidine, to give coloured products. The coupled tetrazonium reaction of Danielli (1947) seems to be the best method for the simple demonstration of proteins in tissue sections.

Determination of the amino-acids which go to make up a particular protein is limited by the procedures available, and by the fact that the behaviour of the amino-acids in denatured proteins in tissue sections differs markedly from that in natural tissue proteins. Danielli (1947, 1950) suggested further reactions to be carried out in association with the coupled tetrazonium reaction, whereby the three amino-acids might be determined. Pearse (1953) has to some extent worked out technical details for the application of the differential reactions suggested by Danielli. These are employed in three ways -

(a) To prevent, by chemical combination, the subsequent staining of a given group: for example, dinitrofluorobenzene is used

to block tyrosine, which fails to react by the coupled tetrazonium method.

(b) To convert or destroy the selected group: such as when tryptophan is oxidised by performic acid, thereafter the indole ring of tryptophan fails to react by the coupled tetrazonium method, whilst tyrosine and histidine continue to do so.

(c) To combine with a given group in the tissues, leaving part of its own molecule reactive, this latter then being made visible by some additional process. This is seen in the reaction between the aromatic hydroxyl group of tyrosine and dinitrofluorobenzene resulting in a colourless substance. If, however, the 4-nitro group of the attached dinitrofluorobenzene is reduced to NH_2, then diazotized with nitrous acid at 0^{o} and finally coupled in cold alkaline solution with a suitable phenol or aromatic amine, the location of the tyrosine-trinitrofluorobenzene compound becomes strongly coloured. This method has been found to be unsatisfactory.

Table 1.

Coupled Tetrazonium Reaction.

Substance	Alone	After dinitro-fluorobenzene	After performic acid	After benzoylation
Tyrosine	+	-	+	-
Tryptophan	+	+	-	-
Histidine	+	+	+	-

Benzoylation - belongs to the first group of 'blocking' reactions, being carried out by treating sections with 10 per cent. benzoyl chloride in dry pyridine for 8-16 hours at room temperature; as a result there is blocking of the reaction with diazonium and tetrazonium salts. Benzoylation, therefore, should prevent the subsequent reaction of tyrosine, tryptophan and histidine by the coupled tetrazonium method.

The reactions for differentiating the amino acids, carried out in conjunction with the coupled tetrazonium reaction, may therefore be summarised as in Table 1 (Pearse, 1953).

Conjugated Proteins. (Nucleoproteins.) Probably the most important conjugated proteins which have to be dealt with are the nucleoproteins, which are combinations of a basic protein with various highly polymerized polynucleotides (nucleic acids). The two types of nucleic acid which are found in the tissues of plants and animals are deoxypentose-nucleic (deoxyribonucleic) acid

of the nuclei and pentosenucleic (ribonucleic) acid of the cytoplasm.

Feulgen and Rossenbeck (1924) introduced a reaction which was reputed to be specific for deoxyribonucleic acid: this has been known as the Feulgen (Feulgen-Schiff) reaction. Unfortunately, in practice it is less useful than supposed, and as a result there has been a great deal of controversy over it. In more recent times a number of extraction techniques for nucleic acids have been used, but even here there is not agreement as to the reliability or specificity of these methods.

Cohen (1944) used trichloroacetic acid for the extraction of deoxyribonucleic acid, and later Schneider (1945) used a 5 per cent. aqueous solution at 90° for 15 minutes to extract nucleic acids from the tissues. This procedure removes both deoxyribonucleic acid and ribonucleic acid but leaves behind a protein component of the nucleus which then has a greater affinity for acid dyes, such as 1 per cent. aqueous light green S.F.

Although not generally regarded as a very useful histochemical reaction, the Gram stain, with modifications, has been used for various histochemical purposes, and some observers consider that nucleoprotein gives a positive result.

Carbohydrates. The carbohydrates may be classified into 4 main groups, viz:-

I. Polysaccharides. Glycogen is the only naturally occurring member of this group which remains in animal tissues after aqueous fixation and paraffin blocking. Baker (1945) has shown that formalin fixation preserves proteins in such a way that glycogen is held by them and is not easily dissolved by water. This view is held also by Vallance-Owen (1948).

II. Mucopolysaccharides. Meyer (1938) defined mucopolysaccharides as polysaccharides containing hexosamine as one component, and occurring either free or as esters of sulphuric acid. They may be either acid mucopolysaccharides or neutral mucopolysaccharides.

III. Mucoproteins and Glycoproteins.

In this group Meyer defined mucoproteins as substances in which hexosamine containing polysaccharide is found in firm chemical union with a peptide. On arbitrary grounds, where the hexosamine content is greater than 4 per cent., a mucoprotein is being dealt with, whilst if the hexosamine content is less than 4 per cent., then the substance is glycoprotein.

For the purposes of this present thesis it is interesting to note that there is general agreement that chorionic gonadotrophin and the precursor substances of the pituitary gonadotrophins (follicle-stimulating hormone and luteinising hormone) are mucoproteins.

IV. Glycolipoids. The principal members of this group are the cerebrosides which are probably of little concern in the present study.

Mention of carbohydrates in histochemistry immediately raises the question of the periodic acid-Schiff (P.A.S.) reaction. Its use in histology was first described by McManus (1946),

but about the same time the method was shown to be suitable for histochemistry; Hotchkiss (1948) showed that by means of the PAS technique a variety of polysaccharide substance could be shown in the tissues. One of the main properties which make periodic acid superior to other reagents currently used in histochemistry is that of being an oxidant of the carbon bond which does not result in further oxidation of the aldehydes which are formed, and these latter can be demonstrated clearly by the combination with Schiff's reagent giving a red coloured substitute dye.

As a result of research and practical application of enzymes to histochemistry, a fairly satisfactory identification and demonstration of the carbohydrate substances can be made. The PAS reaction will give a negative result when applied to tissue from which glycogen has been removed previously by diastase: other forms of carbohydrate, however, which are unaffected by diastase

will continue to react positively to PAS. Thereafter, if sections are now treated with hyaluronidase (commercial 'Hyalase'), the simple neutral mucopolysaccharides are removed, but the acid mucopolysaccharides and other more complex carbohydrates remain and give a positive response to the PAS reaction.

In 1949 Gersh observed that if the tissues were acetylated the oxidation of carbohydrates would be blocked and a subsequent PAS reaction would be negative. If, however, the acetylated sections were saponified, the PAS reaction was restored. This is of some interest, but probably of little importance.

In the present context, when a tissue component is stained so that the resultant tissue-dye complex differs adequately in colour from that of the original dye and from ordinary tissue complexes, then metachromasia is said to be demonstrated. In practice, dyes of the thiazine group are

used, and in this case metachromasia refers to the change of colour from blue to violet or red. In the present investigation, toluidine blue has been employed (Michaelis, 1947). Certain of the carbohydrates may show metachromasia; but it may be noted that the theory, techniques and interpretation of metachromasia still gives rise to much speculation and controversy. It is considered that those carbohydrates which do show metachromasia belong to the complex or acid mucopolysaccharides. Thereafter, those carbohydrates which do not show metachromasia are considered to belong either to the group III mucoproteins or the group IV glycolipoids. These two groups are then differentiated by staining with Sudan black, negative reacters belonging to the group III mucoproteins, and the positive reacters to the group IV glycolipoids.

Lipoids. Although there are different classes of lipoids, for the purposes of the present investigation it has been considered

sufficient to carry out only the general methods for indicating the presence of the substance; that is - the Sudan IV and the Sudan black techniques, together with the physical examination for birefringency.

Staining by thionin (Schmorl's method) has been carried out. This is really a form of metachromasia which may reveal the presence of acid mucopolysaccharides.

Finally, many of the foregoing reactions have been carried out under different extractive conditions, such as the PAS tests for carbohydrates after extraction with acetone and with chloroform. Specific mention will be made where appropriate as to the actual routines carried out.

Full details of the actual methods are given at Appendix A, and these are based entirely on the specific instructions contained in 'Histochemistry, Theoretical and Applied', 1953, by A.G.E. Pearse.

SECTION II.

Histochemistry of Mature Active Human Ovaries.

As indicated in the Introduction, various authors (Dawson and McCabe, 1951; Hökfelt, 1951; Rennels, 1951; Vincent and Dornfeld, 1948) have described a cell in animal ovarian interstitial tissue which appears to contain a large amount of lipoid or lipo-protein, the main feature being a positive reaction to Schiff's reagent. In studying human ovaries the present author has found cells with Schiff positive cytoplasm and resembling to some extent the cells described in animal ovaries. The following account deals with the distribution and structure of these cells, and the cyto-chemical reactions noted with various staining methods, as found in the mature active human ovary.

Material and Methods.

A. Tissue. Ovaries obtained immediately postmortem from patients who had died in pregnancy or the early puerperium

were employed in the first instance. Thereafter, whole ovaries or portions of both ovaries were obtained at operation in non-pregnant patients in the child-bearing age, and in patients at different stages of pregnancy. By this means it was felt that a fairly accurate cross-section of the active human ovary would be obtained, and that any findings would in all probability be accurate and significant for general application. In all, some 59 specimens were employed: of these, 36 were ovaries of 26 pregnant patients, and 23 were ovaries of 19 non-pregnant patients.

B. Histological and histochemical techniques. Some of the specimens were fixed in formol-corrosive, blocked in paraffin, and serial sections cut at 6μ. Others were fixed in formol-saline and serial sections cut on the freezing microtome at 12μ.

Thereafter sections from all specimens were stained by the following techniques as indicated in Section I, and as described in detail in Appendix A, which is based on Pearse

(1953).

(i) Haematoxylin and eosin (H. & E.).

(ii) Periodic acid - Schiff (P.A.S.), or simple Schiff reaction.

In addition to this a series of sections was stained by P.A.S., after treating respectively with diastase, hyalase, acetylation, and acetylation with saponification (Hotchkiss, 1948).

(iii) Repetition of the full range of techniques described in (ii) above after treating sections in acetone for 30 minutes at room temperature (Dawson and McCabe, 1951).

(iv) Repetition of the full range of techniques as in (ii) above after treatment in boiling chloroform and methyl alcohol (Pearse, 1951).

(v) Modified Gram stain (McLetchie, 1944).

(vi) Toluidine blue, for metachromasia (Pearse, 1952).

(vii) Thionin for metachromasia (Schmorl's method).

(viii) Light green, and also trichloroacetic acid - light green, for the detection of

nucleoprotein (Pearse, 1953).

(ix) Sudan IV before and after extraction with warm chloroform.

(x) Coupled tetrazonium, and also benzoylation with the coupled tetrazonium reaction, with, in addition, the coupled tetrazonium technique after performic acid. These were repeated in a few instances after treating for 5-10 minutes in warm chloroform (Danielli, 1947, 1950).

(xi) The Millon reaction.

(xii) Examination for birefringency.

The techniques (iii), (iv), (ix), (x), (xi) and (xii) above were employed only on an arbitrary representative number of sections, principally from the tissues obtained at operation.

Results.

Examination of the sections stained by the H. & E. method reveals a histological picture which is typical of that described so fully by such authorities as Novak (1947) and Smout and Jacoby (1948), that any further

description in the present context is considered unnecessary. There is nothing of significance, apparently, regarding the cells under consideration. In fact, it is almost impossible to distinguish them from other cells in the granulosa and theca layers of the Graafian follicle, or from cells of the normal ovarian stroma.

P.A.S. Excellent results were obtained by this method and Schiff positive material is found widely distributed throughout the ovary. In particular it is noted that a fine basement membrane immediately under the germinal epithelium stains Schiff positive. In the follicles also there exists a basement membrane between the granulosa and theca layers which stains Schiff positive. The zona pellucida of the ovum gives a strong, and relatively broad, positive reaction to the Schiff stain. The blood vessel walls give a positive reaction with the P.A.S. technique. These findings are present in all sections examined, and would appear to be the normal pattern in the organ

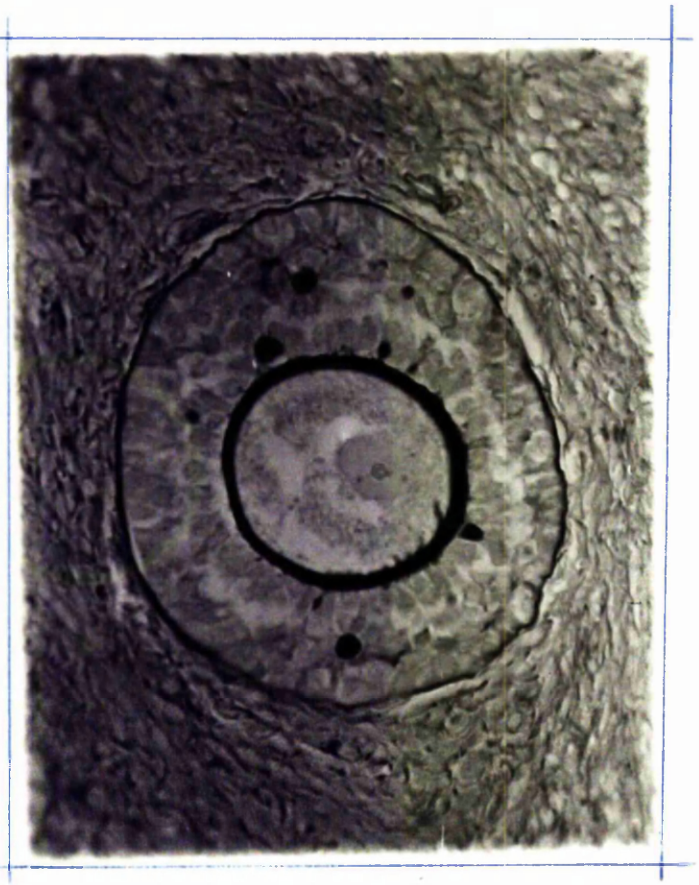


Fig. 1. Section showing early developing follicle with occasional Schiff positive cells in the granulosa layer. The vitelline membrane also gives a strong positive reaction. P.A.S. × 750.

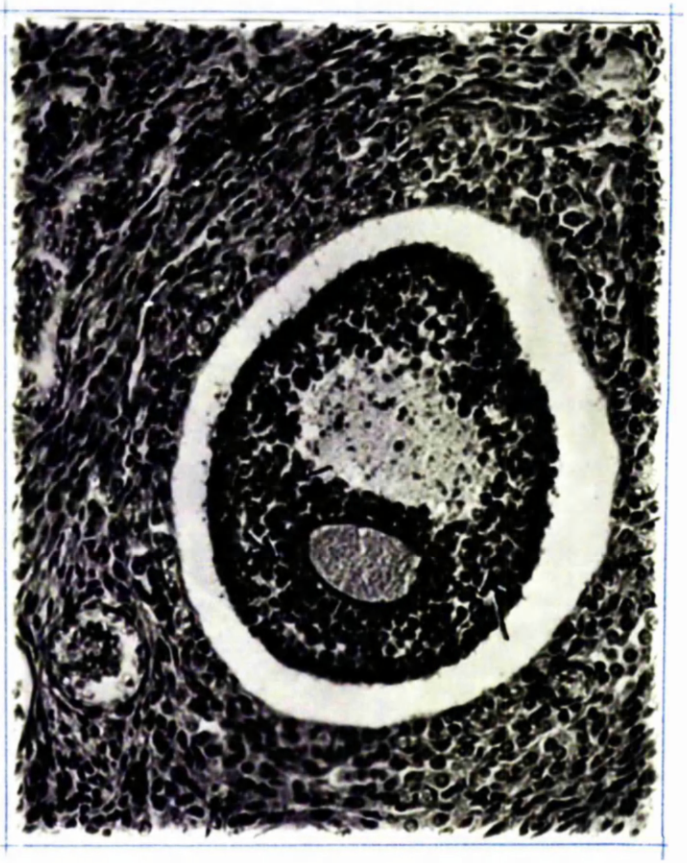


Fig. 2. Section showing follicle with antrum, with Schiff positive cells in the granulosa layer. An indication is obtained also of the distribution. P.A.S. x 280.

under investigation. Throughout this thesis no further reference will be made to these general appearances.

Regarding the Schiff positive cells primarily under consideration, these are found distributed throughout parts of the ovary, as will be described below. These cells are found to vary in shape, mainly according to situation, from somewhat flattened or spindle-shape, to round and polygonal, with a clear generally centrally placed nucleus.

Developing Graafian Follicle. In the sections studied, Schiff positive cells have not been found in association with primordial follicles. In early developing follicles, however, occasional Schiff positive cells are found in the granulosa layer (Fig. 1). These cells are found also in greater numbers in the granulosa layer of the follicles of fuller development and in which the atrium is formed (Fig. 2). In atretic follicles, where many granulosa cells have become disrupted, amorphous Schiff positive material largely fills

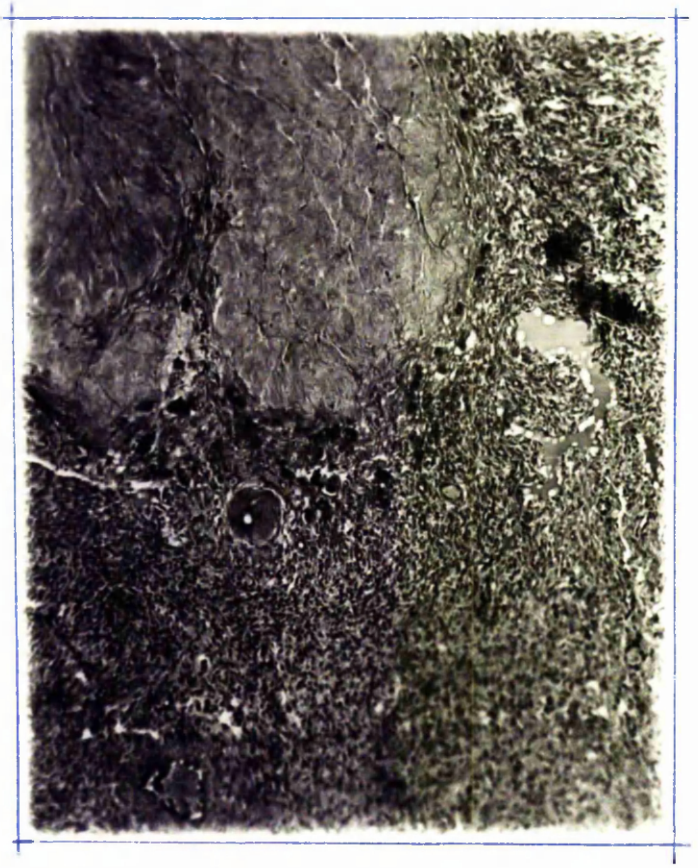


Fig. 3. Section to show Schiff positive cells in the interstitial stroma in close association with one pole of the hyalinised tissue of a corpus albicans. P.A.S. x 200.

the follicle; usually the theca layer is intact, and a few Schiff positive cells are found throughout this layer, extending occasionally into the stroma.

In no instance have these Schiff positive cells been found in the theca layer, except in the case of atretic follicles as noted above. In the granulosa layers these cells are polygonal in shape, and they have a similar appearance in the theca of atretic follicles.

Interstitial Stroma. Schiff positive cells are found in the interstitial stroma, but apparently always in close proximity to follicular structures, such as atretic follicles, corpora lutea, and in particular corpora albicantia (Fig. 3), suggesting an origin therefrom. Otherwise they seem to be absent from the general stroma.

Corpus Luteum. Perhaps the greatest concentration of this Schiff positive material is to be found in the corpus luteum, and affords in this situation what would appear to

Fig. 4. Section showing Schiff positive cells in a corpus luteum. P.A.S. x 500.

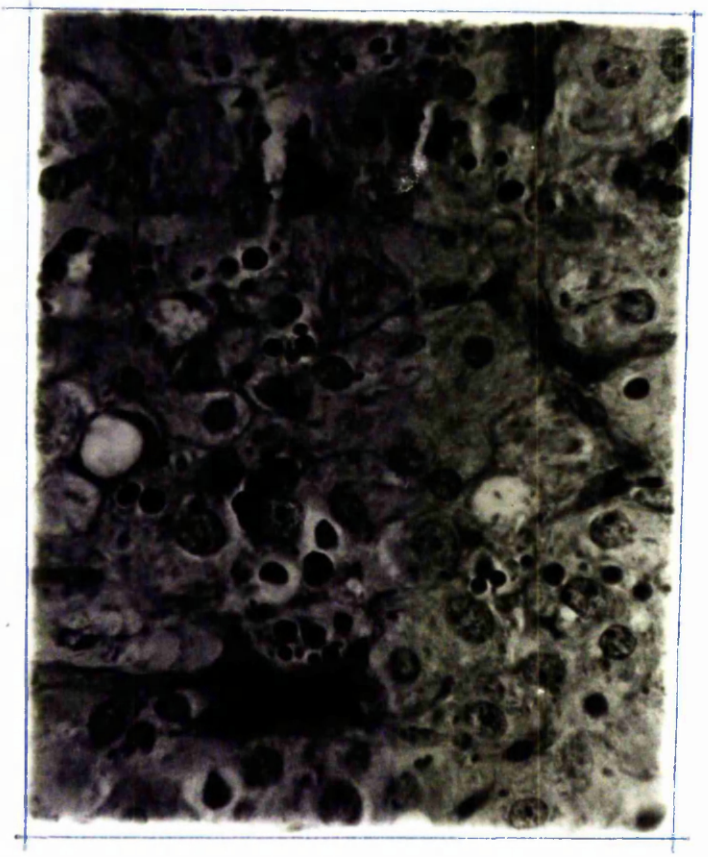


Fig. 5. Section to show globules of Schiff positive material in a corpus luteum. P.A.S. × 750.

be the most fertile region for examination. The most striking feature is the generalised distribution of strongly positive P.A.S. material in the form of what appears to be globules: whether, in fact, these are globules or heavily saturated cells is not clear. Towards the periphery of the corpus luteum, and also in close proximity to the septa therein, numerous cells containing Schiff positive granules are seen. These appear to be flatter or spindle-shaped towards the periphery, as opposed to the more polygonal shape seen deeper within the corpus luteum. Occasionally areas of fine "peppering" of P.A.S. positive granules are seen, the appearances suggesting dehiscence of the granules from ruptured cells. As mentioned above, Schiff positive material is widely distributed in the corpus luteum in the form of what appears to be globules of varying size, occurring in groups, apparently not always contained within the confines of cells (Figs. 4 and 5).

In the stroma immediately surrounding older corpora lutea occasional Schiff positive cells are found.

Corpus Albicans. Except in very old corpora albicantia, Schiff positive cells are always present in association with these bodies. Occasionally single cells are found within the substance of the corpus, but the more usual finding is a bipolar concentration.

Frequency. In the ovaries from non-pregnant patients a considerable number of Schiff positive cells is found in the granulosa layer of the developing follicle. In these the material is in the form of a fine granular suspension. On the other hand, in the corpus luteum the material assumes a globular form, and the number of Schiff positive cells does not appear to be appreciably increased. There are usually, however, many Schiff positive cells in the stroma of the ovary of the non-pregnant patient, but it has not been determined at this stage whether these are related to functioning follicles or merely derived from corpora

albicantia. During pregnancy there appears to be a marked increase in the number of Schiff positive cells of both granular and globular forms in the corpus luteum; stromal cells with Schiff positive material seem to be fewer.

In general, all the foregoing observations apply to the ovary of pregnancy, but with the following modifications. In the follicle granulosa the Schiff positive cells appear to be more definitely concentrated towards the atrial border, and the area towards the theca layer is relatively clear, although when compared with the follicle of non-pregnancy it becomes obvious that there is less evidence in toto of the Schiff positive cells. On examining the interstitial stroma it again becomes obvious that there are fewer P.A.S. reacting cells in the ovary of pregnancy, and those which are present are quite definitely in association with some other body, such as the corpus albicans. On the other hand, the corpus luteum of pregnancy shows the strongest

Table II.

Number or Cases: 26.

Rating	Up to 12/52 pregnant	12/52 - 28/52 pregnant	28/52 - 40/52+ pregnant	Total
+	0 (0%)	1 (11.1%)	2 (15.4%)	3 (11.5%)
++	1 (25%)	3 (33.3%)	3 (23.1%)	7 (27%)
+++	2 (50%)	4 (44.5%)	7 (53.8%)	13 (50%)
++++	1 (25%)	1 (11.1%)	1 (7.7%)	3 (11.5%)
Total	4 (100%)	9 (100%)	13 (100%)	26 (100%)

ARBITRARY RATING SCALE.

1+ = minimal presence of Schiff positive cells.

up to 4+ = high concentration of Schiff positive cells.

concentration of Schiff positive material of any other ovarian body, whether in the pregnant or the non-pregnant state.

As there were these differences between the non-pregnant and pregnant frequency distributions, it was considered necessary to re-examine the pregnancy series of sections to determine if, within this group, there was any difference in the frequency of the P.A.S. positive cells depending on the stage of pregnancy. Accordingly, the sections from the 26 pregnant patients were divided arbitrarily into 3 groups, viz: (a) those under 12 weeks duration, (b) those between 12 and 28 weeks duration, and (c) those over 28 weeks duration. Assessment of the quantitative presence of the Schiff positive cells is not easy, but in general terms it was considered accurate enough to allocate a 1+ for minimal presence of Schiff positive cells, up to 4+ for a high concentration. As will be seen from Table II there is very little difference between the 3 pregnancy ovaries

sub-groups until one is concerned with the highest concentration, at which level there appears to be greatest concentration of the Schiff positive cells in early pregnancy which declines in a diminishing curve as pregnancy advances. It will be noted, however, that arbitrary stages of pregnancy were chosen, a rather relative visual assessment rating was employed, and in particular that the numbers involved are very small: it is possible that these figures are of little significance.

The positive reaction to the P.A.S. technique would suggest that the substance may possess a carbohydrate radicle, or a lipoid. Further tests were carried out, with the following results.

Cytochemistry. According to Rennels (1951) and Dawson and Mc Cabe (1951), the substance in animal ovaries is of lipoid nature, and is removed by acetone, chloroform, etc., and is birefringent. In the present study, it is found that, in the sections examined after

treating with Sudan, a positive reaction is observed in the material already noted as being Schiff positive. This is particularly noticeable in association with the corpus luteum and the early corpora albicantia, the cells being distributed closely around the latter in a bipolar fashion, with occasional positive cells within the corpora. Being Sudan positive, it is taken that lipoid is present in the material. However, after treating the section for 5-10 minutes in warm chloroform and then staining with Sudan, it is noted that the reaction is abolished, indicating that the lipoid has been extracted. But neither acetone nor a mixture of equal quantities of boiling chloroform and methyl alcohol abolishes the P.A.S. reaction, therefore the P.A.S. positive material is not identical with the lipoid fraction.

Diastase and hyalase have no influence on the Schiff positive material, indicating that the reaction is not due to polysaccharides.

Throughout the series no evidence of

metachromasia could be obtained with toluidine blue or thionin (Schmorl's). This confirms the idea that the substance is not a simple carbohydrate. Nevertheless, the P.A.S. reaction is prevented by acetylation, but is subsequently restored by saponification, and according to McManus and Cason (1950) this means that the material is almost bound to possess a carbohydrate radicle. There is thus some evidence that the substance is probably a mixture of mucoprotein and lipoid.

The coupled tetrazonium reaction gives a weakly positive result in the Schiff positive material, indicating the probable presence of amino acids.

The Millon reaction is negative, therefore tyrosine is absent.

The coupled tetrazonium reaction after performic acid is negative, so that possibly tryptophan may be present. This is confirmed by the reaction being negative after benzoylation. (See Table I). These findings would indicate the presence of protein

which contains tryptophan.

The modified Gram's reaction reveals the Schiff positive material to be Gram positive, this being most obvious in the globular state in the corpus luteum. Some observers consider this due to nucleoprotein, but in the present series light green before and after extracting with trichloro-acetic acid showed no difference in the staining reaction, indicating that the substance is unlikely to be of nucleoprotein nature.

Numerous representative sections were examined under the polarizing microscope without any evidence of birefringency.

To summarize these results, it may be stated that, within the human ovary during the child-bearing period, there is a complex substance, detected histochemically in the form of globules, or granules within cells, present mainly in the corpus luteum, and to a lesser extent in the stroma in close proximity to corpora albicantia, and also in the granulosa layer of the follicles. This substance has

the following characteristics. It is Schiff positive and the reaction is not affected by acetone or chloroform/methyl alcohol. Part of the material is sudanophilic but non-birefringent: this part is soluble in chloroform. The material or mixture is Gram positive, but does not appear to contain nucleoprotein. It does, however, give reactions indicating the presence of amino-acids. It would appear from the results of the various techniques that these cells contain a mixture of lipoid and muco-protein, the protein moiety probably containing tryptophan.

COMMENTARY.

In describing his findings in immature rat ovaries, Rennels (1951) has shown that the interstitial tissue possesses intracellular bodies which are sudanophilic, Schiff positive, Schultz positive, birefringent and acetone soluble. These findings, under similar conditions, have been corroborated by others. In no instance, however, has any attempt been

made to determine more specifically the nature of this substance. From the results given here it is obvious that the mature human ovary contains a substance which, although Schiff positive, in many respects is quite different; this substance although Schiff positive and sudanophilic, is insoluble in acetone, and is non-birefringent. Whether or not these two substances are entirely different as between animal and human tissue, or whether the substance is the same in both instances but in a different form, has yet to be established.

It has been shown by Zilliacus (1952a; 1952b) and Zilliacus and Tötterman (1952) that Schiff positive granules and droplets are present in certain pathological cystadenomata in the human ovary. Schiff positive structures, considered by one of these authors (Zilliacus) as being polysaccharide, are present in benign pseudo-mucinous cystadenomata of the ovary. This author further described this Schiff positive substance in pseudo-mucinous

adenocarcinoma, and notes an inverse ratio between the degree of malignancy and the concentration of the Schiff positive material. From a report by the two authors (Zilliacus and Tötterman) in which is described the P.A.S. staining of Brenner tumours, the positive material in one malignant tumour was scattered all over, and was removed by saliva, which is taken as indicative that the material seems to be glycogen. Apart from these reports it would appear that little research has been made into determining the nature of Schiff positive substances in the human ovary.

The results of the present work indicate that there are two substances. As the Sudan reaction is positive this indicates the presence of lipoid, which can be extracted by fat solvents, leaving behind another substance giving a positive reaction with Schiff. This Schiff positive substance, also, is stable when treated with diastase, with hyalase, and after acetylation with saponification; thus

indicating that it is not glycogen, although of carbohydrate nature. Further, the positive reaction obtained by the coupled tetrazonium technique indicates that protein is present, and from the correlation of the Millon, coupled tetrazonium, and performic acid reactions this protein is likely to contain tryptophan. Due to the negative results obtained from the light green reaction before and after extraction with trichloroacetic acid, it is unlikely that nucleoprotein is present.

In short, the material under consideration would seem to be a combination of lipoid plus glyco-protein. From the distribution of the cells containing this material it would appear that they are closely associated with the later stages of follicle ripening and formation of the corpus luteum. Pregnancy, with its greatly hypertrophied corpus luteum, seems to increase the frequency of these cells - and, it may be, especially in the early months of pregnancy. From this

study of the mature human ovary the significance of these cells is not yet apparent, and it was decided, therefore, to investigate immature human ovaries. This forms the subject of the next section.

SECTION III.

Histochemistry of Foetal/Baby Ovaries.

In the previous section it has been shown that the ovary of women in the child-bearing age group contains a complex substance which is considered to be composed of lipoid and muco-protein. It has been suggested that the presence of this material is related to ovarian activity and in particular to the luteal phase of the ovarian cycle. It is, however, difficult to assess the relative importance of this substance in the adult human ovary. The normal ovary contains follicles at all phases of maturation, and, although the presence of this material may be related to certain stages in follicular development, it is equally possible that it is merely a product of degeneration. In addition, the endocrine pattern is complicated in the adult and interpretation becomes doubly difficult. The foetal ovary, on the other hand, is usually inactive and should perhaps

provide some evidence regarding the possible relationship of Schiff positive material to follicular activity.

Although it is generally believed that foetal ovaries are inactive at the time of birth until puberty, cases have been reported from time to time (Novak, 1947; Govan and Mukherjee, 1950) in which varying degrees of follicular maturation have been seen. It was felt that study of similar material might prove of value in interpreting the findings in the adult ovary. At the same time opportunity has been taken to investigate the degree of activity in foetal ovaries in view of the suggestions made by Govan and Mukherjee (1950).

Material and Methods.

A. Tissue. Eighty-eight ovaries from 49 babies were obtained post-mortem, the ages varying from 24 weeks intra-uterine to 9 days postnatal: of these babies, 14 were born alive and died in the early neonatal period; 10 were stillborn at term; 21 were stillborn

between 28 weeks of pregnancy and term; and 4 were stillborn under 28 weeks of pregnancy - miscarriages.

B. Histological and histochemical techniques. Specimens were fixed in formol-corrosive, blocked in paraffin, and sections cut at 6μ. Thereafter the following staining techniques were employed:

(i) Haematoxylin and eosin (H. & E.)

(ii) Periodic-acid - Schiff (P.A.S.)

(iii) Acetone - P.A.S.

(iv) Sudan black.

In addition, sections were examined for birefringency.

Results.

On examining the sections stained by H. & E. it was found that the ovaries could be divided into two groups - those which were apparently completely inactive, and those which showed evidence of activity as judged by multiplication in the granulosa cells, and follicular formation. By this standard 35 ovaries from 21 babies were inactive, and

Table III.

Foetal Age at Death.	Active Ovaries		Inactive Ovaries	
	No. of Cases.	Per cent.	No. of Cases.	Per cent.
Under 28 weeks.	2	7.1	2	9.5
28 weeks - term.	18	64.3	13	62
Neonatal.	8	28.6	6	28.5
Total.	28	100	21	100

53 ovaries from 28 babies showed activity. Most of these ovaries were pairs from single babies, and, invariably, it was found that the changes seen in 1 ovary of a pair were almost identical with those in its neighbour: thus the numbers may be reduced to those of the babies. In other words, activity of varying degree was noted in the ovaries of 28 babies (57 per cent.), while in the remaining 21 babies (43 per cent.) no evidence of activity could be found. In both groups the foetal ages varied from miscarriages to neonatal life, and analysis suggests that activity in these ovaries is not related to age (see Table III).

On further examination, the first evidence of activity is found in the granulosa cells, which become larger, more polygonal, and more numerous. To begin with, no activity is exhibited by the ovarian stroma. When, however, the granulosa layer has reached the stage of about 4 to 8 cells in depth, there is a condensation of the surrounding stroma to

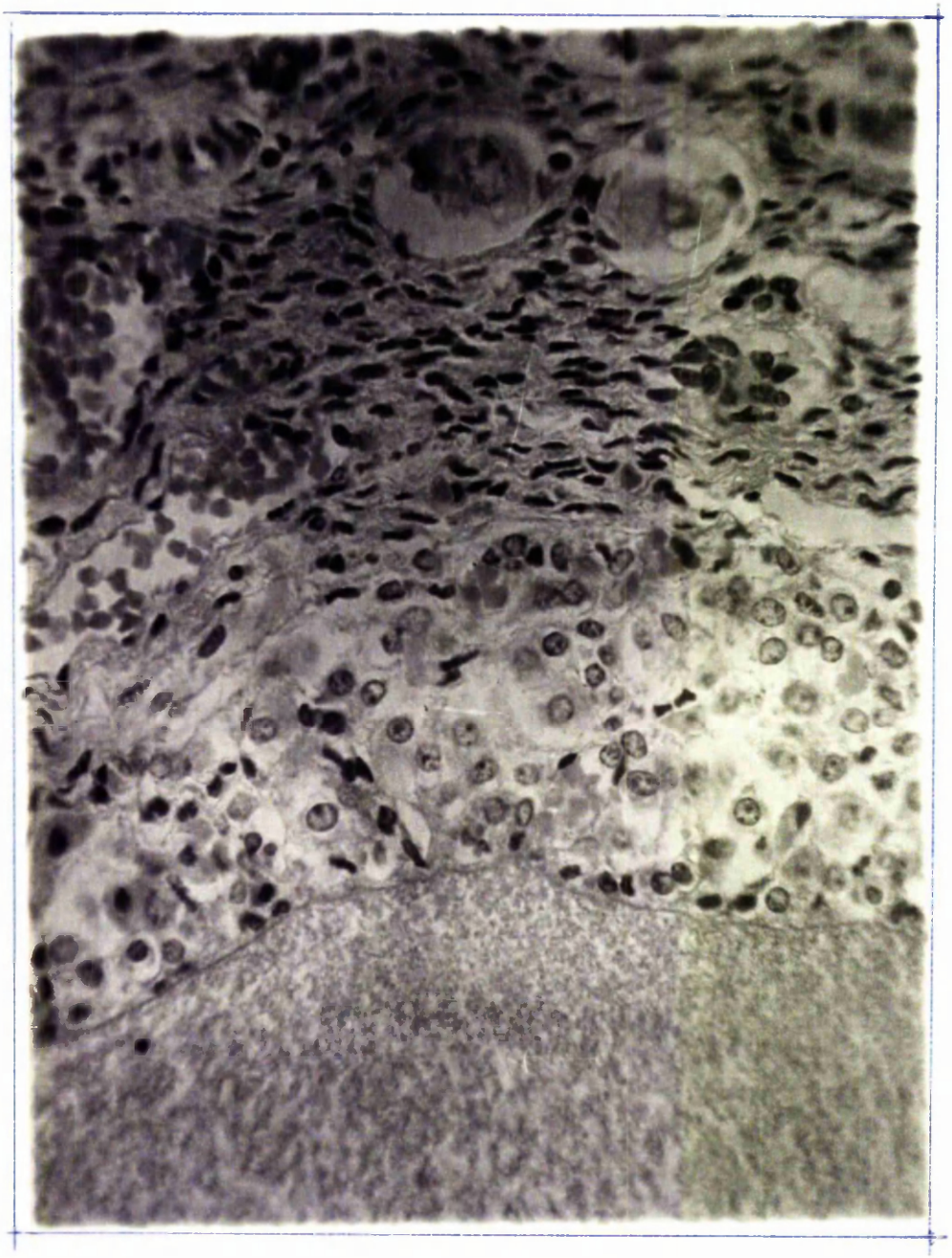


Fig. 6. Section of active foetal ovary to show the type of theca luteinization present: compare with Fig. 7. H.&E. x 840.

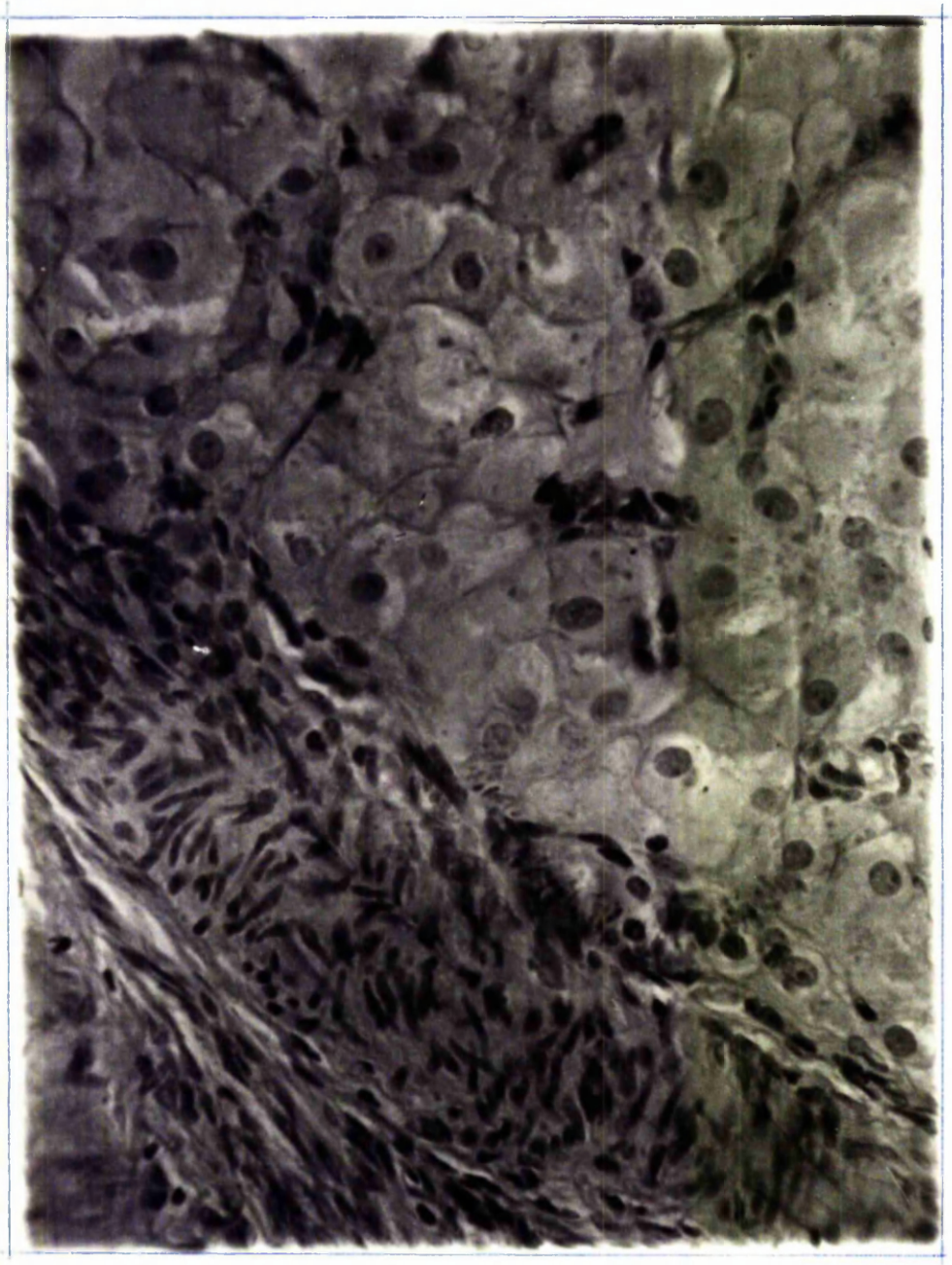


Fig. 7. Section of active adult ovary to show theca-luteinization. Note that the magnification is the same in Figs. 6 and 7. H. & E. × 840.

form a definite thecal layer, and after formation of the atrium in the follicle these thecal cells begin to show evidence of transformation to lutein tissue. This thecal activity, however, does not quite correspond to that seen in the mature functioning ovary; instead of the cells becoming hypertrophied, epithelioid or polygonal, and having a foamy cytoplasm, generally described as theca-lutein cells, they tend to remain spindle-shaped and, although enlarged, never give the appearance suggestive of true luteinization. Where cystic atresia has occurred, the theca layer shows a closer resemblance to theca-lutein cells, but the cells are still relatively small and do not show the usual foamy cytoplasm of the fully developed luteal cells (Figs. 6 and 7).

At this stage, as there did not appear to be any relation between activity and age of the ovaries, some other factor was sought. The maternal clinical histories were studied, and it was found that, in the 28 babies with

active ovaries, 21 were from mothers who had exhibited a toxaemia of pregnancy (pre-eclampsia, eclampsia, high blood pressure, accidental ante-partum haemorrhage with signs of toxaemia), 2 were miscarriages, and the remaining 5 gave maternal histories which did not seem to be relevant (external version with surgical induction, full term with intra-uterine death, foetal congenital abnormality of the bowel, maternal carcinoma of gut, foetal anencephaly with hydramnios). Of the 21 babies with apparently inactive ovaries, the maternal histories in 10 were virtually normal, 2 were miscarriages, 3 were foetal abnormalities (anencephaly), and in 6 there were histories of toxaemia (2 fulminating eclampsia, 4 mild pre-eclampsia). These findings are similar to those reported by Govan and Mukherjee (1950). To consider these findings in greater detail, attention will be given in the first instance to the active ovaries as there are no obvious changes to be found in the inactive ones.

The active ovaries in this present series fall readily into three groups, viz: (1) toxaemia, (2) miscarriages and (3) apparently normal; the sum of these groups being 28 specimens.

1. Toxaemic Group.

Twenty-one ovaries belonged to this group, and according to the degree of activity or follicular maturation observed, they may be considered under three headings.

Early activity. This includes every sign of activity in the developing follicle up to, but excluding antrum formation. It was the commonest finding in all of the ovaries, and was the only change found in 2 cases (9.5 per cent.). The number of follicles of this early type in a single ovary varied from 1 to 45, the average being 10. In addition to these early changes 19 cases (90.5 per cent.) showed more advanced activity.

Advanced activity. Under this heading are included atriated follicles. Nineteen ovaries contained follicles with well developed antra,

the number of such follicles in any single ovary varying from 1 to 36, the average being 7.

Cystic atresia. Follicles which had developed and showed signs of cystic atresia were found in 13 ovaries. In each ovary they varied from 1 to 7, the average being 3.

2. Miscarriage Group.

Two ovaries only fell into this group. In the first of these the only evidence of activity was the presence of 1 early developing follicle. In the second, 2 follicles showed evidence of early activity, and in addition this ovary contained 1 cystic atretic follicle.

3. Apparently Normal Group.

Five ovaries belonged to this group, and the findings in each were as follows. The first ovary contained 8 early active follicles, 2 follicles with antra, and 2 cystic atretic follicles. The second ovary contained 6 early active follicles and 1 each with an antrum and cystic atresia. The third ovary contained 1 early active follicle alone. The fourth ovary

Table IV.

Total Active Ovaries 28.

Follicular Change.	Toxaemic Group 21 Cases. No. of Cases	No. in Ovary. Range	Avge.	Miscarriage Group 2 Cases. No. of Cases	No. in Ovary Range	Avge.	Apparently Normal 5 Cases. No. of Cases	No. in Ovary Range	Avge.
Early activity.	21	1-45	10	2	1-2	1.5	5	1-8	4
Advanced activity.	19	1-36	7	-	-	-	3	1-2	0.8
Cystic atresia.	13	1-7	3	1	1	0.5	3	1-3	1.2
Total.	53			3			11		

contained 3 early active follicles and 3 cystic atretic follicles. The fifth ovary contained 2 early active follicles and 1 follicle with an antrum.

The detailed findings of the above groups are analysed in Table IV. From this Table it is obvious that foetal ovarian activity in all stages may be found in normal and toxaemic pregnancy, but the degree of activity tends to be greater in the presence of toxaemia. This can be seen from the columns dealing with the range and the average for each phase of the developing follicle. The position may be more clearly stated by comparing the incidence of foetal ovarian activity in toxaemic and non-toxaemic pregnancies. For this purpose it is necessary to study the whole group of 49 cases. As previously noted, 27 cases were toxaemic. In 6, or 22 per cent., no evidence of foetal ovarian activity could be found. The remaining 21 cases, or 78 per cent., showed varying degrees of follicular maturation. Twenty-two cases, 4 of them miscarriages, were

Table V.

	Toxaemic (27 cases)	Non-toxaemic (22 cases)
Inactive.	6 cases (22 per cent.)	15 cases (68 per cent.)
Early activity only.	2 cases (7.4 per cent.)	2 cases (9 per cent.)
Advanced activity.	19 cases (70 per cent.)	3 cases (14 per cent.)
Cystic atresia.	13 cases (48 per cent.)	4 cases (18 per cent.)

Fig. 8. Section of foetal ovary from inactive group, to show numerous primordial follicles, absence of activity, and absence of any reaction to the Schiff stain. P.A.S. × 185.

non-toxaemic. The foetal ovaries in 15 of this group (68 per cent.) were quite inactive, and only 32 per cent. exhibited evidence of follicular ripening. The details are shown in Table V. This Table emphasizes the fact that foetal ovarian activity is more common in toxaemic pregnancy and that the activity is of a more advanced degree.

Examination of the sections stained by the P.A.S. technique produced equally interesting results. Here again, there was adherence to the two groups mentioned above. In the 21 cases in which there was apparently no ovarian activity, all sections were Schiff negative for the cells under consideration (Fig. 8). In the 28 cases with ovarian activity, 26 showed Schiff positive material in association with follicles. The zona pellucida gave a strong positive reaction, and in addition globules lying between the granulosa cells, but apparently not contained within them, also reacted with the Schiff reagent. This appearance is similar to that found in developing follicles of mature

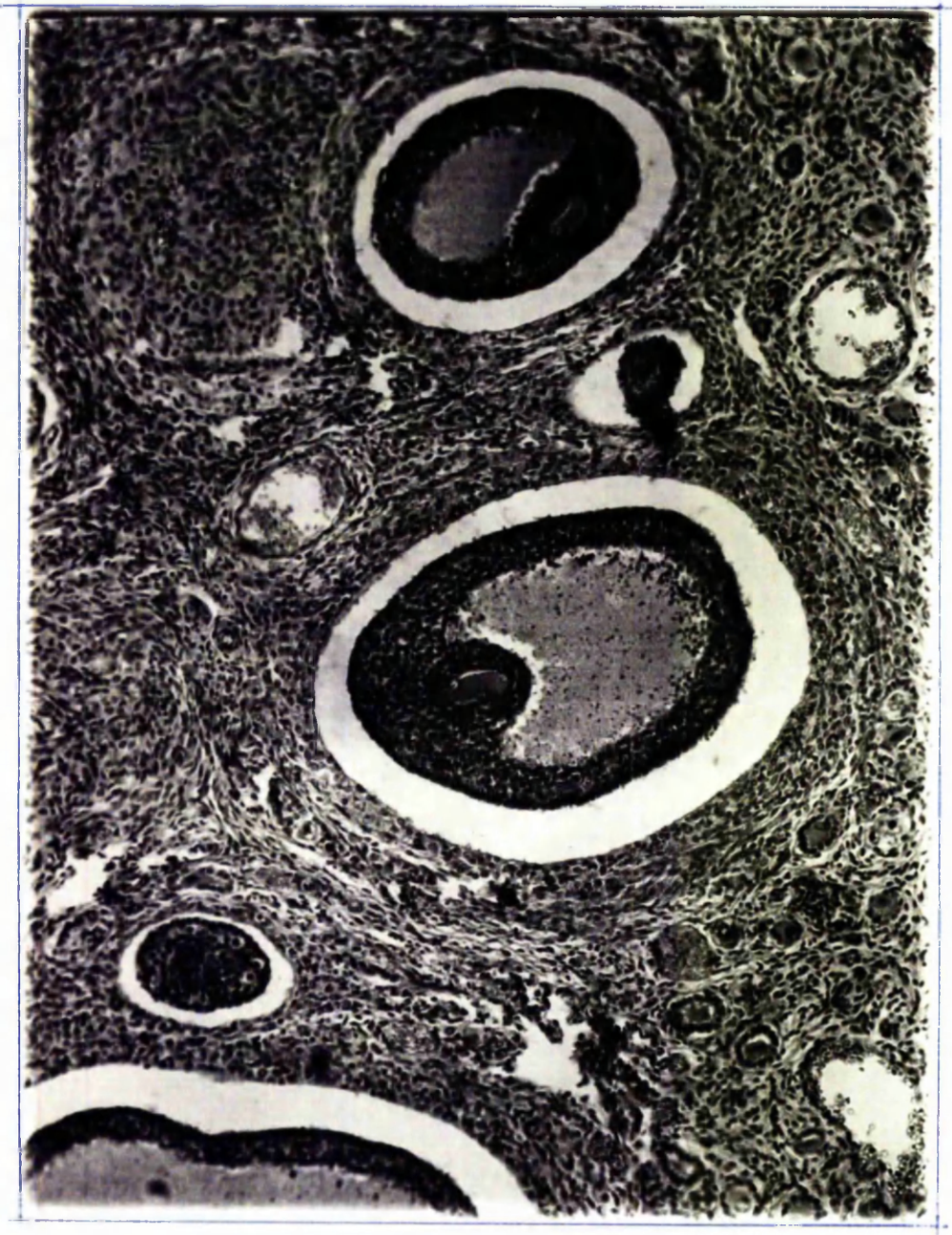


Fig. 9. Section of foetal ovary from active group to show atriated follicle with globules of Schiff positive material in the granulosa layer. P.A.S. × 185.

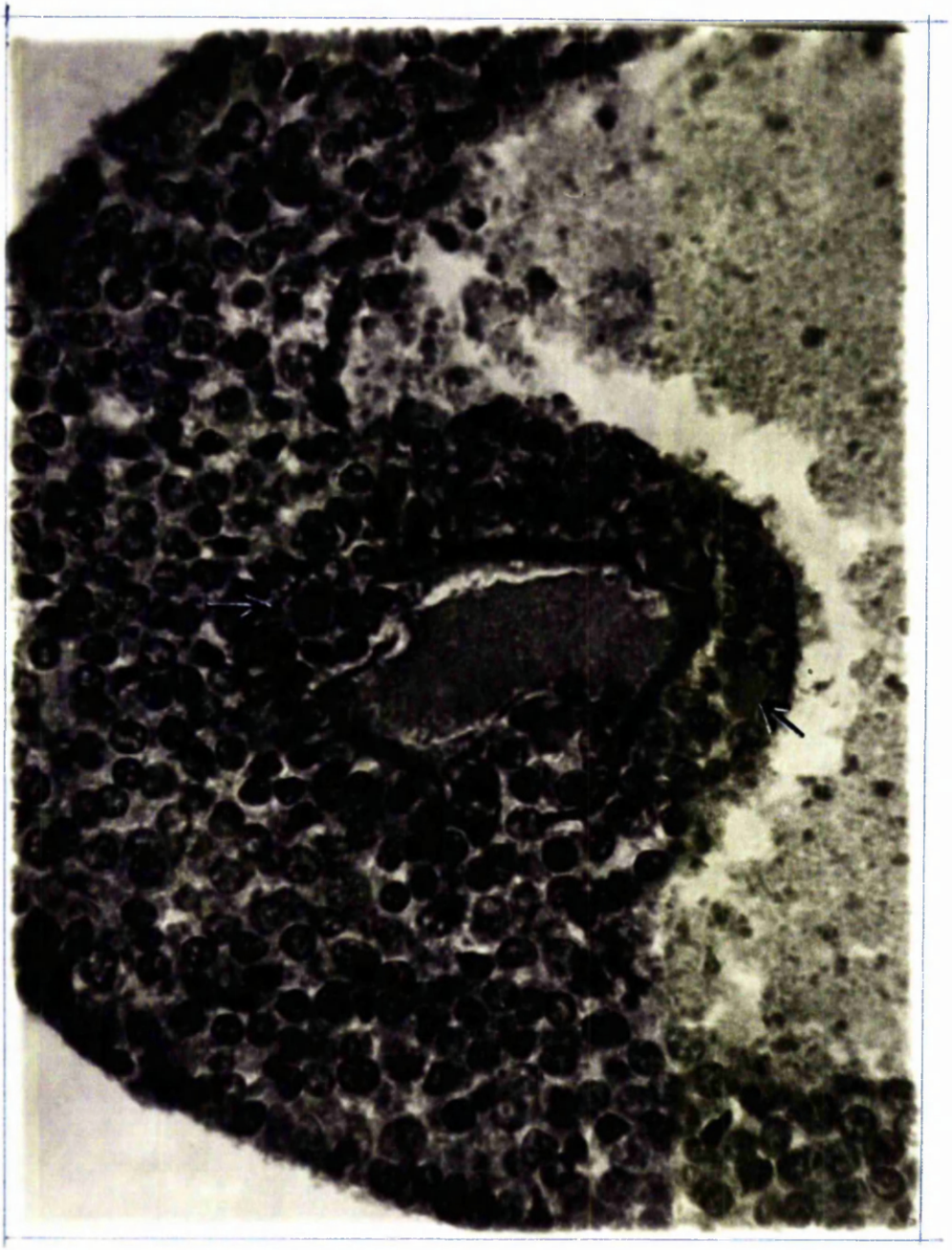


Fig. 10. High power to show cells of the discus proligerus seen in Fig. 9, to show more clearly the Schiff positive globules, two of which are arrowed. P.A.S. × 900.

active ovaries; in no instance was the P.A.S. positive material found in the form of granules, either intra- or extra-cellular, as found particularly in association with the corpus luteum and corpus albicans in the mature active ovary, and as described in Section II, page 28. It was considered possible that these globules may have been derived from break-down of the zona pellucida. However, in all the sections studied this does not seem to be the case, as the strength of the reaction and the breadth of the zona pellucida are unaltered whether Schiff positive globules are present or not in the granulosa layer.

Schiff positive globules were not found until development of the granulosa layer had reached about 6-8 cells deep; this was noted in 8 (28.6 per cent.) of the 28 cases. In 19 cases developing follicles with antra were found, and Schiff positive material was detected in the granulosa layer of 18 (64.3 per cent.) of these, being situated mainly in the region of the discus oöphorus (Figs. 9 and 10).

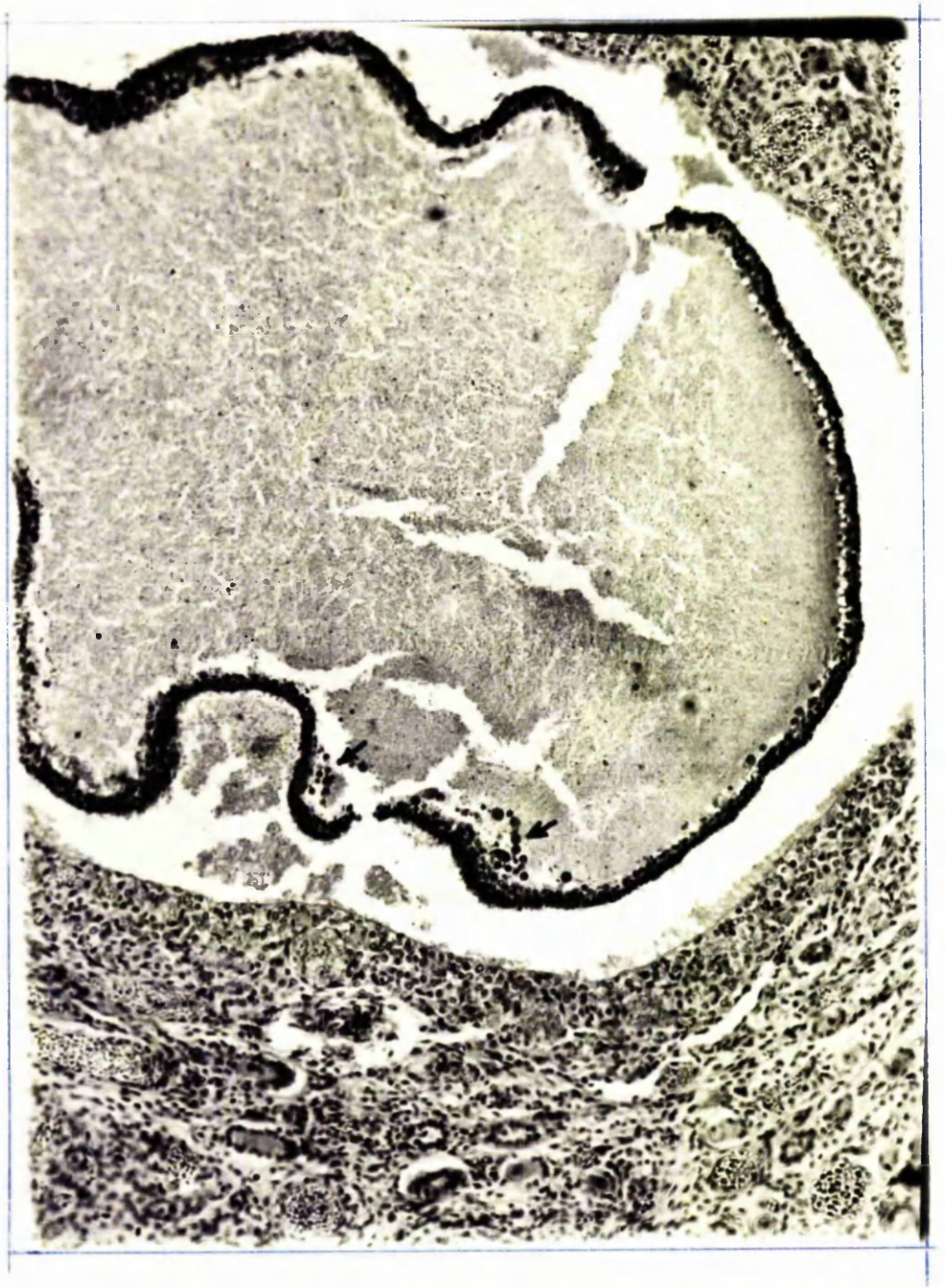


Fig. 11. Section of cystic atretic follicle to show Schiff positive globules in association, (arrowed). P.A.S. x 185.

In 13 cases there were cystic atretic follicles, in 3 of which there was evidence of Schiff positive material (Fig. 11). In short, out of the 28 cases with active ovaries, all but 2 revealed the presence of P.A.S. positive material in the follicular system: in 1 of the negative specimens the mother had suffered from pre-eclampsia, and in the other the condition had been hydramnios associated with anencephaly, pregnancy ending at 36 weeks.

Identification of this material was attempted by examining 11 sections of ovaries from an arbitrarily chosen 7 babies, the sections being stained by the P.A.S. technique after treating with acetone for 30 minutes. In all of these the Schiff reaction remained positive. Further sections from the same ovaries were stained by Sudan black alone, and it was found that the Schiff positive material was Sudan positive. Examination of these sections for birefringency was negative. These findings are similar to those observed in the mature functioning adult ovary (Section II of

this thesis).

From these results it would appear that the material found only in active foetal ovaries is of the same chemical nature as that found in the adult functioning ovary. It is to be noted, however, that the Schiff positive material is in the form of extra-cellular globules, as in the follicular system of the adult ovaries, as opposed to the positive granules contained within cells which have been found in the corpora lutea and corpora albicantia of the active adult ovaries.

COMMENTARY.

Two main facts emerge from this study of the foetal ovary. In the first instance it is obvious that follicle maturation up to the stage of commencing theca luteinization is not an uncommon finding, and secondly this follicular activity is associated with the appearance of Schiff positive material in the granulosa layer.

The follicular activity may be found in either normal or abnormal pregnancy, but the

incidence and degree of maturation is most pronounced in the presence of toxaemia. It is generally accepted that follicle maturation occurs only under the influence of gonadotrophins and usually these are of pituitary origin (Evens, Myer and Simpson, 1932). During pregnancy, and particularly toxaemic pregnancy, there is an enormous increase in the production of gonadotrophin, and according to Smith and Smith (1939, 1940, 1941) the excess in toxaemia is of placental origin. While so-called chorionic gonadotrophin will activate the ovaries of many animals, most observers agree that it has little if any effect on the human ovary (Geist, 1933; Pratt, 1933; Hamblen, 1939; Brown, Bradbury and Metzger, 1941). It is to be noted, also, that in the present series the changes in the foetal ovaries suggest the action of a follicle stimulating hormone, and that true luteinization of the follicular cells did not occur. If Smith and Smith's interpretation is correct, it seems unlikely that the increase in gonadotrophin in toxaemia

can play any part in the activity of the foetal ovaries. Accordingly, one would have to propound a primary foetal mechanism or the production of some other maternal substance which would activate the foetal pituitary. Govan and Mukherjee (1950), however, have suggested that the excess gonadotrophin found in toxaemia is in part at least of maternal pituitary origin, and that it is this substance alone which is responsible for the maturation found in the foetal ovaries. These workers have produced a certain amount of indirect evidence of pituitary hyperactivity in toxaemia of pregnancy (Mukherjee and Govan, 1950; Mukherjee and Govan, 1951; Govan et al, 1951), and more recently other workers have claimed to be able to demonstrate the origin of these gonadotrophins from the pituitary (Lajos, Szontágh and Páli, 1953a, b; Lajos, Szontágh et al., 1953; Agadzhanoff, 1953). Some of these workers have also stated that the gonadotrophins in the late stages of normal pregnancy are also partly of pituitary origin

and this would help to explain the occasional occurrence of foetal ovarian activity in an apparently normal pregnancy. The absence of activity in a few of the toxaemic cases is more difficult of explanation. In 2 cases the toxaemia was fulminating and resulted in the delivery of a macerated stillborn foetus. It is possible that these foetuses died before any manifestation of ovarian activity could develop. In a further 4 cases the toxaemic symptoms were very mild and the circulating gonadotrophin may have been insufficient to promote changes in the foetal ovaries. In any event a reasonably strong case can be made for the presence of gonadotrophins with a pituitary-like activity in these patients.

There seems little doubt that the Schiff positive material detected in these foetal ovaries, similar to that found in adult ovaries, is related to certain phases of follicular development. In the adult ovary, material giving a Schiff reaction was found in the granulosa layer of the developing follicle

and in the cells of the fully developed corpus luteum; but in the present instance the substance was only present in the form of extra-cellular globules in the granulosa layer. Whether, in the globular state, it is responsible for the activity or represents merely some product resulting from this activity is as yet a matter for conjecture. It is possible that in the adult ovary we are dealing with two entirely different substances, although the similarity of their staining reactions would suggest a close chemical relationship. Arguing on this hypothesis it is possible to suggest that the Schiff positive globules detected in the active foetal ovaries may be derived from some breakdown products, although from the findings given here it is unlikely to be from breakdown of the zona pellucida. On the other hand, the Schiff positive globules may be derived from or related to gonadotrophin, which is regarded generally as being responsible for follicular maturation. Again, it may be related to the formation of steroid substances

produced in or by the follicle. In all the ovaries in which activity was noted there was no instance of true luteinization, so that it is unlikely that this material is associated with the progestational phase and formation of the corpus luteum. In fact, as reported in Section II, in association with an active corpus luteum we find Schiff positive intracellular granules in addition to evidence of Schiff positive globules.

SECTION IV.

Histochemistry of Post-Menopausal Ovaries.

In order to give a reasonably complete picture of this study of the human ovary it seemed essential that a certain amount of investigation should be carried out on the ovaries of post-menopausal patients. By so doing it was felt that this would complete an adequate cross-section of the histochemical findings in the human ovary throughout life, although it is realised that this must only be of a general character. No doubt there are other periods in the full life cycle of the ovary, such as at puberty, in which interesting changes or variations may be found, but it was felt that on the whole these probably would not affect the general pattern or conclusions.

This section, therefore, deals with a limited study of the ovaries from post-menopausal patients.

Material.

Thirty-two ovaries obtained at operation

Table VI.

Age in Years.		Age at Menopause.		Interval between Menopause & Operation.	
Youngest	51.	Earliest	35.	Shortest	1 year.
Oldest	73.	Latest	53.	Longest	27 years.
Average	60.	Average	49.	Average	11 years.

from 20 patients were employed. To a certain extent a degree of selection was inevitable: the patients had all been operated upon for a gynaecological condition, such as fibroids, uterine carcinoma, post-menopausal bleeding; they were all post-menopausal; but beyond this the selection was a random one. The youngest patient was 51 years of age, the oldest was 73 years, and the average age was 60 years. The average age at which the menopause occurred in these patients was 49 years, the earliest menopause occurring at 35 years in 1 patient, and the latest occurring at 53 years. The shortest interval between the menopause and operation was 1 year in 1 case, the longest interval was 27 years, and the average interval was 11 years.

These figures may be tabulated as in Table VI for easy reference.

Methods.

These were similar to those employed for the foetal ovaries in Section III, sections

being stained as follows:

(i) Haematoxylin and eosin (H. & E.).

(ii) Periodic acid - Schiff (P.A.S.).

(iii) Periodic acid - Schiff after acetone.

(iv) Periodic acid - Schiff after diastase.

Results.

As in the case of the foetal ovaries, it was found that, out of the total of 32 ovaries examined, where certain changes were seen in 1 ovary of a pair, the other ovary showed similar changes, so that here again the numbers may be reduced to those of the patients, namely 20.

Of the ovaries which were studied after staining with H. & E., 11 did not show any evidence of activity, and in fact were considered to be atrophic, although in several there were corpora albicantia: this group included the patient in whom the menopause had occurred earliest, at 35 years, and also the patient in whom the interval between the menopause and operation was 1 year. A further 5 of the specimens showed small cystic or atretic follicles, associated with corpora albicantia

in otherwise atrophic ovaries. Ovaries from 2 patients contained definite cysts, 1 being multi-locular, and in a further case there was a persistent corpus luteum cyst. In the last of the 20 specimens the ovary was extensively involved in a carcinomatous process.

On examining the sections which had been stained by the P.A.S. method, it was found that of the 11 which did not show any obvious activity, all but 1 were Schiff negative: in this single exception Schiff positive material was seen in association with corpora albicantia, and this case was one of carcinoma of the uterus. Among the 5 specimens which showed atretic follicles, 2 were Schiff negative, and 3 had Schiff positive material associated with corpora albicantia, in one of which P.A.S. positive globules were seen in atretic follicles. In the cases in which the ovaries contained cysts, 1 showed Schiff positive material in relation to corpora albicantia, in the one with the multi-locular cyst the cyst lining epithelium was solidly coloured

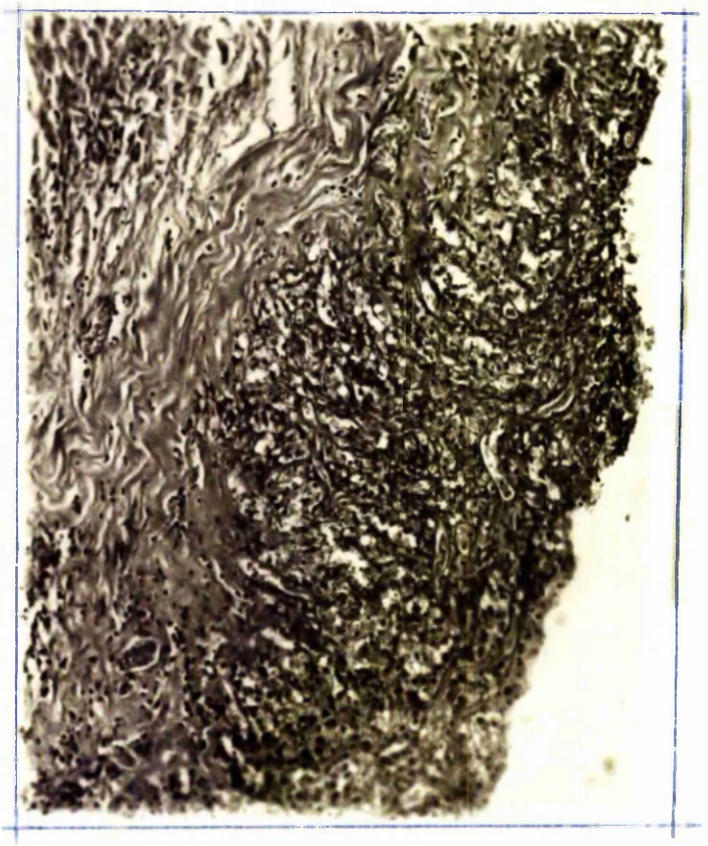


Fig. 12. Section from post-menopausal ovary in which there was a persistent corpus luteum cyst, to show the presence of Schiff positive material in the luteal lining. P.A.S. × 400.

Schiff positive but the section was otherwise negative, and in the persistent corpus luteum cyst P.A.S. positive globules were present in the luteal tissue (Fig. 12). In the ovary with carcinoma Schiff positive globules were extremely numerous and widespread throughout the malignant tissue.

Sections from the ovaries which contained P.A.S. material were studied after treating with acetone and staining by P.A.S., and after treating with diastase before staining with P.A.S., for purposes of identification of the Schiff positive material. In every case the Schiff reaction remained positive after acetone and after diastase, which suggests that the material is not glycogen and is probably identical with the similar material which has been found in the active ovaries of women in the child-bearing age.

Of the 20 cases studied, 9 showed activity histologically, and of these, 7 contained Schiff positive material. In the 2 active cases which were P.A.S. negative the

patients had respectively fibroids of the uterus, and a fibrous mass in the tubes associated with a small follicular cyst of the ovary. From the histories and case records of the 7 patients whose ovaries were active histologically, and in which the Schiff reaction was positive, the clinical conditions were:

Uterine fibroids in 4 cases,

Uterine carcinoma in 3 cases, in one of which the ovary was involved also.

In greater detail, it was found that of the 4 cases of fibroids, in 1 case the patient had had an artificial menopause induced by X-ray therapy 5 years previously at the age of 46 years; in 1 there was a persistent corpus luteum cyst; in 1 there had been a cervical polypus and also an ovarian multi-locular cyst, and in 1 there were ovarian follicular cysts.

The clinical conditions associated with the 11 apparently inactive ovaries were - 'post-menopausal bleeding' in 7; 'post-menopausal bleeding' and fibroid in 1; and uterine carcinoma in 3, in one of which

there was Schiff positive material in the ovary.

COMMENTARY.

Expressed in general terms, these results are of interest from the point of view that in some of the cases the post-menopausal ovaries have shown activity, albeit of a minor degree, which is contrary to the widely accepted concept of the menopause. Having made this statement, however, it cannot be too strongly emphasized that there are at least two important qualifications: first, the number of cases is very small, and therefore would not probably stand up to a statistical analysis; and second, all the cases are gynaecological in origin, so that the findings may not reflect a true general picture of post-menopausal ovaries.

Nevertheless, with these provisos, it seems reasonable to postulate that when comparing these post-menopausal ovaries, histochemically, with the adult and foetal ovaries, the similarity is sufficiently close to warrant the conclusion that the presence of the P.A.S. positive material indicates activity.

This conclusion is strengthened by the purely histological appearance of atretic follicles and cysts in organs which are generally regarded as totally inactive after the menopause.

With the limited number of cases under study, it cannot be stated whether this activity might be responsible for some general effect on the female organism; whether it is some general influence which is producing the activity in these ovaries; or whether the apparent ovarian activity is purely fortuitous. To speculate further, and try to find some reason why in certain cases post-menopausal ovaries show activity, will require somewhat fuller study of the results given above, and in particular of the associated conditions.

It will have been observed that in the cases which show ovarian activity, i.e. 9 in number, fibroids are associated with 6, and uterine carcinoma with 3. Apart from 2 of the fibroid cases, all of these ovaries show the presence of P.A.S. positive material.

This raises the interesting question - is there any relationship between ovarian activity and the production, or at least the presence, of uterine fibroids and of uterine carcinoma?

From time to time the hypothesis for an ovarian hormonal factor being concerned in the aetiology of uterine myomata has been advanced; but in general this has largely been discredited. Novak (1947) has concluded that the evidence for its veracity is unconvincing. Nevertheless, Nelson (1937; 1939) has been able to advance a certain amount of experimental evidence to show that the hypothesis may in fact be well-founded: as a result of experimental oestrogenic treatment he has apparently produced an interstitial nodule which resembles a myoma. Lipschütz and Vargas (1941) have also reported the production of multiple fibroids in experimental animals following prolonged administration of oestrogens.

Similarly, the relationship between oestrogens and carcinoma is frequently cited,

and as an hypothesis for oestrogens being an aetiological factor in the production of carcinoma, it is probably more generally accepted. Certainly, the results of research and experiment seem to substantiate the hypothesis to a large extent (Muir, 1936; Willis, 1948). Further, there is the possibility of a relationship between fibroids and carcinoma of the uterus (Stout, 1932).

As regards the activity found in these post-menopausal ovaries, of the 9 cases described, it is perhaps significant that P.A.S. positive material was found in 7: whereas in the 11 non-active ovaries the P.A.S. reaction was consistently negative apart from 1 case. From this, together with the evidence presented in Sections II and III, it is considered that there can be little doubt that the presence of the P.A.S. positive material is closely related to ovarian activity. Further, from the tests which have been carried out for purposes of identification, it would seem to be beyond reasonable doubt that the

P.A.S. positive material is the same in each case: the similarity in the various ovaries under the same techniques is too close to be denied.

SECTION V.

Animal Experiments.

In the previous sections of this Thesis it has been shown that, apart from positive reactions obtained in blood-vessel walls, basement membranes, etc., the presence of P.A.S. positive material in human ovaries is closely associated with activity in these organs. An attempt has been made to identify the histochemical or chemical composition of this material, and, if the techniques employed are reliable, it would appear that the substance is of a complex nature, being composed of lipoid and mucoprotein fractions, the mucoprotein moiety probably containing tryptophan. From the material and records available, and by the very nature of the tissues examined, i.e. human ovaries, it has not been possible to do more than conjecture as to the exact nature and source of this P.A.S. material. In Section II it has been suggested that the material under

consideration and found in developing follicles is unlikely to be derived from pure break-down products. Indeed, from the results given in that section and in Section III, and partly confirmed by the study of post-menopausal ovaries, it seems very likely that in the developing follicles this material is an active agent: but whether it is directly related to any initiating hormone such as pituitary gonadotrophin, or to an ovarian hormone itself, such as the so-called follicular oestrone, cannot be stated, or for that matter assumed. Nevertheless, it is interesting to recall that there is general agreement that chorionic gonadotrophin and the precursor substances of the pituitary gonadotrophins (follicle stimulating and luteinising hormones) are mucoproteins (page 17). Similarly, regarding the positive P.A.S. material found in the corpora lutea of mature active human ovaries, a definite statement cannot be made.

This being so, it seemed that probably the only way to arrive at any probable

conclusion regarding the exact nature and identification of the substance under consideration was to carry out controlled animal experiments. At the same time it is realised that any observations made as a consequence of animal experiments are not of necessity directly applicable to the human. But if a parallel can be drawn between the species it may be legitimate to draw tentative conclusions.

In the Introduction reference has been made to animal research as reported by such authors as Dawson and McCabe, 1951; Hökfelt, 1951; Rennels, 1951; and Vincent and Dornfeldt, 1948. Of these authors, only Hökfelt, and Rennels have actually reported on experimental results following injections of various hormonal preparations, the former employing gonadotrophin, and the latter employing oestradiol, stilboestrol, androgens, desoxycorticosterone, progesterone, commercially prepared human chorionic gonadotrophin (pregnancy urine), and pregnant

mare serum.

The animal experiments to be detailed in this section have been carried out on lines similar to those used by Rennels. In the first instance mature non-pregnant doe rabbits were chosen as the experimental animals. Thereafter, immature female rats were used; and following on this, immature female mice. The decision to employ these different animals was to eliminate as far as practicable the possibility of species specificity; to ensure that any findings would relate to some general phenomenon and not represent some curious artefact or some characteristic of one species. Various substances and extracts were injected, and animals were killed at regular intervals, the ovaries removed, examined with different staining techniques,, and estimates made of the activity as a result of the injections.

It will be noted throughout these experiments that among the injected substances Extracts A and B from human urine - pregnancy and non-pregnancy - are mentioned: Lorraine

and Brown (1956) have described a method of extracting crude gonadotrophic hormones from urine, and according to these authors Extract A is equivalent to Follicle Stimulating hormone, whilst Extract B represents Chorionic hormone. A saline extract was also prepared to determine if any active principle could be obtained by this means. A description of the method of extraction, based on the work of Lorraine and Brown, is given in Appendix A.

Rabbit Experiments.

Forty-two mature doe rabbits were used. Of this number 2 were taken as controls, the remaining 40 being divided into 8 groups each of 5 animals. In every instance laparotomy under ether anaesthesia was performed to ascertain the state of the ovaries and ensure that each animal was non-pregnant. Thereafter, the animals in each of the 8 groups were injected intravenously with the substance chosen for that group, the injection being made into a vein in the ear.

Injected Substances. Control animals

were not injected at all.

Group I animals were injected with the urine of known pregnant women - 15 ml. being the dose.

Group II animals were injected with commercial "Pregnyl", 1 ml. (1,500 units) being used.

Group III animals were injected with an Extract A of gonadotrophin made from the urine of known non-pregnant women, 1 ml. of the extract being used.

Group IV animals were injected with 1 ml. of an Extract B of gonadotrophin prepared from the urine of known non-pregnant women.

Group V animals were injected with 1 ml. of a saline extract of gonadotrophin prepared from the urine of known non-pregnant women.

Group VI animals were injected with 1 ml. of an Extract A of gonadotrophin prepared from the urine of a known pregnant woman.

Group VII animals were injected with 1 ml. of an Extract B of gonadotrophin prepared from the urine of known pregnant patients.

Group VIII animals were injected with 1 ml. of a saline extract of gonadotrophin prepared from the urine of known pregnant patients.

All injections in each group were made at the same time.

The control animals were killed at the time of laparotomy, the ovaries removed, fixed in formol corrosive, blocked in paraffin, sections cut, mounted, and stained with H. & E., and series sections stained with P.A.S..

In each of the groups, following laparotomy and injection, the first animal was killed 48 hours following injection, the second at 7 days, the third at 14 days, the fourth at 21 days, and the fifth at 28 days. At the time of killing the ovaries were examined macroscopically, removed, fixed, blocked, sectioned and stained in a manner identical to that used for the controls. Thereafter, all sections were examined microscopically, with particular interest to the question of activity and the presence of P.A.S. material.

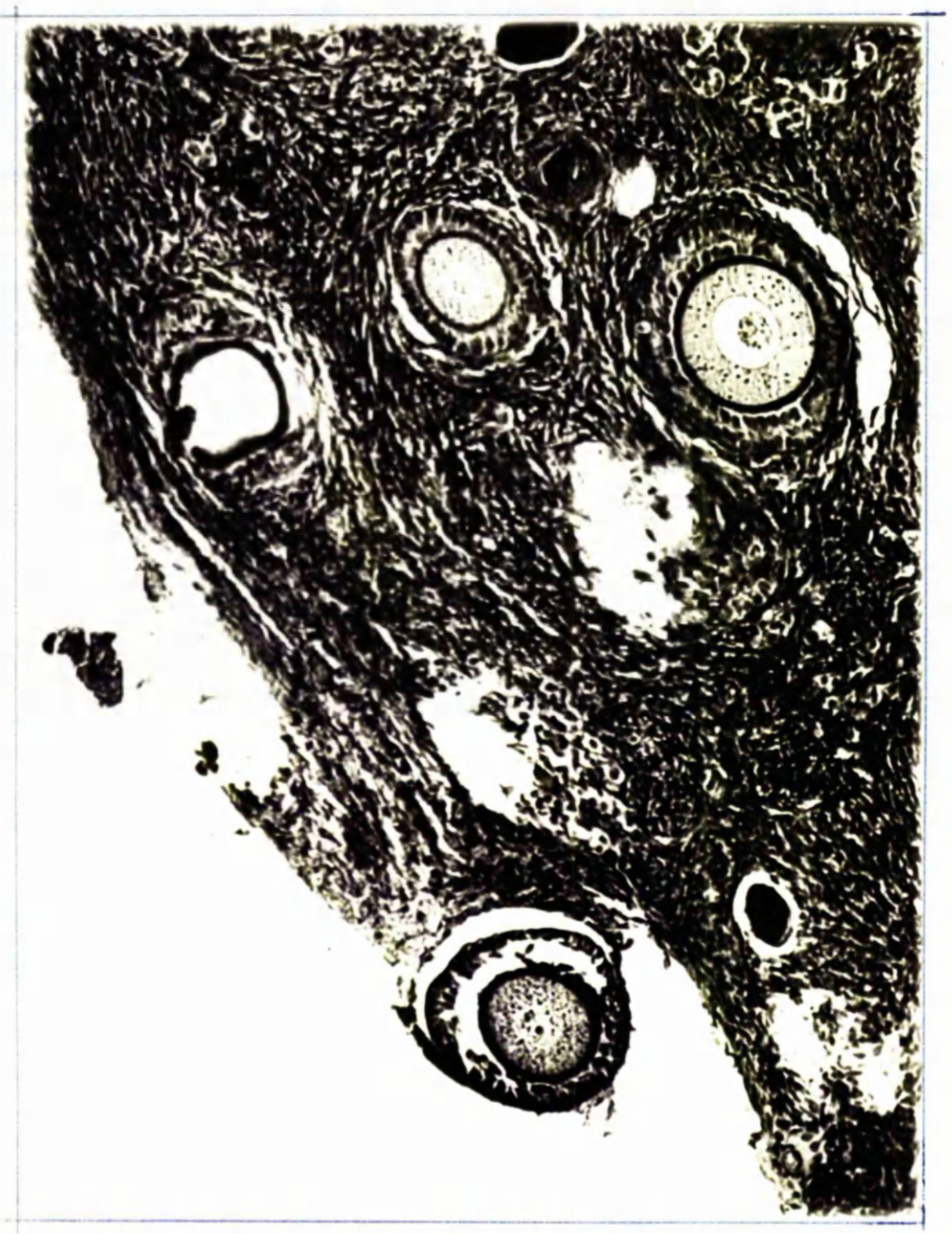


Fig. 13. Section of ovary of control rabbit to show small developing follicles : virtually Schiff negative. P.A.S. × 650.

Results.

Controls. The ovaries in each of the control animals appeared to be normal in size, without vascular injection or congestion, without obvious follicles, and were classed as inactive.

Stained sections revealed the presence of a narrow cortical rim of interstitial tissue which contained several small follicles in different stages of development (Fig. 13): the Schiff reaction was virtually negative - this being understood as negative for the P.A.S. material under consideration in this Thesis, although blood-vessel walls, etc., gave a positive reaction.

Group I. (Pregnant Urine).

Animal (1), (48 hours). Macroscopic examination revealed the ovaries to be about normal in size and appearance, but with the presence of blood-follicles, which were regarded as indicating activity.

Microscopic examination revealed that most of the ovarian stroma appeared "luteal" in form,

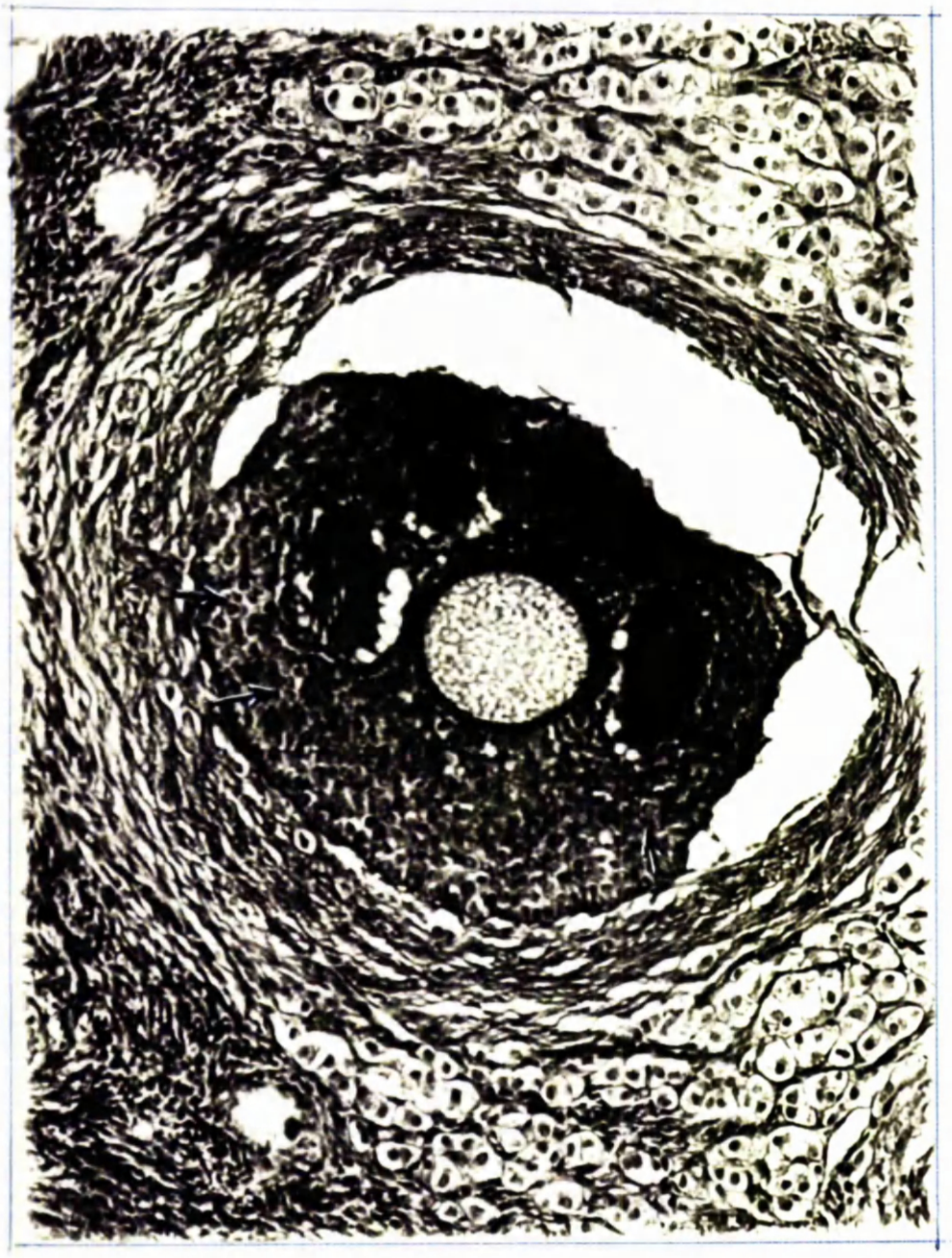


Fig. 14. Section of rabbit ovary to show occasional Schiff positive cells in the granulosa layer of an early atriated follicle, (arrowed). (Human pregnancy urine injected.) P.A.S. × 650.

and containing one or two large follicles which gave the picture of early atresia. In addition there were one or two early developing follicles, but none of these had reached the stage of antrum formation. In these ovaries the Schiff reaction was virtually negative.

Animal (2), (7 days). Macroscopically these ovaries were similar to those of animal (1) above, but blood follicles were more numerous. This animal also was considered to have active ovaries.

Microscopically the general appearance of the ovaries seemed to indicate a further degree of activity over that found in animal (1); there were one or two atretic follicles, several early developing follicles, and one atriated follicle. Study of the sections which were stained by the P.A.S. technique revealed what appeared to be occasional P.A.S. positive cells in the granulosa layer of the early atriated follicle (Fig. 14).

Animal (3), (14 days). When examined macroscopically these ovaries did not contain

any blood follicles, but did in fact contain what appeared to be one large corpus luteum.

Microscopic examination revealed much evidence of luteal tissue - apparently a corpus luteum - in addition to a few early developing follicles. With the Schiff reaction it was found that there were definite P.A.S. positive granules in the corpus luteum. The granulosa cells of the developing follicles were negative for P.A.S. material.

Animal (4), (21 days). These ovaries appeared to be inactive; there were no blood follicles and little evidence of follicular activity: it was noted, however, on macroscopic examination, that the surfaces of the ovaries were very granular, giving them the appearance of white raspberries.

Microscopic examination showed that the ovaries were very similar to those of animal (3) above: the luteal tissue, however, appeared to be somewhat older and contained more connective tissue. The Schiff reaction, also was less marked, there being fewer positive

cells.

Animal (5), (28 days). These ovaries looked large, but otherwise appeared normal and inactive.

Microscopic examination showed very old-looking luteal tissue, with only a very few cells containing weakly positive P.A.S. reacting granules. There was quite a number of large P.A.S. positive globules. On the whole these ovaries were quite similar to those of the control animals.

Summary of these Results.

As a result of injecting the urine of known pregnant women into these non-pregnant rabbits, the inactive ovaries were stimulated into activity - this being the basis of the Friedmann pregnancy test. Within 48 hours blood follicles were produced, and this reaction seemed to be increased up to 7 days. Thereafter, the ovaries gradually reverted to an apparently inactive state by the 28th day following injection. Microscopic examination seems to confirm this sequence of events:

from an apparently dormant state follicular maturation was produced, and by the 14th day corpora lutea were produced. From the 14th day to the 28th day there was then a gradual regression of these changes until the ovaries resumed the apparently inactive, quiescent state. Histochemical study of these ovaries seems to confirm the observations made on the human ovaries that the presence of the Schiff positive material in the ovaries is intimately associated with activity. From negative reactions in the control and early active ovaries, by 7 days, when early atriated follicles were found, P.A.S. positive cells begin to appear in the granulosa layer. By the 14th day there were only early developing follicles noted, and in these the granulosa layer was P.A.S. negative; but there was then a well-developed corpus luteum which did in fact contain P.A.S. positive cells. From then until the 28th day the Schiff reaction again became negative - apart from some rather large positive globules or

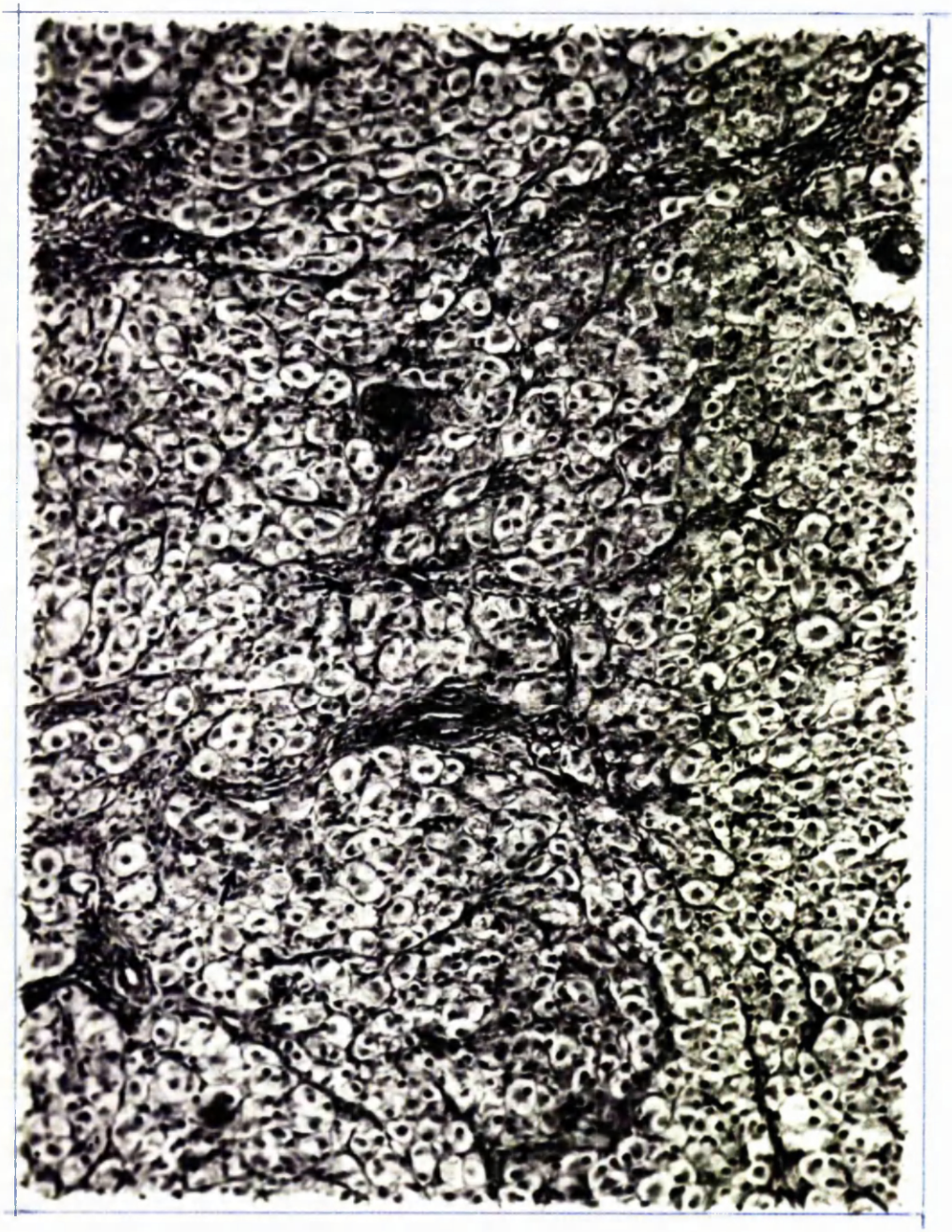


Fig. 15. Section of animal ovary to show the presence of Schiff positive cells in a corpus luteum, (arrowed). (Pregnyl injected.) P.A.S. x 450.

cells, which were so large that they were considered as probably being phagocytes.

Group II. ('Pregnyl').

Animal (1), (48 hours). From the first animal it seemed obvious that the 'Pregnyl' was a much more potent substance than the human pregnancy urine. The ovaries of this animal contained numerous blood follicles, and it was considered as being strongly positive.

Microscopic examination showed the ovarian tissue to be made up largely of fresh corpus luteum. A rim of cortical stroma contained a few early developing follicles; in those in which the granulosa was 10-12 cells deep an occasional P.A.S. positive cell was seen in the granulosa. In the corpus luteum quite a large number of P.A.S. positive cells was noted, particularly in close proximity to the connective tissue septa. In many instances these positive cells had their nuclei displaced towards the periphery. (Fig.15).

Animal (2), (7 days). In all respects the ovaries of this animal appeared to be very

similar to those of animal (1) above. Many blood follicles were seen, and the ovaries were regarded as positive. The microscopic appearances also were almost identical with those of the first animal, and the observations made above apply here.

Animal (3), (14 days). These ovaries were large and pale, and seemed to contain numerous corpora lutea. On microscopic examination the histology was similar to the previous ovaries - much evidence of luteal tissue, and a few developing follicles. However, apart from some P.A.S. positive cells in the follicles of 10-cell deep granulosa layers, these ovaries were virtually negative for the Schiff positive material.

Animal (4), (21 days). Macroscopically these ovaries were very pale, and were hard to the touch, with numerous pale coloured tubercles - regarded as probably corpora lutea. Microscopically these ovaries closely resembled those of animals (1) and (2) above: there were several developing follicles, all the

larger of which contained P.A.S. positive material in the granulosa layers: fresh corpus luteum tissue also contained much P.A.S. positive material.

Animal (5), (28 days). Here again, the ovaries were very similar to those of animal (4) above. The most notable feature was the presence of P.A.S. positive granules, cells, and globules in the corpus luteum, some areas of which were degenerating.

Summary of these Results.

These results are in all ways similar to those obtained in the Group I animals, but the reactions seemed to be more rapid, and stronger. The impression is that 'Pregnyl' is a more potent substance than human pregnancy urine, and produces stronger reactions more rapidly, and the effect seems to last longer. Beyond this, it will be noted that these results would appear to confirm the findings in the previous animal experiment; and together, further confirmation is given, apparently, for the hypothesis indicated by the

results obtained on the human ovaries regarding the association of the Schiff positive material with ovarian activity.

Group III. (Gonadotrophin - Extract A - Non-Pregnant urine).

Animal (1), (48 hours). On inspection the ovaries were small and apparently inactive. Microscopically the most obvious and striking feature was the presence of an old corpus luteum which was in an advanced stage of organisation and widely invaded by ingrowth of connective tissue from the stroma. In one or two places in this corpus luteum there were large, open, clear cells peppered with fine P.A.S. positive granules. The stroma at the periphery of the corpus luteum also contained cells in which there were P.A.S. positive granules. One or two early developing follicles were present in the cortical stroma, but these were negative for the P.A.S. material.

Animal (2), (7 days). Both in macroscopic and microscopic appearance these ovaries resembled in all respects those of animal (1)

above. There was, however, less evidence of the P.A.S. positive material.

Animal (3), (14 days). As before, these ovaries were small and seemed to be inactive. Microscopically there was no evidence of luteal tissue. Numerous developing follicles were seen in different but early stages of maturation. Throughout, the P.A.S. reaction was virtually negative.

Animal (4), (21 days). In all respects these ovaries were fundamentally identical to those of animal (3) above. If any difference, it was macroscopic, in so far as animal (4) ovaries were minute in size.

Animal (5), (28 days). Here again, the ovaries of this animal were essentially identical to those of animals (3) and (4) above.

Summary of these Results.

It would appear that the gonadotrophic extract A made from the urine of non-pregnant women does not contain any factor which will stimulate true activity in the ovaries of the experimental animals. In the first 2 animals,

the presence of corpora lutea could hardly be ascribed to the action of the injected experimental substance; the corpora were too obviously old, degenerated, and well infiltrated with connective tissue. It is probable that the large cells containing P.A.S. positive granules were phagocytes. Apart from this the ovaries of the entire series of animals were essentially inactive and free from any evidence of the P.A.S. positive material under consideration. A different explanation is, of course, possible - it may well be that the extract failed to produce signs of activity simply because it did not contain any active principal due to some fault in the extractive technique.

Group IV. (Gonadotrophin - Extract B - Non-Pregnant Urine).

Animal (1), (48 hours). At laparotomy it was noted that these ovaries were small and apparently inactive. Microscopic examination revealed a picture which resembled that seen in the ovaries of animal (1) in the previous

group: organisation of old corpus luteum tissue was quite advanced. There was practically no evidence of P.A.S. positive material.

Animal (2), (7 days). The ovaries of this animal were small and, apart from one or two small follicles on the surface, seemed to be inactive. Microscopic examination revealed that there was practically no evidence of luteal tissue. In the stroma there were several follicles in different stages of development, most of which showed some slight evidence of P.A.S. positive material in the granulosa layer. Apart from this the ovaries were virtually negative to the Schiff reaction.

Animal (3), (14 days). As in animal (1) above these ovaries were small and inactive on macroscopic examination. Microscopy revealed the presence of some luteal tissue which, however, was markedly invaded by connective tissue and well-organised. Two cells only were found to contain P.A.S. positive granules, and these cells were situated in the connective

tissue invading the luteal body. Otherwise the ovaries were Schiff negative.

Animal (4), (21 days). On inspection these ovaries were considered to be inactive. The microscopic appearance was similar to that noted for animal (2) above - viz., mainly stroma with developing follicles at different stages; there seemed to be some evidence of P.A.S. positive cells in the stroma.

Animal (5), (28 days). In all respects these ovaries were similar to those of animal (2) above - viz. - small, inactive ovaries; little evidence of luteal tissue; a few early follicles in different stages of development. There was no evidence of any P.A.S. positive material.

Summary of these Results.

Probably the most notable feature in this group is the fact that the ovaries were all small and apparently inactive. The mere presence of luteal tissue in some of the ovaries is probably of little significance, especially as it was so markedly organised. Further, the

presence of so few P.A.S. positive cells in these ovaries, and in particular in association with the follicular system, is probably more fortuitous than due to any influence of the injected substance. In short, the overall impression is that the gonadotrophic extract B from non-pregnant urine has little if any effect on the ovaries of the experimental animals.

Group V. (Gonadotrophin - Saline Extract. Non-Pregnant Urine).

Animal (1), (48 hours). These ovaries were small and inactive. There was little of note on microscopic examination: the stroma was dense, and there were one or two relatively early developing follicles. Further, the ovaries were virtually negative to the Schiff reaction.

Animal (2), (7 days). Almost identical to the ovaries of animal (1) above, both macroscopically and microscopically, these ovaries were virtually P.A.S. negative.

Animal (3), (14 days). Gross examination

of these ovaries showed them to be small and inactive. Microscopic examination revealed them to be largely made up of well-advanced organising old luteal tissue. Occasionally through this tissue there were a few P.A.S. positive cells, and one or two small areas showing faint dusting with P.A.S. positive granules. Apart from this there was little else of note.

Animal (4), (21 days). These ovaries appeared normal and inactive. Stained sections were similar to those of animal (1) above. Negative.

Animal (5), (28 days). Here again, both on macroscopic and microscopic examination, these ovaries were almost identical to those of animal (1) above. Negative.

Summary of these Results.

Fundamentally, the ovaries of this group are small and inactive. Any evidence of activity which is present would seem to be the result of stimulation which had occurred prior to the initiation of the current experiment:

it appeared to be old-standing. Apart from those of animal (3), all the ovaries were negative for the P.A.S. material under consideration: in this one exception, the presence of the Schiff positive material was slight and may have been degenerative in origin as it was found in a well-advanced organising corpus luteum. It seems probable, therefore, that the injected substance did not have any effect on the ovaries.

Group VI. (Gonadotrophin - Extract A - Pregnancy Urine).

Animal (1), (48 hours). The ovaries of this animal were large, and both showed evidence of activity in so far as they contained obvious follicles, of which 2 in one ovary (right) were blood follicles. Surprisingly enough, the microscopic appearances were disappointing in comparison to the macroscopic findings: there was little of note apart from the large Graafian follicles and, in one ovary, a small organising corpus luteum. The P.A.S. reaction was virtually negative.

Animal (2), (7 days). These ovaries also were enlarged and gave the impression that they were active, although blood follicles were not seen. There did appear to be numerous corpora lutea. Microscopic examination confirmed that these ovaries were active. In each ovary there were several large follicles and 3 recently formed corpora lutea. In each of the luteal bodies there was evidence of the P.A.S. positive material, and in all of the follicles there was also P.A.S. positive material which was confined to the granulosa layer.

Animal (3), (14 days). On macroscopic examination these ovaries were small, apparently normal, and inactive. Microscopic examination, however, revealed them to be quite active. There were follicles in all stages of development right up to the late stages of maturation, and there were also early, recently formed corpora lutea. Schiff positive material was present in the most mature follicles and in the corpora lutea.

Animal (4), (21 days). These ovaries, although larger, were in other respects similar to those of animal (3) above. There was strong evidence of activity, many of the follicles were large and mature, and there were one or two quite large, recently formed corpora lutea. There was much more evidence of the presence of the P.A.S. positive material - sometimes globular in form - in the granulosa layers of the follicles, and in the corpora lutea, where it was both granular and globular in form. There was an occasional P.A.S. positive cell in the stroma.

Animal (5), (28 days). In all respects these ovaries were almost identical with those of animal (4) above. On gross examination they were of about the same size and appearance. On microscopic examination the sections were also closely similar to those of the previous animal, and indeed there was even more evidence of the P.A.S. positive material in association with the follicles and corpora lutea.

Summary of these Results.

This group, which represents the results of injecting gonadotrophin - extract A - from pregnancy urine, reveals that the injected substance contains some factor which is capable of initiating ovarian activity in the experimental animals. The follicular system is stimulated to growth and maturation, up to the point of rupture of the follicle, to be followed by corpus luteum formation. Throughout these stages of maturation there is increasing evidence of the presence of the P.A.S. positive material under consideration.

Group VII. (Gonadotrophin - Extract B - Pregnancy Urine).

Animal (1), (48 hours). At the original laparotomy, prior to injection, it was especially noted that these ovaries were very small. Now, 48 hours following injection, they were seen to be greatly enlarged, very congested and injected, and contained several blood-follicles. The microscopic appearances were similar to those of the ovaries of animal (1) in Group VI,

viz. - there were several developing follicles, some of which were mature Graafian follicles, and there was some organising luteal tissue: in this animal, however, there was evidence of P.A.S. positive intra-cellular granules in the old corpus luteum and in the stroma.

Animal (2), (7 days). These ovaries also were large and appeared to be active; both contained blood-follicles. On microscopic examination, apart from the presence of one or two mature follicles, there was little of note to indicate great activity. The P.A.S. positive reaction was virtually negative.

Animal (3), (14 days). The ovaries of this animal were quite large and embossed, and there seemed to be great numbers of corpora lutea. However, on microscopic examination they did not appear to be very active. There were one or two large follicles, including 2 blood-follicles in which there was faint evidence of P.A.S. positive material. Other than this there was little of note.

Animal (4), (21 days). Again, these ovaries were quite large and embossed. The microscopic findings were similar to those described above for animal (3), but with the addition of fresh corpus luteum tissue, in which there was evidence of a fine suspension of P.A.S. positive granules.

Animal (5), (28 days). The ovaries of this animal were large, markedly injected and congested. On microscopic examination they appeared more truly active than any of the previous ovaries in this group: they contained follicles in different stages of development, and one or two corpora lutea. There was more evidence of the presence of P.A.S. positive material in the stroma, the corpora lutea, and slight evidence of the Schiff positive material in the granulosa layers of the developing follicles.

Summary of these Results.

In this group the macroscopic appearances consistently suggested activity, but the microscopic picture was variable and inconstant.

There seems little doubt that the injected substance did in fact promote follicular maturation, but it was only in the later animals (4 and 5) that there was any real evidence of corpus luteum formation. The presence or otherwise of the P.A.S. positive material in these ovaries has been found to be too bizarre and inconsistent to warrant any observations.

Group VIII. (Gonadotrophin - Saline Extract - Pregnancy Urine).

Animal (1), (48 hours). The ovaries of this animal were average-sized, normal looking, and apparently inactive. Microscopic examination revealed them to be completely inactive, and negative for the Schiff material.

Animal (2), (7 days). These ovaries also were normal and inactive in appearance. Microscopy revealed the presence of a few follicles, some with antra, but otherwise little of note. Apart from one or two large, possibly phagocytic, cells which contained P.A.S. positive granules, and present in the stroma,

the Schiff reaction was negative.

Animal (3), (14 days). On gross examination these ovaries were small and apparently inactive. Examined by the microscope, the ovaries were seen to contain numerous follicles in all stages of development: in those which were atriated and in which the granulosa layer was about 5 or 6 cells deep or more, there was some evidence of P.A.S. positive cells in the granulosa.

Animal (4), (21 days). In many ways these ovaries were similar to those of the previous animal (3): they were small and apparently inactive. Microscopically they were less active, there were fewer elements, and the stroma was dense. Likewise, there was much less evidence of any P.A.S. positive material.

Animal (5), (28 days). These ovaries also resembled those of animal (3) above in general, but there was still less evidence of activity, and practically no evidence of the

P.A.S. material.

Summary of these Results.

Throughout this group there is a fairly close resemblance between all the ovaries, and on the whole these are inactive, although there is some slight evidence of the injected substance perhaps having stimulated a minor degree of follicular activity as seen in the animal killed at 14 days; after which there is steady diminution of activity - it being noted that there was never any marked degree of activity even when at its greatest in animal (3).

Rat Experiments.

The technique in these animal experiments followed the general pattern of that employed for the Rabbit experiments. All the rats employed were immature, and each weighed 70 gms. For controls, 3 animals were employed and for each of 8 groups for injection, 3 animals were used, so that the total was 24 animals plus 3 controls. The animals were killed at 48 hours, 5 days, and 10 days after injection.

In addition to these experimental animals, 2 mature pregnant rats were selected at random and killed within 30 minutes of ratting, laparotomy performed, and the ovaries removed for sectioning.

Injected Substances. Control animals were not injected at all.

Group I animals were injected with pooled urine from known pregnant women: the 3 rats in this group had 10 ml. of pooled pregnancy urine injected into the peritoneal cavity. In order to prevent, as far as possible, this quantity of urine being too great or too toxic on a single injection, it was administered in a divided dose - 4 ml. on the first day, and 6 ml. on the day following, each of these being divided into a morning and an afternoon dose.

Group II animals were treated and injected in the same way, but the substance used here was pooled urine from known non-pregnant women.

Group III animals were injected with

an extract A of gonadotrophin made from the urine of known non-pregnant women, 1 ml. of the extract being used and administered as a single intra-peritoneal dose.

Group IV animals were injected with a single dose of 1 ml. of an extract B of gonadotrophin prepared from pooled urine of known non-pregnant women.

Group V animals were injected with a single dose of 1 ml. of a saline extract of gonadotrophin prepared from the urine of known non-pregnant women.

Group VI animals were injected with 1 ml. of an extract A of gonadotrophin prepared from the urine of known pregnant women.

Group VII animals were injected with 1 ml. of an extract B of gonadotrophin prepared from the urine of known pregnant women.

Group VIII animals were injected with 1 ml. of a saline extract of gonadotrophin prepared from the urine of known pregnant patients.

All injections in each group were made

at the same time.

The control animals were killed at the same time as the animals from Group I, the first animal from each group being killed 48 hours after injection. Thereafter, as stated above, in each group the second and third animals were killed 5 and 10 days respectively after injection.

At the time of killing the ovaries were examined macroscopically, removed, weighed, fixed in formol-corrosive, blocked in paraffin, sectioned, mounted, and stained according to the P.A.S. technique. The sections were then examined microscopically.

Results.

Controls. Although none of these animals had received any injection, they were killed at intervals corresponding to that for the injected animals, i.e. the second animal was killed 3 days after the first, and the third animal 5 days later: whether this had any bearing, from a growth and development aspect, on the weights of the ovaries, cannot be stated, but it was of interest to note that the ovarian weights did

in fact increase with the longer life of the second and third animals.

Control (1). The ovaries appeared to be inactive. After removal, the right ovary weighed 20 mgm. and the left ovary weighed 40 mgm. giving an average of 30 mgm. Microscopy revealed that in each ovary there were several early developing follicles: the granulosa layer showed development, and in some of the larger follicles there was a definite theca layer which, however, was dense and did not reveal any evidence of theca luteinisation. All sections were virtually negative for the P.A.S. material.

Control (2). In one ovary - the left - a small serous follicle was noted before removal of the organs. After excision, both ovaries weighed 35 mgm. which is 5 mgm. above the average for the first control. Microscopic examination revealed the ovaries to be very similar to those of control (1), the only point of difference being rather equivocal theca - luteinisation. The Schiff reaction was

virtually negative.

Control (3). These ovaries appeared quite inactive, and when removed, they were found to weigh 45 mgm. and 70 mgm., the average being 57 mgm: this is a considerable increase over the previous control ovaries. Microscopic examination revealed a picture which was almost identical to that of control (1). The P.A.S. reaction was virtually negative.

Summary of these Results.

The ovaries of each of the control animals appeared to be inactive, yet, on sectioning and examining under the microscope, they were all found to contain small early developing follicles. Throughout, the P.A.S. reaction was virtually negative. An interesting point was the progressive increase in weight of the ovaries with the longer life of the animal.

Group I. (Pooled Pregnancy Urine).

Animal (1). At laparotomy the ovaries of this animal were very injected, and seemed to be active, with the presence of apparent follicles - none of which were blood follicles.

The ovaries weighed 50 mgm. each. Microscopic examination revealed obviously active ovaries - there were several large follicles with signs of early luteinisation of the theca. The P.A.S. reaction was virtually negative.

Animal (2). These ovaries also appeared to be active, with small clear follicles and moderate injection. The weight of the ovaries was rather surprising - each weighed only 20 mgm. On microscopic examination, activity was confirmed by the presence of a few follicles and what appeared to be several corpora lutea. The P.A.S. reaction was virtually negative.

Animal (3). In all respects these ovaries were very similar to those of animal (2) above. The ovaries seemed active, and each weighed 20 mgm. Microscopy confirmed activity, there being follicles in different stages of development, and in addition there were corpora lutea. The P.A.S. reaction was virtually negative.

Summary of these Results.

The outstanding features of interest with

regard to this group were, first, the variation in weight of the first animal as compared to the second and third; second, the anatomical evidence of the presence of activity in all the ovaries; and third, the fact that throughout this group the P.A.S. reaction remained virtually negative.

Group II. (Pooled Non-Pregnancy Urine).

Animal (1). These ovaries appeared to be inactive, and each weighed 10 mgm. Microscopically one or two small, early follicles were noted in a rather loose stroma. The P.A.S. reaction was virtually negative.

Animal (2). The ovaries appeared to be inactive, and each weighed a fraction under 10 mgm. On microscopic examination there was no evidence of activity, and the P.A.S. reaction was virtually negative.

Animal (3). These ovaries also seemed to be inactive. Each weighed a fraction under 10 mgm. Microscopically they also appeared inactive, and the P.A.S. reaction was virtually negative.

Summary of these Results.

The results given above seem to be consistent: the weight of the ovaries, to all intents and purposes, do not show any variation, and the macroscopic and microscopic appearances, together with the negative P.A.S. reaction, seem to demonstrate that the intra-peritoneal injection of non-pregnancy urine does not have any effect in promoting activity in the ovaries.

Group III. (Gonadotrophic Extract A from Non-Pregnancy Urine).

Animal (1). The ovaries from this animal appeared to be inactive: they weighed 10 mgm. and 50 mgm., the average being 30 mgm. On microscopic examination the ovaries bore quite a close similarity to those of the control animals - there were a few early developing follicles, and the P.A.S. reaction was virtually negative.

Animal (2). In all respects these ovaries were quite similar to those of animal (1) above: they appeared to be inactive; they weighed 35 mgm. and 15 mgm., the average being 25 mgm:

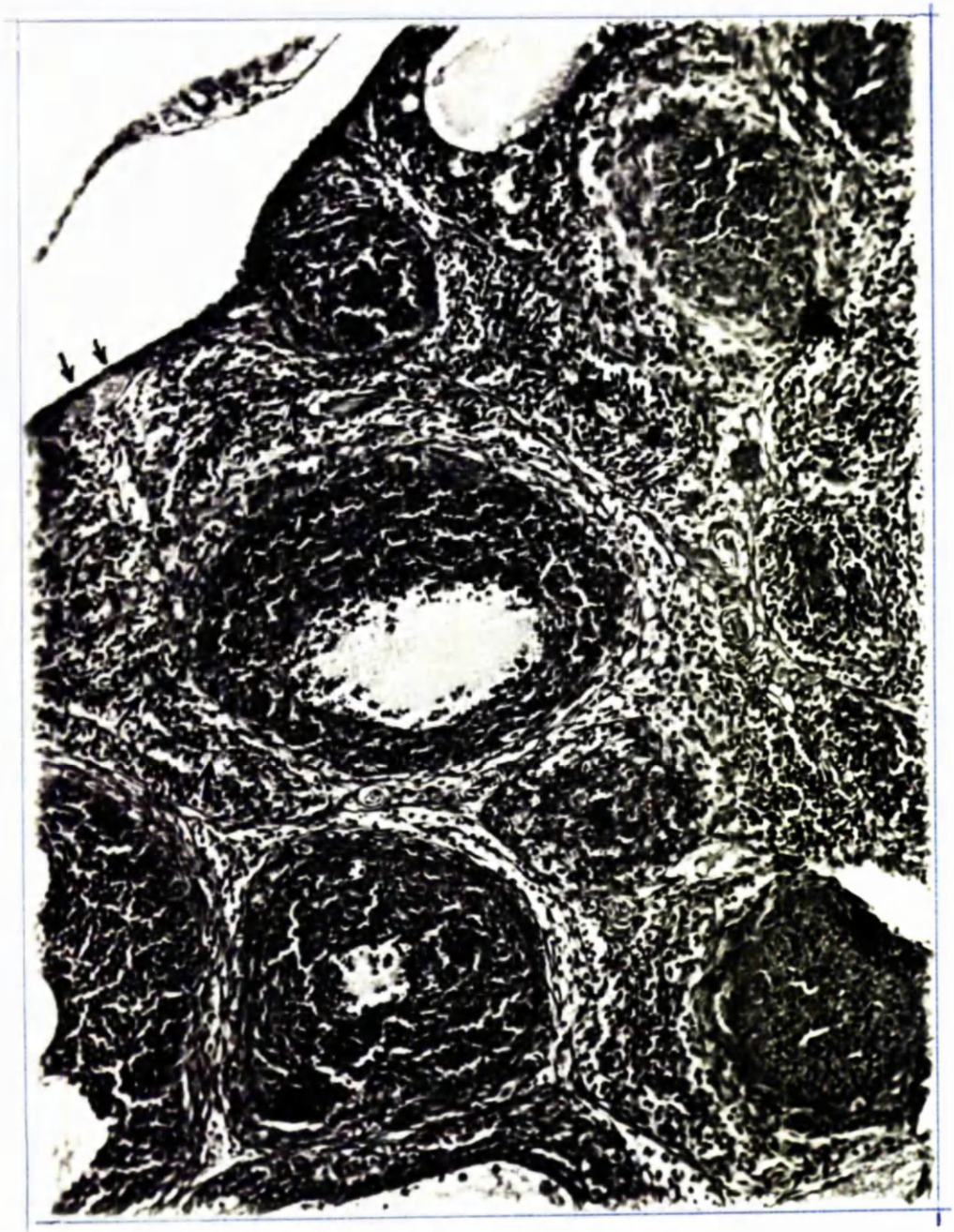


Fig. 16. Section of rat ovary to show an occasional Schiff positive cell in the stroma close to follicles, (arrowed). P.A.S. × 450.

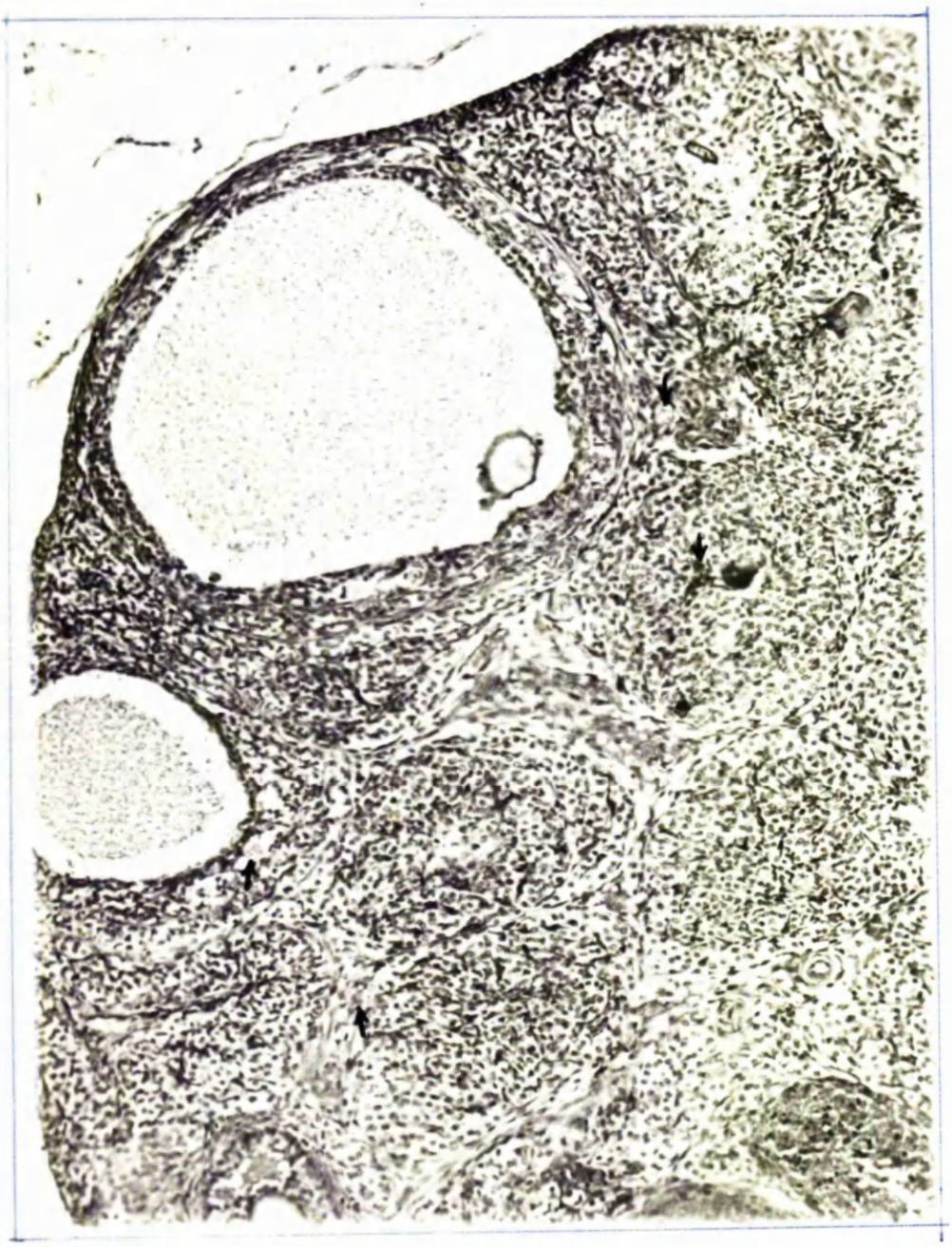


Fig. 17. Section to show Schiff positive cells in the stroma close to follicles, (arrowed). P.A.S. × 450.

there were several small follicles in different stages of early development. The reaction to the P.A.S. staining, however, was of some interest; in general this was regarded as negative, but there was an occasional granular P.A.S. positive cell in the stroma close to the follicles. (Fig. 16).

Animal (3). Again, in these ovaries there was a similarity to those of the two previous animals. The ovaries appeared to be inactive, and weighed 60 mgm. and 70 mgm., the average being 65 mgm. The microscopic appearance, however, gave the impression of greater activity - the follicles were larger, some of them showing evidence of early luteinisation of the granulosa, and the theca layer was defined although without luteinisation. There seemed to be an occasional P.A.S. positive globule in the granulosa layer of the larger follicles, and there was more evidence of P.A.S. positive cells in the stroma. (Fig. 17).

Summary of these Results.

From these results it is rather difficult

to arrive at any definite summary. It would appear that, although the ovaries appeared to be inactive their weights did increase (more than could be accounted for even by assuming that some fat had been left attached at dissection), and there was some evidence - no doubt minimal - of some follicular activity, with the appearance of P.A.S. positive cells (again minimal) in association with the follicular system. All this in spite of the fact that the injected substance was prepared from non-pregnancy urine.

Group IV. (Gonadotrophic Extract B from Non-Pregnancy Urine).

Animal (1). The ovaries in this animal were very small and seemed to be inactive: they weighed 10 mgm. and 25 mgm., the average being 17 mgm. On microscopic examination there was an occasional small, apparently degenerated follicle. The P.A.S. reaction was virtually negative.

Animal (2). Although these ovaries were a little larger than those of animal (1) above,

they seemed to be inactive: each weighed 18 mgm. On microscopic examination the appearances were similar to those of the previous animal, but the tissues looked fresher and the occasional follicle which was present was noted as being more normal in appearance. The P.A.S. reaction was virtually negative.

Animal (3). Like the ovaries of animal (2) above, these organs were moderately large, and each weighed 40 mgm. The microscopic findings were almost identical to those found in animal (2) above.

Summary of these Results.

It seems reasonably obvious that the injected substance did not produce any great evidence of activity in this group. Throughout, the P.A.S. reaction was virtually negative.

Group V. (Gonadotrophic Saline Extract from Non-Pregnancy Urine).

Animal (1). These ovaries appeared to be inactive and they weighed 20 mgm. and 15 mgm.,

the average being 17 mgm. It was surprising to find that, on microscopic examination, in spite of the ovaries being very small, they contained quite well-developed follicles, some quite large and with antra: but there was virtually no evidence of P.A.S. positive material.

Animal (2). Although these looked quite well developed ovaries, they seemed to be inactive in situ. They weighed 15 mgm. each. The microscopic appearances were similar to animal (1) above - numerous follicles in all stages of development. The P.A.S. reaction was virtually negative, although there was an occasional P.A.S. positive cell in association with a degenerating follicle.

Animal (3). In all respects these ovaries were similar to those of (1) and (2) above. Each weighed 50 mgm. The microscopic appearances also were almost identical to animals (1) and (2). In this instance, however, the P.A.S. reaction was virtually negative.

Summary of these Results.

Although there was a certain amount of

evidence of some follicular activity, there was virtually little evidence of any P.A.S. positive material. It cannot be stated whether any activity which was present was due to the injected substance.

Group VI. (Gonadotrophic Extract A from Pregnancy Urine).

Animal (1). On macroscopic examination these ovaries seemed to be inactive. There was quite a difference in the weights of the two organs - 30 mgm. and 65 mgm. the average being 47 mgm. Microscopically the appearances were similar to those of the control animals although more advanced - follicles in all stages of development, good development of the granulosa, with differentiation of a theca layer. The P.A.S. reaction was virtually negative.

Animal (2). These ovaries appeared larger and seemed to contain follicles. The ovaries were injected, and injection was also noted in the tubes. The ovaries weighed 70 mgm. and 50 mgm. (average 60 mgm.). Apart

from being larger ovaries, the microscopic findings were almost identical to those in animal (1) above. Here, also, the P.A.S. reaction was virtually negative.

Animal (3). On inspection these ovaries were about the same size as those of animal (2), and appeared to be active in-as-much as follicles were present. They weighed 50 mgm. and 40 mgm. (average 45 mgm.). On microscopy they looked much more active; there were several follicles in different stages of development, and in one area there was what appeared to be well developed luteal tissue - probably a corpus luteum. There was a good response in the granulosa layers of the follicles, and there was formation of a theca layer. There were occasional P.A.S. positive cells - granular - in the granulosa layers of the larger follicles, and also in the "corpus luteum" tissue.

Summary of these Results.

The appearances described here seem to indicate quite definitely that the injected

substance occasioned a progressive series of changes in the ovaries. Schiff positive cells were also found.

Group VII. (Gonadotrophic Extract B from Pregnancy Urine).

Animal (1). These ovaries were relatively small and appeared to be inactive: they weighed 20 mgm. each. The microscopic appearances were very similar to those of animal (2) in the previous group (VI). The P.A.S. reaction was virtually negative apart from one small area in the interstitial tissue in which there were P.A.S. positive granules.

Animal (2). Although the ovaries appeared to be quite inactive, both tubes were seen to be very oedematous and hypertrophied, and they were mildly injected. The ovaries weighed 40 mgm. each. Microscopically there was a good reaction: the ovaries contained numerous follicles in all stages of development, some of them being quite large. There were also corpora lutea in each ovary. The P.A.S. reaction was positive, the Schiff positive material being

present both in the granulosa layers and in the corpora lutea.

Animal (3). These ovaries also appeared to be inactive, but it was noted that there was an increase in their weight - 80 mgm. and 70 mgm. average 75 mgm. Again, microscopically, there were appearances of activity - greater than in the previous animal. There were several large, well developed follicles with much evidence of P.A.S. positive material in the granulosa layers. There was one old corpus luteum. There were also one or two P.A.S. positive cells in the stroma immediately surrounding one follicle.

Summary of these Results.

From these results it would appear that the injected substance caused a progressive series of active changes with which there was associated increasing evidence of the appearance of the P.A.S. positive material.

Group VIII. (Gonadotrophic Saline Extract from Pregnancy Urine).

Animal (1). These ovaries appeared to

be inactive. They weighed 15 mgm. and 30 mgm., average 22 mgm. On microscopic examination they resembled those of the controls in that they contained one or two small follicles with defined thecal layers. The P.A.S. reaction was virtually negative.

Animal (2) and Animal (3). These were in all respects similar to animal (1) above. The ovaries weighed 15 mgm. each in the case of animal (2), and 35 mgm. and 50 mgm. (average 42 mgm.) in the case of animal (3). The P.A.S. reactions were negative.

Summary of these Results.

It would appear that the injected substance employed for this group did not produce any activity in the ovaries.

Mature Pregnant Rats killed within 30 minutes of Ratting.

Two animals were used. At laparotomy the ovaries in each animal were large and gave the surface appearance of activity.

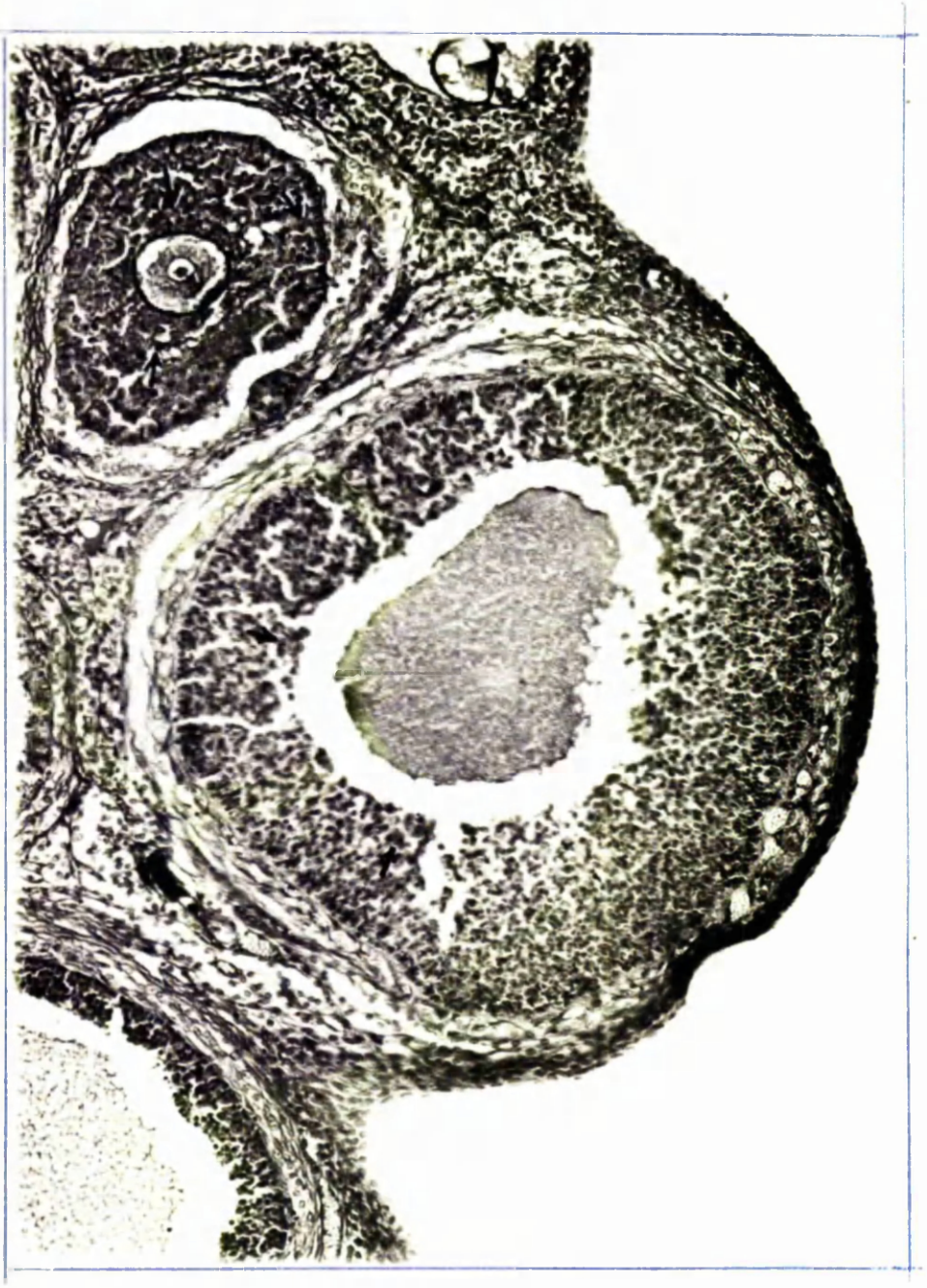


Fig. 18. Section of pregnant rat ovary to show the presence of Schiff positive cells in the granulosa layer of follicles, (arrowed). P.A.S. × 450.

Microscopically there were numerous follicles in all stages of development, together with a few atretic follicles. There were, also, apparently active corpora lutea.

After staining by the P.A.S. technique, in many ways these ovaries resembled the "pregnant" human ovary. There were well developed corpora lutea with P.A.S. positive cells and a few free granules - globules were entirely absent. Schiff positive cells and granules were also found in the stroma, near the corpora lutea and the follicles. There were occasional P.A.S. positive cells in the granulosa layer of the follicles. (Fig.18).

MICE.

In these experiments 22 immature mice were employed: they were all of approximately the same size, the average weight being 7 gms. Six of the animals were used as controls; they were not given any injections, and they were all killed at the same time. Of the remaining 16 animals, they were divided into

2 groups each of 8 animals, these being put in pairs within each group so that any loss by death during the course of the experiment might be covered. Animals in Group I were given intra-peritoneal injections of "Pregnyl" 3 i.u. per ¼ ml. which was the volume injected: the mice were thereafter killed in pairs at intervals of 2, 5, 7 and 10 days after injection. A Follicle Stimulating Extract was the substance used for injecting into the animals of Group II - 1 ml. of the extract being used - all injections were given at the same time. The animals in Group II were killed in pairs at intervals as in Group I.

When killed, laparotomy was performed and the ovaries of each animal inspected. Thereafter the ovaries were dissected free, removed, and weighed. Sections from each of the ovaries were then prepared by the usual technique, stained with H. & E. and also according to the Schiff technique, after which they were examined.

Results.

Controls. In every instance the ovaries appeared to be quite negative and inactive. Although there was slight variation in ovarian weight in individual animals this was considered to be insignificant, as was any difference in the ovarian weights between different animals: the average weight of a single ovary was 5.5 mgm. Examinations of sections from each ovary of each animal did not give any indication of activity - there was an occasional small, early developing follicle, but nothing more. Throughout, the P.A.S. reaction was virtually negative.

Group I. ("Pregnyl").

Animals (1 and 2). Of the first 2 animals killed, the ovaries appeared to be inactive, although in one animal the tubes were injected and slightly oedematous; in this animal each ovary weighed 9 mgm. whilst in the other animal each ovary weighed 6 mgm. Microscopic examination revealed a picture similar to that of the controls, viz. - virtually inactive

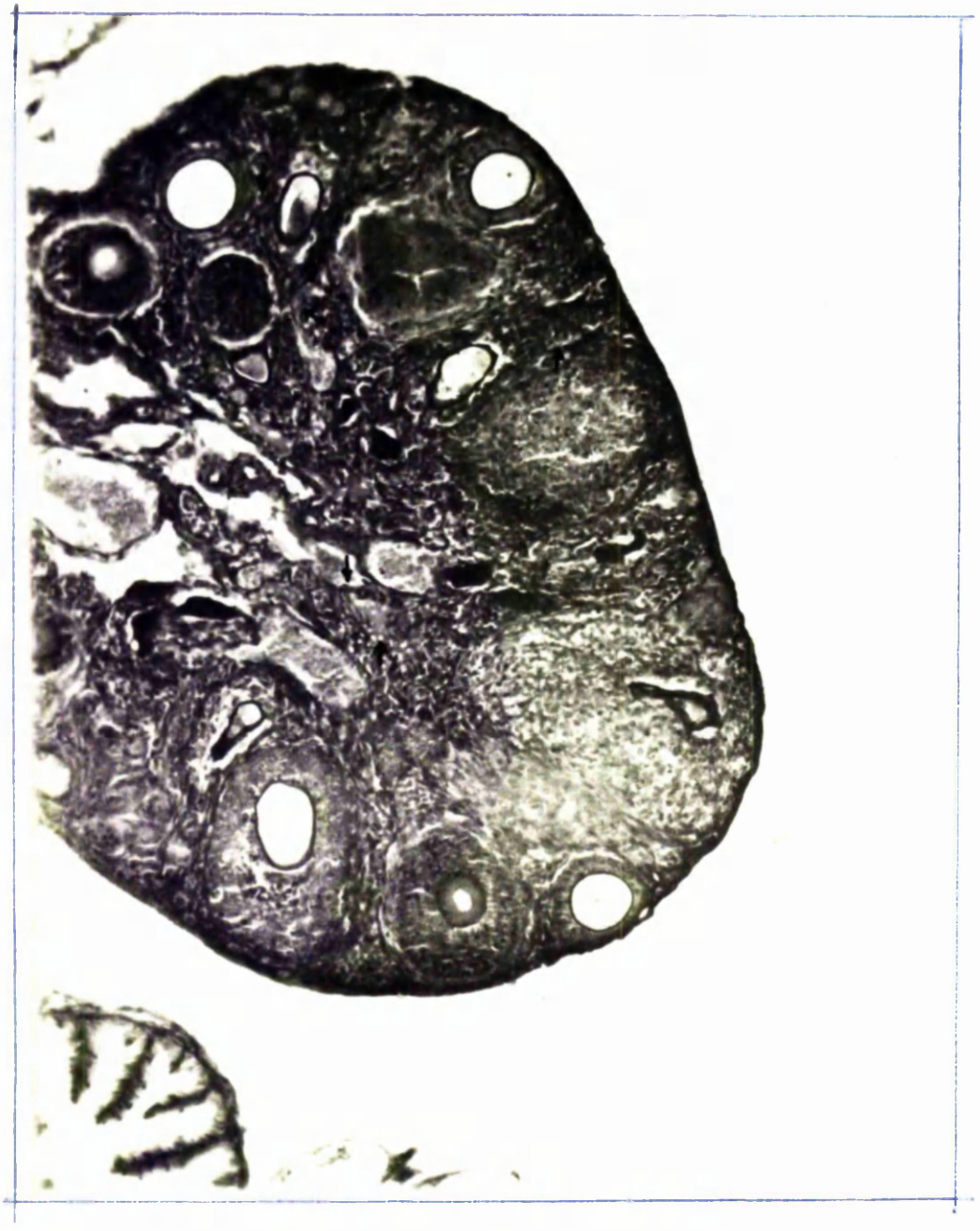


Fig. 19. Section of mouse ovary to show the presence of Schiff positive cells, mainly in the stroma, (arrowed). P.A.S. x 650.

ovaries with a few small, early developing follicles: the P.A.S. reaction was negative.

Animals (3 and 4). In both animals the tubes were markedly oedematous: in one animal the ovaries were questionably positive, whilst in the other the ovaries were obviously active in-so-far as they contained blood follicles. In the first animal the ovaries weighed 10 mgm. and 15 mgm. - average 12.5 mgm., and in the second the weights were 10 mgm. and 11 mgm. - average 10.5 mgm. On microscopic examination true activity was confirmed; there were good developing follicles, some being quite large, and there were blood cysts in each ovary. The Schiff reaction was mildly positive for the material under consideration, being situated mainly in the stroma. (Fig. 19).

Animals (5 and 6). The ovaries from these animals all appeared to be inactive, and the tubes were neither oedematous nor injected. The ovaries weighed 5 mgm. and 6 mgm. (average 5.5 mgm.), and 6 mgm. and 8 mgm. (average 7 mgm.). Microscopic examination revealed rather poor

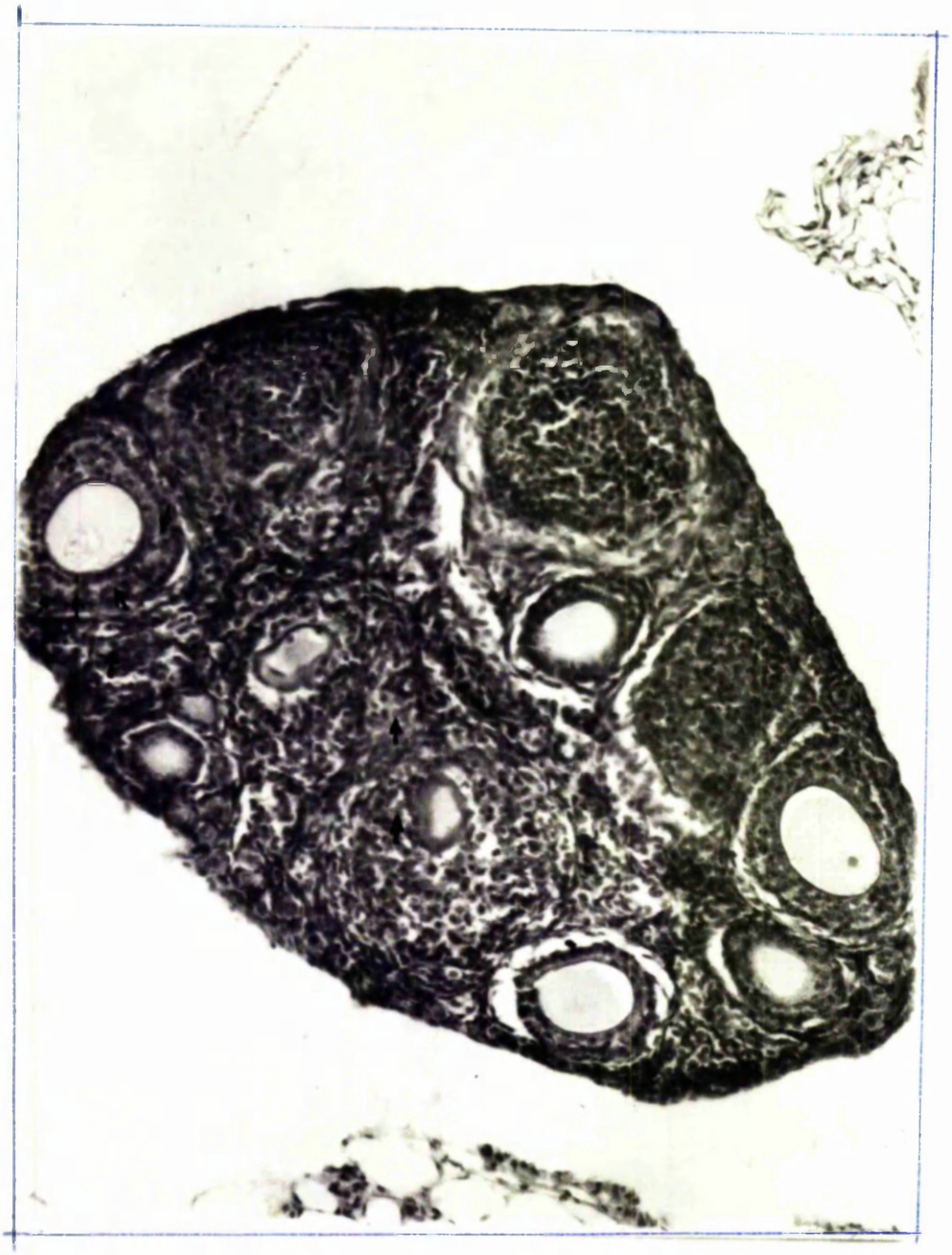


Fig. 20. Section to show the presence of Schiff positive cells in the follicle granulosa and in the stroma, (arrowed). P.A.S. × 450.

follicular development in each of the ovaries. In the ovaries of one animal there was an occasional P.A.S. positive cell in the stroma, but the ovaries of the other animal were virtually negative.

Animals (7 and 8). The tubes in these animals were apparently negative, whilst in each of the ovaries the surface was rather granular, giving the impression of old activity. The weights of the ovaries were 3.5 mgm. and 4 mgm. (average 3.75 mgm.), and 7 mgm. and 8 mgm. (average 7.5 mgm.). The microscopic appearances were much more interesting: in each of the ovaries there were follicles in all stages of development, early theca formation being evident in the larger follicles, whilst in the heavier ovaries there were blood cysts. In each of the ovaries there were P.A.S. positive cells in the stroma and in the granulosa layers of the larger follicles. (Fig. 20).

Summary of these Results.

From this series it is interesting to note that the first obvious changes were noted in the

tubes: thereafter macroscopic evidence of activity was seen in the ovaries. The microscopic evidence of activity in the ovaries also became evident, but there would appear to be a short time lag before the Schiff positive cells appeared.

The injected substance ("Pregnyl") would appear to have been responsible for the activity found in the ovaries of this series.

Group II. (Follicle Stimulating Hormone Extract).

Animals (1 and 2). Macroscopically these ovaries appeared to be inactive. After removal they weighed 3 mgm. and 5 mgm. (average 4 mgm.) and 3.5 mgm. and 2.5 mgm. (average 3 mgm.). Microscopically there was only slight evidence of activity - several early developing follicles; there was also slight but definite evidence of P.A.S. positive cells in the stroma near the developing follicles. The granulosa cells were negative for the P.A.S. reaction.

Animals (3 and 4). Here again, the ovaries appeared to be inactive. On weighing, they were found to be somewhat lighter than the

previous ovaries, viz. - 2 mgm. and 3 mgm. (average 2.5 mgm.), and 2.25 mgm. and 2 mgm. (average 2.12 mgm.). Microscopically, however, there was evidence of greater activity: there were follicles in all stages of development, and there was good evidence of P.A.S. positive cells in the granulosa layers of the larger follicles.

Animals (5 and 6). The ovaries appeared to be inactive. They were heavier than the previous ovaries - 3.5 mgm. and 6.5 mgm. (average 5 mgm.) and 4.5 mgm. each (average 4.5 mgm.). Microscopically, activity was evident from the presence of follicles in all stages of development, apparently greater than in the ovaries of the previous animals. A few large P.A.S. positive cells were present in the granulosa layers, but from their size it was considered that these might be degenerative products.

Animals (7 and 8). In all respects - macroscopically and microscopically - these ovaries were closely similar to those of

animals (5 and 6). They weighed 3 mgm. and 2.5 mgm. (average 2.75 mgm.) and 3 mgm. each.

Summary of these Results.

In this group it would appear that the Follicle Stimulating Hormone Extract was responsible for promoting development of the follicles in the ovaries, but could not produce further maturation.

Commentary on the Animal Experiments.

As indicated in the introductory remarks to this section (page 71) the main purpose of these particular experiments was to make an attempt at discovering what substances, if any, when injected into the experimental animals, would result in an acceptable degree of ovarian activity, and also to pay especial attention to the appearance of the Schiff positive material which has been found to be associated with such activity in the human ovary. By such means it was hoped to determine the role played by the Schiff positive material in ovarian activity, and also to give some more definite indication

of its source and any association it might have with actual hormone principals.

When comparing the ovaries of the experimental animals one with another, and with the controls, certain general facts seem to emerge. In all the control animals, although these were immature, a minor, early degree of follicular activity was noted: in no instance, however, was there any evidence of the presence of the Schiff positive material under consideration. With this in mind, and using the controls as the criterion on which to base the estimate of activity, it has been shown that:

(1) Pooled urine from pregnant women, when injected into the animals, promotes ovarian activity. As the animals were immature, and the response followed so closely on the injection, it would appear probable that the injected urine contained some factor or factors which acted directly on the animal ovaries. As there was follicular maturation with, in many cases, ultimate corpus luteum formation, it further seems probable that the injected pregnancy urine

contained at least two factors, viz. - a follicle stimulating hormone-like substance, and a luteinising hormone-like substance. With the appearance of histological ovarian activity, after a short time lag, there is also the appearance of the Schiff positive substance under consideration which is regarded as histochemical evidence of activity.

(2) When used, "Pregnyl" likewise occasions a similar series of progressive changes, from early follicular development, through full maturation to corpus luteum formation, with the appearance of the Schiff positive material in the follicular and luteal bodies. It is noted, further, that, judged by the appearance of the various bodies in the ovaries, the "Pregnyl" seems to be a more potent source of the factors responsible for the activity produced.

(3) With regard to the extracts from pregnancy urine used in the rabbit and rat experiments, here again activity is produced by the extracts A and B, but not by the saline extract. The degree of activity produced by

extracts A and B is similar to that produced by the pooled pregnancy urine. In the same way, the P.A.S. positive material appears in these ovaries.

(4) The injection of pooled human non-pregnancy urine, or of extracts made from non-pregnancy urine, does not occasion any obvious response in the ovaries of the experimental animals, and the ovaries remain virtually negative for the Schiff reaction.

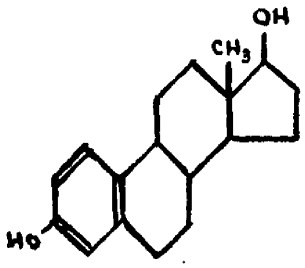
(5) When Follicle Stimulating Hormone is injected into immature mice, activity in the ovaries is initiated, but only up to the stage of mature follicles; after a short time lag the P.A.S. positive material makes its appearance in the follicular system. In no case was a corpus luteum formed.

From these findings, it has been shown that the ovaries of the experimental animals - whether adult but inactive animals or immature animals - are not in any way affected by the injection of non-pregnancy urine or of extracts made from non-pregnancy urine. In these

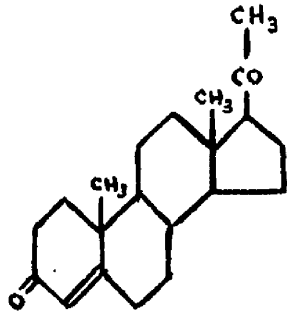
instances the ovaries remain negative both for activity and for the presence of the P.A.S. positive material under consideration.

With the exception of the saline extract from pregnancy urine, all the other substances used for injection - pregnancy urine, extracts A and B from pregnancy urine, "Pregnyl" and Follicle Stimulating Hormone - seem to be responsible for stimulation of the ovaries with resultant activity, and ultimately with the appearance of the P.A.S. positive material. It would appear to be of some significance that a time lag has been noted between the initial production of activity and the appearance of the Schiff positive substance. This finding would seem to indicate that it is only after the ovaries have been stimulated to activity that they then are responsible for producing this substance which histochemically is revealed as being Schiff positive. If this is not a break-down product, then it seems highly probable that it represents the actual ovarian hormones or their precursors, rather than the hormonal principles which

initiate the ovarian activity. The recognised ovarian hormones are dihydro-oestrone from the follicles, and progesterone from the corpus luteum: but these bodies are closely related, one being derived from the other, and in both the Schiff positive material is found. But the hormones produced by these bodies are also similar one to the other -



Dihydro-oestrone.



Progesterone.

and if it is allowed that the histochemical techniques, in particular the Schiff reaction, may not be too selective, it would not be surprising that the two hormones should give the same histochemical reaction.

DISCUSSION AND CONCLUSIONS.

In this study an attempt has been made to investigate, by histochemical means, part of the functions of the human ovary: as a result it is hoped that a contribution will have been made towards a fuller understanding of the physiology of this important organ. As indicated in the Introduction, it seemed to the author that no truly comparable investigation had ever before been attempted; consequently there is a meagre literature to which reference can be made.

A short historical summary has been given on the development of histochemistry as a means of investigating the functions of body organs and tissues. From this it may be inferred that, old as this particular science may be, changes in, and modifications of methods, and the introduction of new techniques do take place from time to time: on this basis it may be argued that some of the techniques employed may be open to doubt or question. Nevertheless, on present day knowledge the fundamentals of the method are

generally accepted by all authorities. In particular this would seem to apply to the use of the Periodic Acid-Schiff (P.A.S.) techniques which have been used so extensively in the present investigation. On this assumption it is believed that the findings now presented may well be a significant, if small, contribution to the study of the human ovary. At the very least this may perhaps be regarded as a basic point from which fuller investigation and study may emanate.

It is realised that the presentation of observations is one thing, whilst the interpretation of these observations is quite another. With this in mind, and accepting the significance of the histochemical techniques which have been employed as reliable, it is now proposed to discuss the findings here reported and attempt an interpretation.

In the earlier part of this study a large variety of staining techniques was employed, some of which gave negative results whilst others gave positive results.

There was little of significance detected in sections stained by H. & E.

The Schiff reaction revealed the presence of some substance in the ovary, which aroused much interest, and especially when the various qualitative modifications were employed.

The Sudan reaction gave a positive result in this Schiff positive material, although this did not persist after extraction, whilst the Schiff reaction continued to be positive. At no time was birefringency demonstrated.

Metachromasia was not shown at any time when using toluidine blue or thionin.

Although a positive result was obtained by the modified Gram's reaction - and some observers consider that this indicates the presence of nucleoprotein - it was particularly noted that the more generally recognised method for demonstrating nucleoprotein by staining with light green before and after extraction with trichloro-acetic acid gave negative results, and this was taken as indicating the absence of nucleoprotein.

As the study continued it was considered that all the techniques should not be repeated, especially those which initially appeared to give negative results: many interesting facts may well have been revealed, but it was felt that this study might become overloaded and involved if attention was given to too many factors. As the Schiff reaction gave such good results it was decided to pay particular attention to it, and it therefore assumes a position of prime importance throughout this investigation. In effect, the main technique employed has been the Schiff reaction, with its various modifications, and to a lesser extent the Sudan reaction. This is reflected in the later parts of the study.

As shown in Section II, the mature active human ovary exhibits the presence of some substance which would appear to be a mixture of lipoid and muco-protein, the protein moiety probably containing tryptophan. Also from this section it has been shown that this complex substance is especially associated with the

follicular system, in so far as it is found in the granulosa layer of the follicle, in the substance of the corpus luteum, and in close proximity to the corpus albicans. But what is the significance of this substance?

The first consideration is that the appearance of this substance as shown by the histochemical techniques may be purely adventitious. If this is so, then it does seem strange that an artefact should recur with such regularity throughout the comparatively large numbers of sections examined: and further, that it should continue to appear in the same sites. On these grounds, it can only be concluded that the appearance of this lipo-muco-protein is not an event of chance but is indeed of significance as indicating some process in, or result of the cycle of events which take place in the human ovary. That there is an association between activity in the mature human ovary and the appearance of this Schiff positive material in the ovary seems to be established beyond reasonable doubt.

At this stage it seems pertinent to observe that the substances which are being studied in the mature, the foetal/baby, and the post-menopausal human ovaries give similar histochemical staining reactions, and appear in the same situations. The obvious conclusion seems to be therefore, that in each instance it is the same substance which is being studied.

In the commentary dealing with the study of the foetal/baby ovaries, it has been stated that it is possible to suggest that the Schiff positive substance may be derived from break-down products: and this could apply equally to the mature active ovaries, and, indeed, also to the post-menopausal ovaries. But as pointed out in Section III, it seems unlikely that this lipo-muco-protein is a break-down product. We are dealing here with a complex substance, and in nature it is found that break-down substances are of relatively simple character: for example, lipoids break down to fatty acids, carbohydrates give glucose,

and proteins result in amino acids and urea. This would enhance the probability that the substance being studied is not in fact the result of break-down.

If, then, the substance under investigation is not an artefact, and is not a break-down product, it must therefore be derived as a result of some positive active phase in the ovarian cycle.

Much has been written regarding the so-called interstitial cells of the ovary, and Wright (1937) has stated that they are polyhedral in shape: that they may contain numerous granules, chiefly of a lipoid nature, which may represent their secretory product: but that in about half the specimens examined these cells are absent. In the present study it has been shown that cells similar to the interstitial cells have occasionally been found in the interstitial tissues of the human ovary in close proximity to the corpus albicans, and this would seem to indicate a strong probability that these are in fact the so-called interstitial cells. But

these same cells are found in greater numbers in the corpus luteum, the active secretory body which after degeneration forms the corpus albicans. There are therefore logical grounds for suggesting that when the corpus luteum degenerates, some of the Schiff positive cells are absorbed, whilst others, before absorption can be effected, are extruded from the forming corpus albicans into the interstitial tissue to be absorbed in due course probably by phagocytosis. This would certainly explain the findings reported here, and would also explain the presence of so-called interstitial cells. If this view is accepted then it would appear that the so-called interstitial cells of the human ovary probably play a relatively minor part - or even no part at all - as truly secretory cells, but merely represent an end stage in a process which has taken place for the main part in the corpus luteum; and which process itself has already started in the developing follicle.

There is general agreement that the ovary is completely under the influence of the

pituitary gland: that the ovary only grows, matures and becomes active under the influence of the pituitary hormones, and in particular of the Follicle Stimulating Hormone and the Luteinising Hormone, formerly referred to as Prolan A and Prolan B respectively. The theory which receives greatest credence is that the ovaries are immature and remain quiescent until the anterior pituitary hormones exert their influence on them at or about the time of puberty. From then on the ovaries assume an active role, under the influence of the pituitary, developing follicles and corpora lutea, liberating ripe ova, and as a result of this elaborating the ovarian hormones in the form of dihydro-oestrone from the Graafian Follicle, and progesterone from the corpus luteum. This process, one of continuity, takes place continuously until the ovaries cease activity at or about the time of the menopause. The fact that during their active life the ovaries show the follicular system, including corpus luteum formation and degeneration, in all stages at the

same time makes it difficult to assess the significance or role of the Schiff positive material under consideration: this material is found to be present in the granulosa layer in the later stages of follicle ripening, and in the corpus luteum.

So that this aspect of the investigation could perhaps be simplified and some evaluation made of the significance of the Schiff positive substance, study of foetal/baby ovaries was decided upon. In effect, as stated above, such ovaries should be immature and inactive, and presumably should not show any evidence for the presence of the Schiff positive material which has been shown to be associated with ovarian activity. But cases of activity in foetal ovaries have been described from time to time, and it was thought that if any such cases could be found it might be possible to show the presence of the Schiff positive substance and at the same time demonstrate it in simpler surroundings.

As reported in Section III of this

Thesis, amongst the foetal/baby ovaries studied, there were some which did indeed show activity, with the presence of the Schiff positive material. As in the adult ovaries, this material is associated with follicular activity, being found in the granulosa layer of the developing follicle: but here the similarity ends. Two findings which may be significant are, first, the maturation of the follicle, unlike that in the adult, only reaches a stage of commencing theca luteinisation, and second, that the Schiff positive material in the granulosa layer of the follicle seems to be entirely globular in form.

Although in the present foetal/baby series follicular activity has been found in specimens from both normal and abnormal pregnancies, there is no question of the preponderance of the active ovaries being associated with abnormal pregnancy in the form of pre-eclamptic toxaemia, and as discussed in the commentary to Section III it seems more than likely that it is an excess of maternal pituitary gonadotrophin which, crossing the placental barrier, acts directly

on the foetal ovaries to bring about the follicular maturation in these cases. With such immature tissues and organs which form the foetal organism, it seems most unlikely that one could propound a primary foetal pituitary origin for the production of the gonadotrophin, either as a direct initial activity, or as a secondary response to the action of some maternal factor. From the evidence presented the present author concludes that these foetal/baby ovaries which show activity do so as a result of the direct action on them of maternal pituitary gonadotrophin, and in particular of the Follicle Stimulating Hormone.

As in the case of the foetal/baby ovaries it was considered that the post-menopausal ovaries ought not to show any evidence of activity, and although only a small number of specimens was examined (20 patients), it was of considerable interest to find that in not a few (9 cases) ovarian activity was in fact demonstrated: and further, that in 7 of these

there was associated Schiff positive material. Note has been made of the apparent association of the clinical conditions of fibroids and carcinoma with activity and the presence of the Schiff positive material in these ovaries. It would seem that a certain amount of support is given for the hypotheses which are advanced for an ovarian hormonal factor being concerned in the aetiology of fibroids and also for the relationship between the oestrogens and carcinoma, but from the numbers involved in this study it would be unwarranted and most unwise to draw any conclusions.

From all the results given in Sections II, III, and IV it may, however, be concluded that the Schiff positive material found in these human ovaries, directly or indirectly plays some definite part in the cyclical activity of these ovaries. The actual role played by this substance, however, still remains far from clear.

On further reflection of the results given, in the mature human ovary the Schiff

positive material first appears in the granulosa layer of early developing follicles, even before antrum formation, and from then on the concentration of the material increases. In the foetal/baby ovaries which show developing follicles, the Schiff positive material appears after the granulosa layer has reached a depth of about 6 to 8 cells. As, however, in the ovaries from post-menopausal women, activity was only revealed by the presence of atretic follicles and cystic follicles, it is not possible to indicate at what stage the Schiff positive cells first appear in them. Nevertheless, from the younger ovaries, it seems quite definite that there is a slight delay between the initiation of follicular maturation and the appearance of the Schiff positive material. This would suggest at least two possible mechanisms. First, it is possible that any excess of pituitary gonadotrophin - Follicle Stimulating Hormone - after the initiation of follicular activity, may be deposited in the granulosa of the

follicle to await removal or destruction, presumably by phagocytosis. But if this is the case, it might have been expected that any excess would also be deposited in the ovarian stroma, and as shown in Sections II and III this does not in fact appear to happen. Further, as stated above with regard to break-down products, it might be anticipated that any deposition of excess gonadotrophin would be in the form of simpler break-down products: but the histochemistry of this substance reveals it to be of a complex nature. This therefore seems unlikely to be the explanation for the appearance of the Schiff positive material. Second, an alternative explanation which may be offered is that after the pituitary gonadotrophin has initiated follicular activity, the follicle itself then elaborates its own hormone - dihydro-oestrone, and it is this substance or its precursor, or perhaps a carrier substance which is being visualised. This no doubt is a somewhat expectant or optimistic explanation, but would

certainly interpret the site of the material and the time-lag in its appearance. Either of these explanations might account for the appearance of the Schiff positive material in the granulosa layer of developing and mature follicles, but would they necessarily hold for its presence in the corpus luteum? For its study in this site we are confined to the mature active ovary - and it will be remembered that here the Schiff positive material is in two forms - globules and granules.

If the substance present in the developing follicle is related to Follicle Stimulating Hormone, it may further be inferred that when the follicle ruptures to liberate the oöcyte the Schiff material detected in the corpus luteum may be related to Luteinising Hormone which brings about the luteinisation, and may represent an excess or break-down product of the Luteinising Hormone: in which case the two forms of the Schiff positive material would represent Follicle Stimulating Hormone and Luteinising Hormone. This seems somewhat

unlikely, for the reasons given above, and also, because in the normal cylical series of events which take place as an established rhythm, it seems extraordinary that nature should be so prodigal as to produce such excess. On the other hand when the corpus luteum is formed, it elaborates its own hormone - progesterone. It seems more likely that the Schiff positive material found in the corpus luteum may be more intimately related to progesterone. As regards the two forms of the material, it may well be that one is associated with progesterone, whilst the other is associated with dihydro-oestrone, the production of which continues though to a reduced extent, in the corpus luteum, (McGregor, 1955).

If this latter explanation is correct, then it can be assumed that the Schiff positive material found in the atretic follicles of the post-menopausal ovaries may be closely allied to ovarian hormone production in these ovaries.

It is only too fully appreciated that the work carried out, and the results given, in this study are too small to warrant the presentation of any dogmatic statements; nevertheless, from the above arguments it seems not unreasonable to suggest the formulation of a tentative working hypothesis regarding the Schiff positive material under investigation, and which is associated with activity in the human ovary.

Tentative Working Hypothesis. In the human ovary follicular activity is initiated and controlled under the influence of the pituitary gonadotrophin, Follicle Stimulating Hormone, either directly as in the mature woman, or in the case of the foetal/baby ovaries as a result of maternal pituitary gonadotrophin crossing the placental barrier to act directly on the foetal ovary. Following a short time-lag after the commencement of follicular maturation, the Graafian follicle then becomes active and elaborates dihydro-oestrone in the granulosa layer and one form or other of this steroid is visualised by the Schiff technique. Thereafter,

when ovulation takes place, the pituitary gonadotrophin, Luteinising Hormone, exerts its influence to promote transformation of the follicle to the corpus luteum, and initiates this body to elaborate the hormone progesterone, which steroid in one form or another is visualised by the Schiff technique, together with dihydro-oestrone, the production of which continues to a reduced degree in the corpus luteum.

It can only be emphasised, however, that this would form a purely working hypothesis, and further investigation would be required in order to obtain substantiation or otherwise. For this reason the animal experiments were carried out.

One obvious fact which emerges from the animal experiments is that these ovaries exhibit the presence of Schiff positive material in a manner similar to the human ovaries; and further, if the histochemical techniques employed are to be relied upon, then the material detected in the animal ovaries must

bear a very close relationship to that found in the human ovaries - if, indeed, it is not identical. On these grounds it may be reasonable to assume that any conclusions arrived at with regard to the Schiff positive material in the animal ovaries may very probably be applied to that found in the human ovaries. It should be noted that the findings, particularly the characteristics of the Schiff positive material found in these animal ovaries, do not correspond completely to the findings reported by Rennels (1951).

The mature rabbits used in the initial experiments were confirmed non-pregnant animals, and the investigation revealed them to bear a close similarity to the active mature human ovaries, both as regards general histological appearances and the presence of Schiff positive material. Injection of urine from known pregnant women resulted in the promotion of activity in these ovaries: likewise the injection of "Pregnyl", and of follicle stimulating hormone and luteinising hormone

extracted from human pregnancy urine also seemed to be responsible for producing activity in the animal ovaries. The general impression gained from study of the animal ovaries where the injected substance was a saline extract from known pregnancy urine, and the fractional extracts of Follicle Stimulating and Luteinising Hormones, together with a saline extract, from known non-pregnancy urine suggests that these substances have little or no effect on the animal ovaries.

The experiments carried out on the immature rats perhaps show more clearly the pattern resulting from the injection of the various substances. Here again, the overall impression is similar to that described as occurring in the rabbit: injection of human pregnancy urine, and of Follicle Stimulating and Luteinising Hormones extracted from human pregnancy urine results in ovarian activity and the appearance of the associated Schiff positive material, whilst in general negative results are obtained when a saline extract from pregnancy

urine, and other substances - non-pregnancy urine and extracts made therefrom - are employed. The very close similarity of the mature rat ovary of pregnancy to the human active ovary would seem to enhance the conclusion that the changes found in the human ovary are closely parallelled by the experimental animal ovary.

The experiments carried out on the immature mice do not bring anything further to light. They conform to the pattern and merely confirm the findings reported on the other animals.

In the comments to the section on the Animal Experiments it has been stated that certain general facts seem to emerge, and it is unnecessary to repeat these in detail here. It may, however, be stated that urine from pregnant women; extracts (Follicle Stimulating and Luteinising Hormones) from human pregnancy urine; and "Pregnyl" seem to be responsible for stimulating the animal ovaries to activity, with the ultimate appearance of the Schiff positive material in the granulosa layers of

the follicles and in the corpora lutea. The time-lag between the initiation of ovarian activity and the appearance of the Schiff positive material - as also found in the study of the human ovary - would appear to be significant. It may be pertinent to observe that in some instances the Follicle Stimulating and Luteinising Hormone fractions extracted from human pregnancy urine each appeared to fulfil the double role of causing follicle maturation and bringing about corpus luteum formation: it seems most probable that this should be explained as due to some error in the actual extractive technique such as would lead to incomplete fractionation. In any event, it is unlikely that this would vitiate the general results obtained. Although certain suggestions have been made regarding the possible origin of the Schiff positive material, it can only be emphasised that these are merely suggestions, and the present animal experiments fail to resolve the problem of its true identity and origin. What may be

accepted, however, is that the similarity between the human and the experimental animal ovaries is sufficiently close as to warrant the carrying out of further animal experiments and of applying any findings and conclusions to the human ovary.

From this Study, then, the following conclusions would appear to be justified:

1) The human ovary, when active, exhibits the presence of some substance which is demonstrable by histochemical procedures, in particular the Schiff reaction and its various modifications.

2) This Schiff positive substance is complex and would appear to be a mixture of lipoid and muco-protein, the protein moiety probably containing tryptophan.

3) This Schiff positive substance is especially associated with the follicular system of the ovary - the granulosa layer of the maturing follicle, the corpus luteum, and the corpus albicans close to which it may also be found in the stroma.

4) It seems unlikely that this substance is

either an artefact or due to simple break-down products, but is in fact representative of some positive active phase in the ovarian cycle.

5) It seems more than likely that what in the past have been referred to as ovarian interstitial cells are in fact a terminal stage of the Schiff positive substance which is now reported as being intimately associated with activity of the follicular system of the human ovary, and that as interstitial cells they probably play a relatively minor part, or no part at all, as truly secretory cells.

6) A tentative working hypothesis has been advanced, but from the study of the human ovaries it has not been possible to substantiate this hypothesis.

7) Animal experiments show that the ovaries of rabbits, rats, and mice respond to stimulation in a way similar to that of the human ovary, and exhibit Schiff positive material under the same conditions, in similar sites, and as a result of similar forms of stimulation. It may be that the Schiff positive material demonstrated

in the animal ovaries is identical to, or very closely related to that demonstrated in the human ovary.

8) Unfortunately, the animal experiments which were carried out, the results of which have been described in this thesis, have not been successful in resolving the problem of final identification of the Schiff positive material. Further set and controlled experiments will be required.

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APPENDIX.

With so many different techniques employed in this study it was felt that to describe them in detail in the main body of the thesis would tend to break the continuity of the theme, and for this reason it was considered that the purpose of description would be better served by grouping the techniques together in the form of an appendix.

Fixation and cutting of the section material employed has been mentioned within the context of the thesis, and such techniques are normal routines in laboratory methods that it seems unnecessary to give further details here. Likewise, the technique for staining with H. and E. is also a standard routine in the laboratory, and will not be described.

With regard to the histochemical methods, the descriptions which follow are taken mainly from Pearse (1953): the debt incurred and the help obtained are fully acknowledged by the present writer.

As mentioned in the introductory remarks to the animal experiments, the extraction of gonadotrophins (A and B) from human female urine - pregnancy and non-pregnancy - has been carried out according to the methods described by Lorraine and Brown (1956) by Mr. J. Sommerville, F.I.M.L.T., in the histochemistry section of the Research Department, Royal Maternity and Women's Hospital.

Techniques:

1. The Periodic Acid-Schiff Technique.

Having brought the sections to water, mercury is removed, and they are then rinsed in 70 per cent alcohol. The sections are then immersed in periodic acid solution for 5 minutes, and again rinsed in 70 per cent alcohol. After immersion in the reducing bath for 1 minute, the sections are again rinsed in 70 per cent alcohol. Thereafter they are immersed in Schiff's solution for 20 minutes, and washed in running water for 10 minutes. The nuclei are then stained lightly with celestin blue for 2 - 3 minutes, followed by Mayer's haemalum

for 2 - 3 minutes. Differentiation in 1 per cent acid alcohol is carried out, and the sections washed in running water for 30 minutes. Sections are then counterstained with orange G for 10 seconds, washed in water (about 30 seconds), and dehydrated in alcohol, cleaned in xylene, and finally mounted in DPX.

Results: Mucoproteins and neutral muco-polysaccharides, deep purplish-red; glycogen, purplish-red; glycoproteins, usually pale red or pink; various lipids, pale red or pink. (This list is abbreviated).

2. Prevention of the P.A.S. Reaction by Acetylation.

Three sections are used in each case, 2 being controls. All three sections are brought to water. Sections 2 and 3 are treated in acetic anhydride in pyridine for 45 minutes, and washed in water. Section 3 is now treated with 0.1 N KOH for 45 minutes at room temperature, and then washed in water. All three sections are then stained by the P.A.S. routine.

Results: A positive result in sections

1 and 3 with a negative result in 2 confirms the carbohydrate nature of the material.

3. Modified Gram.

Sections are brought to water, and stained in carmalum for ½ hour. After rinsing in water they are stained with crystal-violet 0.5 per cent for 2 minutes, followed by Lugol's iodine for 2 minutes, followed by Na Cl 10 per cent for 5 minutes. They are then blotted dry, and decolourised in aniline oil-zylol, cleared and mounted in DPX.

Results: Gram positive material, blue-black.

4. Toluidine Blue - for metachromasia.

Sections are brought to water and covered for 30 seconds with toluidine blue solution: they are then rinsed with 0.25 per cent borax solution. A solution (previously saturated with mercuric chloride and filtered) of 0.5 per cent potassium dichromate is applied for 15 seconds, and fresh dichromate sublimate solution added and left for 2 minutes. After blotting dry, the section is immersed in

absolute alcohol for 10 seconds and then immersed in clean xylene for 30 seconds. Absolute alcohol is used for rinsing, and, having drained, the section is covered with aqueous dichromate-sublimate solution for 30 seconds: it is then rinsed in colophony-alcohol, cleared in two changes of benzene, and mounted in Canada balsam.

Results: Metachromasia is shown by a red colour.

5. Thionin - for metachromasia.

Sections are brought to water, and treated with saturated aqueous mercuric chloride for 30 seconds. After washing quickly in water, they are stained in dilute aqueous thionin for 5 - 15 minutes. They are then washed in distilled water and mounted.

Results: Metachromatic substances are red, the nuclei blue.

6. Light Green - for deoxyribonucleic acid.

Bring the sections to water, and treat with 4 per cent trichloroacetic acid at exactly 90° for 15 minutes. Wash and stain with

1 per cent aqueous light green S.F. Both types of nucleic acid are extracted by this procedure. Dehydrate in alcohol, clear in xylene, and mount in DPX.

Results: Nucleic acids do not stain with acid dyes, but after extraction they leave behind a protein component of the nucleus which can be demonstrated by its increased affinity for acid dyes.

7. Sudan Black B - for lipids.

Bring the sections to 70 per cent alcohol and then stain for 30 minutes at room temperature in saturated Sudan Black B in 70 per cent alcohol. Remove excess dye by rinsing quickly in 70 per cent alcohol, and then wash in running water. Counterstain in Mayer's carmalum for 16 hours, wash in water, and mount in glycerine jelly.

Results: Lipids stain black, and even a brownish-black stain may indicate the presence of lipid or lipo-protein.

8. Sudan III and IV - for neutral fats.

Cut frozen sections into distilled water

or 1 per cent formalin. Rinse in 70 per cent alcohol and stain for not more than 1 minute in the Sudan mixture (Sudan III and IV). Rinse in 50 per cent alcohol to remove excess stain, and then in water to remove all alcohol. Counterstain in dilute Ehrlich's haematoxylin for 1 - 3 minutes. If necessary, differentiate in 0.5 per cent HCl in 50 per cent alcohol. Wash in distilled water containing a few drops of ammonia, and mount in glycerine jelly.

Results: Lipids (neutral fats) stain orange-red to orange.

9. Coupled Tetrazonium - also with Benzoylation, and with Acetylation - for tyrosine, tryptophan and histidine.

Bring sections to water and remove mercury, thereafter immersing them in freshly tetrazotized benzidine at 4° for 15 minutes. Wash in water and in three changes of veronal acetate buffer at pH 9.2 for 2 minutes in each change. Immerse in a saturated solution of H-acid in veronal acetate buffer at pH 9.2 for 15 minutes. Wash in water for 3 minutes, and dehydrate in alcohol.

Clear in xylene and mount in balsam or DPX.

Results: Most tissue components stain reddish brown, indicating the presence of tyrosine, tryptophan or histidine. If the coupled tetrazonium reaction is preceded by mild heat and benzoylation (see below) positive structures now stain either a deep red-brown or purplish red.

Benzoylation. After removal of mercury, sections are brought to water and then to absolute alcohol. They are then immersed in petroleum ether for 3 minutes, removed and allowed to dry in the air. Heat may then be applied at 60° in an incubator, to dry the sections. The dried sections are placed in 10 per cent benzoyl chloride in dry pyridine for 10 - 16 hours at room temperature, after which they are rinsed in absolute acetone followed by immersion in absolute alcohol. Sections are then taken to water and the coupled tetrazonium reaction carried out.

Acetylation. The general details as described above, but instead of placing sections in 10

per cent benzoyl chloride in dry pyridine, immerse them in 10 per cent acetic anhydride in dry pyridine and heat for 4 - 8 hours, at approximately 100°.

Results: The results obtained and the differentiation have been described in the body of the Thesis. By the differentiation it is probable that tyrosine, tryptophan and histidine can be distinguished.

10. Performic Acid - Schiff.

Bring sections to water with removal of mercury. Treat with performic acid solution for 10 minutes, and wash in water for 2 - 5 minutes. Then immerse in Schiff's solution for 30 - 60 minutes, wash in warm running water for 10 minutes, dehydrate, clear and mount.

Results: Structures containing SS groups (sulphur containing amino acids - cystine) appear pink or red.

11. Millon Reaction. - for proteins containing tyrosine. Remove wax from paraffin or freeze dried sections with light petroleum, rinse in absolute acetone, and allow to dry in air.

Next, cover the section with Millon reagent and allow to stand at room temperature until the maximum colour develops (this may be speeded up by placing in a covered Petri dish in the 60° incubator, when the reaction is completed in about 60 minutes). Rinse in cold or warm 2 per cent nitric acid; dehydrate rapidly in 70 per cent and absolute alcohol; clear in xylene, and mount in Canada balsam.

Results: Proteins containing tyrosine stain orange to rose-red.

Extraction Method for Crude Hormone Preparations: Urine.

(a) Saline Extract.
(b) Gonadotrophin - Extract A (Follicle Stimulating).
(c) Gonadotrophin - Extract B (Chorionic).

The twenty four hour specimen of urine was adjusted to pH 4.0 with 20 per cent HCl - all pH measurements made using a glass electrode and Marconi pH meter. Kaolin (BDH acid washed) was then added to the urine, in the proportion of 2 gms. Kaolin per 100 mls. urine and the mixture stirred for one hour. The kaolin was

allowed to sediment in the refrigerator. The supernatant urine was now sucked off and the kaolin centrifuged down, the supernatant being discarded.

(a) Extraction of Saline Fraction. The kaolin sediment was now washed with saline, three times, to give a total volume of 90 mls. of saline washings. This saline extract was adjusted to pH 5.5 with HCl and 5 volumes of cold acetone added. This mixture was allowed to stand in the refrigerator overnight to flocculate the precipitate. The precipitate which formed was centrifuged down and then washed with cold acetone (twice), ether (twice), and allowed to dry at room temperature.

(b) Extraction of Gonadotrophin (A) Fraction. Distilled water 100 mls. was added to the kaolin sediment and NaOH added until the pH was 11.5, and mixed by hand for 15 minutes. The mixture was centrifuged, after which the supernatant was collected. HCl was now added to this supernatant until the pH was 5.5, five volumes of cold acetone were added and the mixture

allowed to remain in the refrigerator overnight. On the following day the precipitate was centrifuged down and washed with cold acetone, ether, and allowed to dry. When the powder was free of ether it was dissolved in water and adjusted to pH 8.5 with sodium hydroxide. Tri-calcium phosphate gel suspension was now added and the mixture stirred. After 15 minutes the gel was centrifuged down and the supernatant collected. This supernatant is the Gonadotrophin (A) fraction, and was precipitated with 5 volumes ice cold acetone. After allowing to fully precipitate in the refrigerator overnight the precipitate was centrifuged down, washed with acetone, then ether, and allowed to dry off.

(c) Extraction of Gonadotrophin (B) Fraction. The gel was now mixed with 0.02 M $Na_3 PO_4$ solution (pH 11.7) stirring until a fine mixture resulted; this was stirred for 15 minutes. The mixture was now centrifuged to sediment the gel, and the supernatant kept. The gel was washed three times with

0.02 M $Na_3 PO_4$, 20 mls. each time. The supernatant washings contain the Gonadotrophin (B) - presumed to be chorionic gonadotrophin. The washings were adjusted to pH 5.0 and precipitated with cold acetone; the precipitate was washed with acetone, then ether, and allowed to dry.

By these methods of extraction it was possible to obtain from 1000 mls. of female urine the following weights of fractional extracts:

	Non-Pregnancy. mg. dry powder.	Pregnancy. mg. dry powder.
Saline Extract	57.7	46.9
Gonadotrophin-A	137.9	412.5
Gonadotrophin-B	212.1	499.3

For the animal experiments described in this Thesis the following weights of extracts were contained in 1 ml. saline injection:

RABBITS.

	Non-Pregnancy.	Pregnancy.
Saline Extract	0.6 mgm.	0.5 mgm.
Gonadotrophin-A	1.4 mgm.	4.1 mgm.
Gonadotrophin-B	2.1 mgm.	5.0 mgm.

RATS.

	Non-Pregnancy.	Pregnancy.
Saline Extract	0.6 mgm.	0.5 mgm.
Gonadotrophin-A	1.4 mgm.	4.1 mgm.
Gonadotrophin-B	2.1 mgm.	5.0 mgm.

MICE.

	Non-Pregnancy.	Pregnancy.
Saline Extract	0.6 mgm.	0.5 mgm.
Gonadotrophin-A	1.4 mgm.	4.1 mgm.

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