STUDIES ON THE HISTOCHEMICAL IDENTIFICATION OF MUCOPOLYSACCHARIDES.

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SYNOPSIS OF THESIS

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The initial intention was to investigate the effect of hyaluronidase on growing connective tissue and to elucidate its relationship to inhibition of connective tissue formation by adrenal corticosteroids. It has been shown that persistent application of the enzyme to granulating tissue causes rapid invasion of the surface exudate by fibroblasts and that the normal formation of bundles of reticular tissue is impaired. Investigation of the effects of a single application of hyaluronidase and the relationship of these effects to simultaneous corticosteroid application was not carried out as the histological techniques being used in the investigation were unsatisfactory.

Investigation of these techniques, the background of which is described, led to the adoption of newer ones. One of the techniques adopted was that of using water soluble polyethylene glycol wax as an embedding medium. It permits identification of lipids in serial sections and reduces losses and distortion of tissue constituents during embedding. While using this wax difficulties in sectioning were encountered and investigation showed that these were caused by high atmospheric relative humidities causing absorption of water by the very hygroscopic wax.

In order to eliminate chemical loss or alteration, or physical change in the tissue, freeze-drying was adopted as a method of preparation. Due to inadequate knowledge at that time of the problems involved the theory of tissue freeze-drying was entered into in some detail and a new apparatus, based on the theoretical findings, was designed and built.

Because of the lack of specificity of the histochemical chniques available for the identification of hyaluronic acid attempts were made to develope a new one. These attempts are described. The fact that exposure of conventionally prepared sections to sodium hydroxide, before periodic acid oxidation, greatly enhances the colour reaction obtained in certain tissues with the periodic acid-Schiff technique, was noted. The investigation of this effect is described and it is shown that formalin appears to affect certain substances, possibly by polymerizing them, so that they only react weakly with this technique. The alkali reverses this formalin effect so that they react strongly to the periodic acid-Schiff technique again. The relationship between this formalin effect and the chemical structure of the usbstances involved is discussed.

The conclusion reached is that hyaluronidase has a definite effect on fibroblastic infiltration and fibre formation in growing connective tissue but the relatioship of this function to the action of adrenocorticosteroids is not clear. In a histochemical study of this type it is essential to ensure that the substances involved in the study, hyaluronic acid in this case, are not being lost during preparation for microscopic examination, and that there is available a reliable technique for identifying them. As these two criteria were not satisfied in this case freeze-drying and the use of poly-ethylene glycol waxes were adopted in order that the first would be. Investigation of the second has shown that although there is no histochemical method for identification of hyaluronic acid available it might be possible to develope one based on the alkaline decomposition of its hexosamine content. Then it might be possible to return to a

accurate study of the inter-relationship of hyaluronidase and hyaluronic acid function in fibroblastic activity.

"My business is to teach my aspirations to conform themselves to fact, not to try to make facts harmonise with my aspirations. Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever nature leads, or you will learn nothing."

- Thomas Huxley.

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Preface.

The initial intention was to study the effect of hyaluronidase on growing connective tissues (Chapter I). By introducing the enzyme into granulation tissue it was hoped that some modification of hyaluronic acid production would occur and this would result in an alteration in fibre formation. Experiments based on this plan are described and it is shown that an effect is produced but technical difficulties prevented conclusions being reached. These difficulties were concerned with the preservation and identification of hyaluronic acid and similar substances, and it was felt that the conventional methods used were inadequate. Therefore an entirely new approach using different technique of preservation, embedding and staining was planned. Chapter II outlines the reasons for using these newer techniques.

One of the difficulties encountered was in the use of water soluble polyethylene glycol waxes as embedding media in identifying hyaluronic acid like substances and a short investigation into their use had to be carried out (Chapter III). The conventional methods of fixation were not considered adequate for the preservation of the substances of interest in the initial investigation and therefore I decided to use freeze-drying for preserving the tissues. As is explained in the text I had to carry out a considerable amount of personal research on the theory of tissue freeze-

drying, because at the time when the machine was planned there was not available any concise treatment of the subject. These theoretical and practical considerations are given in Chapter IV.

As the techniques available for the identification of hyaluronic acid in sections were considered to be inadequate an attempt was made to develop a new one. The use of an alkaline ferricyanide method for this purpose is described and the result, which showed that exposure to alkali has an effect upon the periodic acid-Schiff staining intensity of certain substances, are described in Chapter V.

In the last part of the thesis (Chapter VI) I have described the further investigation of the effect of alkali on periodic acid-Schiff positive substances and shown the relationship to formalin fixation. In it I have also made some suggestions about the possibility of developing new histochemical methods for saccharides.

The entire investigation has shown that although hyaluronidase has an effect on growing connective tissue it is not possible to determine the role played by hyaluronic acid. It is hoped however that, following the lines of investigation laid down here, some method of doing so may be developed.

The work described entailed the preparation, examination and reporting of over two thousand histological sections. Most of these are filed and are available for examination.

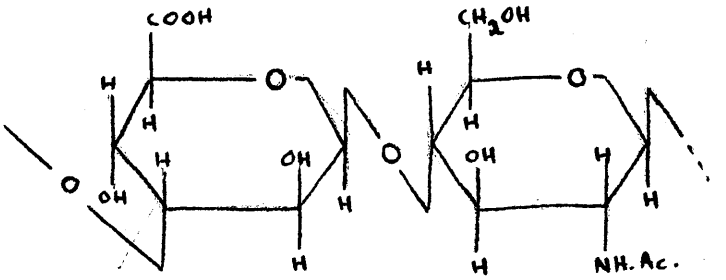
To produce photographs of the morphological and histochemical variations shown in these sections would be excessively expensive and time consuming, and would be pointless where a negative reaction is shown. Thus I have chosen only those microscopic illustrations which demonstrate the main theme of any particular section of the thesis.

I

EFFECT OF HYALURONIDASE ON THE GROUND SUBSTANCE OF CONNECTIVE TISSUE.

(a) The structure and function of hyaluronic acid.

The ground substance of connective tissue is believed to have a gel-like structure. (Clark and Clark, 1918, 1933; Laguesse, 1921; Bensley, 1934a; McMasters and Parsons, 1939;) Hyaluronic acid is a simple acid mucopolysaccharide found in that ground substance. (Meyer and Palmier, 1936; Meyer and Chaffee, 1941; Meyer, 1946; Day, 1947;) Its structure was first suggested by Meyer and Palmier (1936) and it is considered to be a protein free polysaccharide composed of glucuronic acid and N-acetylglucosamine in equimolecular proportions. The formula given below is that of Meyer and Fellig (1950).



Chondroitin sulphate which is often found in conjunction with hyaluronic acid is of very similar construction (Meyer, Odier and Siegrist, 1948).

Hyaluronic acid is thought to be produced by young fibroblasts (Meyer, 1946.) which secrete it into their surrounding space along with a precursor of collagen and a chondroitin sulphate. By local acidification in the immediate neighbourhood of the cells the first fibres are produced by the polysaccharide from the native soluble

collagen, which denature into the insoluble fibres, on the surface of which lies a sheet of polysaccharides. With ageing of the fibres, the polysaccharide layer becomes thinner and the hyaluronate is replaced more and more by chondroitin sulphate. Only in metabolically very active connective tissue like that of skin, does hyaluronic acid production continue in appreciable quantities.

Thus hyaluronic acid is seen to be part of the ground substance of actively growing connective tissue and is also found on the surface of immature collagen fibres. It is thus partially, perhaps incidentally, a 'spacer' lying between fibres.

Hyaluronic acid however is not the only mucopolysaccharide component of ground substance. According to Meyer and Rapport (1951) there are five components. In addition to hyaluronic acid there are three types of chondroitin sulphate and a hyaluronsulphate but of these only hyaluronic acid and chondroitin sulphate-C are found in skin.

(b) <u>The origin and function of hyaluronidase</u>.

Hyaluronidase is the name given to a series of enzymes of varying origin all of which can depolymerize hyaluronic acid. This group of enzymes was originally known as the 'spreading factor' of Duran-Reynals (1928) because of its ability to depolymerize the ground substance of connective tissue and permit easy penetration of the tissue by fluids. (Hobby et al, 1941).

Hyaluronidase has been isolated from testis (Duran-Reynals, 1928; McLean, 1930), from skin (Claude and Duran-Reynals, 1934; Meyer, Chaffee, Hobby and Dawson, 1941) from leech extract (Claude, 1939), from snake venom (Chain and Duthie, 1940; Favilli, 1940), from bacteria (Duran-Reynals, 1933; McLean, 1936; Meyer, Dubos and Smith, 1937; Meyer, Hobby, Chaffee and Dawson, 1940; McLean, Rogers and Williams, 1943; and Crowley, 1944), and from schistosoma (Levine, Garzoli, Kurtz and Killough, 1948.)

According to Meyer, Hobby, Chaffee and Dawson, (1940) testicular hyaluronidase affects sulphate-containing and sulphate-free mucopolysaccharides while the bacterial hyaluronidase affects particularly the latter. According to them hyaluronidase acts on hyaluronic acid in two different ways. It first causes a rapid depolymerization and second a slow hydrolysis. Meyer (1947) has suggested that two enzymes might be necessary for this action and the differences between testicular and bacterial actions might be due to the relative amounts of these two present in the parent substance. This is supported by the work of Hahn (1945), Rogers (1946,1948), Madinavietia, Todd, Bacharach and Chance (1940), Meyer (1950) and Dorfmann (1950).

The contamination of the earlier testical preparations with other enzymes (Meyer and Linker, 1951) has now been eliminated. M'Cullagh, Cassidy, Valentine, and Tolksdorf

(1949) provide information on the stability and stabilisation of testicular hyaluronidase.

The action of hyaluronidase is known to increase the permeability of connective tissues when the enzyme is applied locally (Seifter, Baeder, and Dervinis, 1949; Duran-Reynals, Bunting and Wagenen, 1950; Sprunt, 1950) and it has been suggested (see section (c)) that this function is required to allow rapid penetration of cells in actively growing tissue.

(c) The effect of hyaluronidase on growing connective tissue.

Hyaluronidase is present in physiological resting skin (Meyer and Chaffee, 1941; Meyer, 1947; Day, 1947.) where its function is probably to remove the hyaluronic acid from the surface of newly formed collagen fibres and permit its replacement by chondroitin sulphate-C. Bensley (1950) has shown that injection of hyaluronidase into resting connective tissue causes a proliferation of fibroblasts. In actively growing connective tissue its function is probably to depolymerize the ground substance and permit easy penetration and thus rapid growth of the organising tissue by blood vessels and fibroblasts, in the early stages. Its reduction in amount causes consolidation in the later stages and permits the hyaluronic acid removal from the surface of the new fibres.

Gersh and Catchpole (1949) have shown that fibroblasts

contain granules which may be the precursors of the ground substance of connective tissue. They state that in rapidly growing tissues the ground substance changes from a water insoluble to a water soluble compound and the fibroblasts present are rich in granules. In resting tissues they suggest that there is a constant depolymerization and removal into the blood stream, of part of the ground substance, and this is replaced by secretion from fibroblasts. In very actively growing connective tissues they suggest that this mechanism is increased.

It has been suggested (Duran-Reynals and Stewart, 1931; Boyland and McLean, 1935; and McCutcheon and Coman, 1947.) that hyaluronidase action plays a part in the rapidly invasive action of certain tumours and this is supported by the fact that water soluble alcohol insoluble mucopolysaccharides, similar to the breakdown products of ground substance, are present in large amounts in the circulating blood of patients having certain tumours. (Siebert et al, 1947; Winzler, Devor, Mehl and Smyth, 1948). In relation to this it has been noticed that the rapid passage of intravital dyes, into regions of inflammation and tumour formation (Duran-Reynals, 1939; Menkin, 1940; Cope and Marrow, 1943) may be an indication of depolymerization of the ground substance (Gersh and Catchpole, 1949).

Because of this relationship between hyaluronidase and rapid growth of tissues it was decided to study the effect of putting hyaluronidase into actively growing

connective tissue to see if it caused more rapid growth. It thus might produce more rapid healing of wounds and thus show that rapid formation of loose tissues produced a less dense granulation tissue and thus a softer scar.

The term granulation tissue was originally applied to the deep-red tissue of somewhat granular aspect, seen in the floor of a healing ulcer. Such tissue is comparatively cellular and very rich in young blood vessels, and the term is now applied to any newly formed tissue with these characters. The repair of a gap in the skin surface, however,produced, or of a wound which has failed to unite by primary union is affected by the growth of granulation tissue from the deeper parts. In the floor of the wound there is an active formation of new capillaries from the pre-existing blood vessels in the manner already described, and they grow upwards as vascular loops at right angles to the surface; at their upper extremity they often have a coiled arrangements. Along with them there grow a large number of fibroblasts which are at first mainly arranged parallel to the new capillaries. Active emigration of leucocytes, chiefly polymorpho-nuclear, takes place from the new capillaries, especially in their upper part, and these cells pass to the surface, where they play an important part in checking the growth of bacteria, and thus in the healing of the wound.

The growth of the vascular tissue is directed to filling up the gap, and when this process has gone on for some time,

the fibroblasts in the deeper part arrange themselves more or less parallel to the surface, that is, at right angles to the vessels, and collagen fibrils appear between them. As they mature they contract markedly and thereby reduce the surface area of the wound which has to be epithelialised. When the gap has been sufficiently filled up, the cells of the epithelium at the margin begin to proliferate and grow over the young vascular tissue. When the young tissue has been covered by epithelium, the growth of blood vessels comes to an end, and a process of obliteration of the new vessels, or devascularisation, sets in. This is accompanied by the formation of abundant collagen fibres which gradually become orientated along the lines of stress, and ultimately the tissue becomes dense and fibrous, and is comparatively avascular. This forms the 'white' scar tissue.

(d) Materials and Methods.

(i) Production of wounds. Standard wounds were produced in the necks of black and white rats using the method of Hutcheson (1943). This entailed making a small incision into the skin and subcutaneous tissues in the midline of the nape of the neck and inserting a small sterile cup of celluloid-like plastic into the wound. These cups, produced by the Viscose development company, are of a standard size. They were prepared as described by Hutcheson. They were plugged with cotton wool and held in place in the wound by stitches on either side, which also help to close

the wound. With practice the cups can be inserted easily and firmly and after the rats have recovered from the anaesthetic, given to them during the operation, they cannot remove them because of the position of the wound. Eventually, if no mishap occurs, the cups will be pushed out of the wound by the granulating tissue. Strict aseptic conditions must be observed as infection, apart from any general effect, will introduce a source of hyaluronidase locally which will invalidate the experiment.

(ii) Insertion of hyaluronidase. This method of standard wound production was originally designed to permit study of the effect of various chemotherapeutic agents on granulating tissue. These agents were applied by dropping a solution of them into the cotton wool plug in the centre of the cup and letting them diffuse through the semipermeable wall. In this case the large molecular weight (unknown but high) would prevent the diffusion and thus an alternative had to be used. The method chosen was very slow intradermal injection of the solution at a definite position in the wound area.

(iii) Hyaluronidase used. Hyalase (Benger) a testicular extract of hyaluronidase, was used. 500 units of this preparation were injected into the wound area as described. Control solutions were prepared by heating active solutions at 80 °C. for 20 minutes.

(iv) Method of carrying through the experiment. Ten male black and white rats of approximately the same size and age were weighed before the experiment started and then each was reweighed again before being killed. (Table 1.) All of them were operated upon as described above and one half received hyaluronidase and the other half received inactivated hyaluronidase. The injections were given immediately after the operation and every two days thereafter except on the day on which the animal was killed. A rat, from each series, was killed and the wound area dissected out for examination every second day after the operation. Thus a series of practically identically produced wounds, varying in age, by two day intervals from 2 to 10 days old was obtained.

A second series was carried out with sixteen male black and white rats of the same age and size (table.2.) These were injected each day and one from each series was killed each day. Thus a series of treated and controlled wounds was obtained varying, by 1 day intervals, from 1 - 10 days old.

Wounds were examined each day for infection. The rats were kept in individual cages and were fed with a stock diet.

(v) Histological methods used. As tough granulating tissue was being used the wounds were fixed in 12% neutral

formalin and carried through by a phenol and methyl benzoate method (Appendix.1.) in order to prevent undue hardening of the tissue.

The following staining techniques were used (Appendix.2.)

Haemalum and Eosin.

Mallory.

Weigert's elastica.

Gordon and Sweet's reticulum.

Periodic acid-Schiff.

Toluidin blue.

Sections from each block at various levels in the block were carried through these methods and were dehydrated in ethanol, cleared in xylol and mounted in D.P.X.

(e) Results.

Depending on the age of the wound being examined and irrespective of its treatment there were differences in the amount of granulation tissue formed. This variation corresponded to the normal process of granulation described above. With the Weigert, Periodic acid-Schiff and Toluidin blue methods no difference could be detected between the wounds treated with active hyaluronidase and the controls. In the Haemalum and Eosin, Mallory, and Reticulum methods there did appear to be a difference.

The treated wounds showed a tendency for the

advancing granulation tissue margin to have an infiltrating appearance. The fibroblasts instead of advancing in a compact line, as in the control wounds, appeared to send out 'scouts' into the surface serous exudate which was being organised. (figs. 1 - 4).

In the reticulum series there were obvious differences between the two types of older wounds. In the control series the reticulum assumed a more compact arrangement and the individual bundles appeared thicker and seemed to be composed of numerous smaller fibres lying side by side. Treated wounds had a very diffuse form of reticulum which appeared to consist of an irregular arrangement of fragmented individual fibres. (figs. 5 - 8).

The illustrations are characteristic of different stages in both series. There is apparent fibroblastic invasiveness in the early stages and a lack of consolidation of reticulum in the late stages of the treated wounds.

(f) Discussion.

The relationship between hyaluronic acid and hyaluronidase has already been discussed and it has been stated that injection of hyaluronidase into resting connective tissue causes a proliferation of the fibroblasts present (Bensley, 1950). Gersh and Catchpole (1949) discuss the variation in the organisation of ground substance in relation to fibroblastic activity and show that there is an interdependance.

Now it is known (Baker and Whittaker, 1949; Baker, Ingle, Li, and Evans, 1948; Ragan, Howes, Plotz, Meyer, and Blunt, 1949; Castor and Baker, 1950; Baker and Whittaker, 1950; Opsahl, White and Duran-Reynals, 1950; Pirani, 1951.) that adrenal cortical steriods have an inhibitory effect on the formation of connective tissue and stop invasion of the tissue by fibroblasts. It is also known (Layton, 1951) that cortisone has a retarding effect upon chondroitin sulphate synthesis and that adrenal corticosteroids reduce capillary permeability. (Menkin, 1940; Seifter et al, 1949; Opsahl et al, 1950.)

Thus the overall effect of corticosteroids is to reduce the production of new connective tissue. Benditt, Schiller, Wong and Dorfman, (1950) suggest that adreno-corticosteroids produce this effect indirectly by acting through an intermediate.

Now hyaluronidase is inhibited in vivo by adrenocoricosteroids (Seifter et al, 1949; Shuman and Finestone, 1950; Opsahl et al, 1950; Benditt et al, 1950). Thus it is possible that adrenocorticoids may be responsible for delay in wound healing under conditions of shock where there is always an excess of circulating adrenocorticoids, (Selye, 1947), because of their depressant effect on formation of new connective tissue. They may be producing this effect by inhibition of hyaluronidase (Seifter et al, 1949) which normally loosens ground substance, increases

capillary permeability and causes fibroblastic proliferation, all of which produce a loose actively growing type of connective tissue.

By giving hyaluronidase to the rats in this experiment it was hoped that the connective tissue depressant effect of the adrenocorticoids, released during the stress produced during and after the operation, would be overcome. This would then cause more fibroblastic proliferation, loosen ground substance, and increase capillary permeability and produce more rapid healing with a looser more pliable scar..

The experiment shows that there is a more diffuse and widespread infiltration of fibroblasts into the serous exudate being organised and that the reticulin formed in the granulation tissue is of a finer and looser type. This latter effect may be due to the persistent injections causing a continued fibroblastic stimulation thus preventing consolidation, because the fibres were not allowed to mature.

The experiment was inconclusive however for the following reasons.

(a) Physiological.

It would be necessary to study the effect of a single injection immediately after wound formation, since this might produce initial fibroblastic infiltration and then permit quicker consolidation. The question of the different hyaluronidase having different effects would also require to

be investigated, and the relationship between them and local and parenteral administration of adrenocorticoids would also have to be clarified.

(b) Technical.

It was hoped that the P.A.S. and toluidin blue techniques would give some information regarding variations in hyaluronic acid and chondroitin sulphate in the ground substance produced by the experiment. Very little reaction was observed in the ground substance at any time with these techniques and it was decided that this lack of reaction may have been due to

(i) loss of these materials during fixation and subsequent methods.

(ii) lack of sensitivity of the methods used.

Consequently it was decided that if any useful information was to be gleaned about the behaviour of these substances in connective tissues reliable techniques would have to be found and used. Thus the theory and technique of histochemical identification of tissue polysaccharides and of tissue preparation was entered into in some detail and the results of that investigation are reported in the rest of this thesis. The use of these new methods entailed a departure from conventional histology and I have outlined the justification of this in part II of the thesis.

II.

HISTORICAL INTRODUCTION TO DYNAMIC HISTOLOGY.

Although the magnifying properties of lenses have been known since the times of the ancient Egyptians it was not until Borellus of Pisa, in 1656, made some observations on pus corpuscles, that any form of microscope was used in examining the tissues of higher animals. Further observations on blood corpuscles were made by Swammerdamm (1658), and on the circulation and the minute structure of many tissues by Malpighi (1686). Leeuwenhoek (1674) made many observations on microstructure and is probably the best known of early microscopists.

The observations of these early investigators were concerned with function and one is constantly struck, in reading their reports, with the dynamic approach to morphology adopted by them and their immediate successors. Cameron (1952) presents and excellent review of early microscopy and histology and in it he outlines the work of these first investigators and shows how their living approach led first to the formulation of the cell theory and then to the classification of the tissues and organs. This classification produced the science of morphological hitology and it is to Schwann (1847) and Virchow (1860) that we owe a debt for this work.

Leeuwenhoek was the first person actually to stain tissue when he described, in 1714, the colouring of muscle fibres with saffron. Vieussens (1684) and Magnol (1709)

had used coloured injections to outline vessels but this cannot really be considered staining. Lewis (1942) has produced an excellent account of all the early staining methods and perusal of it will show how this first method led to the development of staining with natural dyes such as madder (Belchier, 1736), pernambuco wood (Riechel, 1758), carmine (Gleichen, 1778) and logwood (Knight, 1803).

It was also noted at this time that only dead tissues would stain and that alum could be used as a mordant to improve staining (Cotta, 1806). Thus the relationship between fixation and good staining was established.

During this time and in the ensuing years many natural dyes were used in identifying and classifying structures but the real era of morphological histology was not embarked upon until anilin dyes were introduced as stains.

Shortly after Perkin isolated 'mauve', Bencke introduced 'lilac anilin', which was probably the same dye, as a hiltological stain in 1856. Other dyes such as rosanilin, paris blue, and anilin (Waldeyer, 1863), fuchsin (Onimus, 1865), parme (Frey, 1868), daylia (Zupinger, 1874) and subsequently many others were introduced. Conn (1948) reviews the literature extensively and has placed the introduction and development of the use of these synthetic

dyes in their proper perspective.

Interesting publications were those of Cornil (1875) on metachromasia, Schwartz (1867) on double staining, Ehrlich (1879) on the difference between acid and basic dyes, and Van Gieson (1889) on triple staining. The development of these and many other techniques of a similar nature led to the appearance of a large number of publications on morphological observations.

While these investigations into the use of stains were progressing Walther Flemming (1882) was carrying out his researches into the effect of fixatives on structure and staining reactions. Baker (1945) in his excellent book on cytological technique reviews some of this earlier work which led to the appearance of the large number of fixative now in use. Among these are the investigations of Fischer (1897, 1899) which pointed out the great variation in appearance that could be produced by the use of different fixatives.

Combined investigations using the different fixatives and stains available led to the apprecation of artefact formation as distinct from true structure in the tissues and a vast literature on morphological histology appeared.

While these morphological techniques were appearing in large numbers a smaller but equally important number of publications on specific techniques were appearing. It is acknowledged (Baker, 1943, 1945; Pearse, 1953) that Raspail can be considered the founder of histochemistry.

He used, in microscopic studies on flowers (1825a, 1825b, 1829), the iodine reaction for starch first described by Colin and de Claubery (1814) and used by Caventou (1826) in microscopic studies. He also used the xanthoproteic test for protein (1829) the furfural test for carbohydrate (1829), microincineration and tissue pH testing techniques. His brilliant example was followed slowly but persistently by a small band of histochemists. The line of thought and investigation in these early times may be followed in the publications of Meischer (1874), Frey (1874), Lehmann (1851) and Bunge (1887). The early histochemical techniques outlined in these publications showed that at least some histologists were interested in the chemical structure of tissues and their physiological relationship to one another. The relatively small number of these publications was swamped however by the large number of morphological reports which were important in classifying the tissues and permitting the differentiation of the various abnormalities.

Mann's 'Physiological Histology' (1902) and Macallum's 'Methoden und Ergebnisse der Mikrochemie' (1908) with their details of the methods then in use, were two of the few publications on histochemical techniques to appear for many years. Those of Parat (1927), Patzelt (1928), Klein (1929), Hartwig (1929), Chamont and Mason (1930), Romeis (1932), Policard and Okkels (1932) and Linderstrom-Lang (1936) listed most of the specific techniques which

had appeared in the previous thirty years, but no great amount of work on histochemical techniques was carried out until Lison published his 'Histochemic Animale' in 1936. This book had a profound effect and marked the beginning of an era of notable advance in the science of histochemistry. From then until the present day a large number of investigations have been carried out on this subject and there are now available some excellent reference books (Glick, 1949. Danielli, 1953.) and text-books (Gomori, 1952. Pearse, 1953.)

The development of these techniques during the past twenty years showed that people were not just interested in what the morphology of a tissue looked like. They wanted to know of what it was built. There was also an awareness that histological studies were of little value unless they were correlated with the various cyclic physiological changes that occur in all organisms. Thus many of these studies were concerned with the influence of these changes on the result obtained with one or more techniques.

It was also appreciated that if important conclusions were to be drawn from tissue studies then fixation artefacts, of morphological and chemical nature, should be reduced to a minimum. Thus freeze-drying (Gersh, 1932) was developed to eliminate these artefacts. The problems of diffusion or non-reaction of tissues

components before and after fixation were investigated and Danielli (1953) has presented a valuable monograph on the subject.

While these histochemical techniques were being developed many other related methods appeared. Tissue cultureing methods became widespread and resulted in many interesting morphological and biochemical observations. Phase microscopy (Zernicke, 19) proved invaluable in qualitative studies of living and fixed specimens, and the ultra-violet absorption technique of Caspersson (1940) introduced a quantitative method for identifying nucleic acid and its precursors in intact cells. Visible light absorption methods were introduced by Stowell (1942), who used a spectrophotometric method for measuring the amount of nucleic acid in Feulgen stained tissue, and Engstromm (1946) re-introduced, in a more accurate and useful form the technique of X-ray absorption spectrophotometry. Autoradiography is by no means a new technique but it has become increasingly important in the past decade because of increasing availability of radio-active elements. We thus have available a method of accurately identifying the position of a fair number of elements in tissues.

All of these methods give a reasonable amount of quantitative data regarding the distribution of certain types of substances in cells. When used in comparing living and dead cells they provide some interesting

information regarding cyclic physiological variations, and losses during different types of fixation. The introduction of interference microscopy (Davies and Wilkins, 1952) as a quantitative method in histology opens up an entirely new field of research of this type.

Thus it appears that there is a return to the dynamic approach to histology (Bourne, 1953). Specific methods, with adequate controls, are being used. Fixation artefacts are being reduced, and attempts are being made to correlate microscopic identification of substances in tissue with physiological and biochemical data.

The development of electron microscopy during this time has again caused the pendulum to swing towards the side of morphology, but the problems of fixation, diffusion, etc., are tremendously increased in this technique and conclusions as to physiological significance must be guarded.

In order to understand these varied techniques a research student must be part chemist, part physicist, and part histologist. Without a histological training there is no foundation of knowledge on which interpretation of results may rest and new theories be built. It is the correlation of findings in these various fields and the elimination of errors, that were previously considered inevitable, that is the task of the histologist today and thus an unrivalled opportunity of carrying out interesting

and valuable research is offered.

In this thesis I hope to show how I have attempted to follow this plan in applying histological, physiological, biochemical and biophysical techniques to solve a particular problem.

III.

USE OF WATER SOLUBLE WAX IN THE IDENTIFICATION OF HYALURONIC ACID-LIKE SUBSTANCES.

(a) Classification of P.A.S. positive substances.

As described in chapter one, one of the methods used for identifying hyaluronic acid in tissue sections is the period acid-Schiff method of McManua (1946). This technique (Lillie, 1947; Hotchkiss, 1948; McManus, 1948.) is really a Malaprade reaction. (Lillie, 1950). It identifies .CHOH.CHOH.,CHOH.$CHNH_2$., and .CHOH. CHNHR. groups in polysaccharides, mucopolysaccharides, mucoproteins, glycoproteins, and glycolipids by production of aldehydes, through periodic acid oxidation, which colourize the Schiff aldehyde reagent. (See Section V(a) for discussion of the significance of this technique.)

(b) Identification of glycolipids.

If these substances are to be identified in a tissue it is necessary to use a method of preparation for microscopy which will not effect the lipid content of that tissue. Using frozen sections would permit this if only the P.A.S. method was used. However, some of the other staining methods being used, e.g. Weigert's elastica, preclude this. There would also be the added difficulty of obtaining serial frozen sections. An alternative is to use one of the newer water soluble waxes which permit fixation of the tissue in an aqueous solvent and transfer of it to the wax without subjection to dehydration and clearing. Because of the elimination of the use of alcohol and chloroform,

or other dehydrating and clearing agents, which are fat solvents, one can obtain a tissue, still containing its lipids, embedded in wax. This tissue block can then be serially sectioned in the normal way and any of the staining techniques applicable to paraffin sections used. There is some disagreement as to the efficacy of lipid preservation in water soluble wax sections (Firminger, 1950a and 1950b; Gersh, 1952; Rinehart and Abul-Haj, 1951a, Hack, 1951) and Miles and Linder (1953) state that although the lipid seems to be well retained in the tissue with their technique its staining reactivity seems to have undergone some change.

(c) Review of the use of water soluble waxes.

According to Carsten (1947) 'Polyethylene glycols are particularly suitable embedding agents because of their chemical inertness, noncorrosive nature, good thermal stability, high aqueous solubility, high molecular weight and low hygroscopicity. With increasing molecular weight the aqueous solubility and the hygroscopicity decrease and the melting point rises.' They were introduced by Richards, Anderson and Hance (1942) as embedding media and since then have been used by Carsten (1947), Blank (1949), Blank and M'Carthy (1950), Berlin and Brines (1951), Firminger (1950a and b), Van Horme and Zopf (1951), McLane (1951), Rinehart and Abul-Haj (1951a), Hack (1951), Gersh (1952) and

Wade (1952).

The polyethylene glycol ester waxes, which are not so water soluble, have been used by Orton and Post (1932), Cutler (1935), and Steedman (1945, 1947, 1949). They were introduced as an alternative to paraffin wax for routine preparations and their main advantages are that they produce less shrinkage during the preparation of the block and less compression of the section during cutting. They do not assist in the preservation of lipids.

The mode of action of the polyethylene glycols is that they infiltrate the wet tissue and progressively replace the water in it. The hygroscopic nature of the waxes permits this and it is sometimes referred to as a dehydration (Rinehart and Abul-Haj, 1951a; Van Horne and Zopf, 1951) although it cannot really be considered so (Miles and Linder, 1953). Depending on which particular wax is used the individual steps in the embedding technique vary. In most cases the initial steps are usually carried out at room temperature with low melting point waxes, or mixtures of wax and water, and the penultimate and final changes carried out at around 37°C. so that when the block cools the wax solidifies. Because the greater part of the technique is carried out at low temperatures, and because of the elimination of dehydrating and clearing agents, there is a minimum of shrinkage and hardening of the tissue. (Miles and Linder, 1953.) as compared with paraffin blocks.

(Patten and Philpott, 1921; Baker, 1945; Bolles Lee, 1946; Brain, 1949.) A further advantage in the use of the wax is a possible diminution of diffusion, loss or chemical alteration of tissue constituents normally caused by dehydrating and clearing agents and prolonged incubation in wax at higher temperatures. (Stafford and Atkinson, 1948; Firminger, 1950a and b; McLane, 1951; Miles and Linder, 1953.)

(d) Description of their use.

The method used was essentially that of Blank and McCarthy (1950) except that their mixture of 'Carbowax' 4000. (4 parts), and 'Carbowax' 1500. (1 part), was replaced by an identical mixture of 'Hydrowax'. This was the only polyethylene glycol of the same molecular weight available in the country at that time. (Polyethylene glycol "900" and Nonex 6B are now used.)

The tissue being prepared was fixed in 10% neutral formalin or any other aqueous fixative, until fixation was presumed to be complete. It was then removed and washed in running tap water for several hours. After washing, the tissue, suitably trimmed, was transferred to the molten mixture of wax and was agitated occasionally to assist mixing of wax and water. At least two changes of the wax, kept molten in a 37°C. oven, are desirable in an infiltration period of 1-3 hours. This period of time is sufficient for most tissues. Carsten (1947) and Van Horme

and Zopf (1951) have recommended the use of wetting agents to permit easier embedding, but I have not found them necessary.

After infiltration the specimen is transferred to an L-mould or paper box and fresh molten wax is added to form a block. The block in its container, is transferred to a cool dry place, such as a refrigerator, and allowed to harden for five to ten minutes. Contact with ice or water must be avoided because of the solubility of the wax. No difficulty was encountered with the crystal formation in the wax discussed by Hardkarni, Meyers and Zopf, (1949).

The hardened block is trimmed and fastened to a microtome specimen holder. During cutting the block and knife must not be chilled lest condensation interferes with the very hygroscopic sections. Numerous methods of mounting and flattening polyethylene glycol wax sections have already been described by the various investigators already mentioned. In this case it was found that the best method for mounting sections was to produce a very fine film of water, preferably with a spreading agent added, on a slide and to mount the sections on this cold slide and then dry them in a 37°C. oven. This method was found to be the least difficult and was accepted as routine practice.

While cutting these blocks, it was found, not infrequently, that it was impossible to cut satisfactory sections. It was found that they either crumpled up as a

homogeneous ridge on the edge of the knife or else disintegrated to a flat watery sludge on the broad part of the blade. As this only occurred on some occasions and not on others the cause of the variation was sought. It seemed not improbable that high humidity might lead to absorption of water by the surface of such blocks making cutting and subsequent manipulation of the sections impossible. Thus the following procedure was planned to elucidate the problem.

(e) Investigation into the difficulty of handling polyethylene glycol sections.

Tissues were prepared as described above. Sections were always cut from blocks prepared from the same tissues carried through at the same time by identical processes. The same knife was used throughout, being assiduously cleaned and stropped before and after each attempt to cut sections. A Unicam flat cutting rocker microtome was used. The blocks were sectioned in a windowless room sealed off from all outside contact except for one closely fitting door of refrigerator type. This door opened into a small anteroom separated by a small door from the corridor of a centrally heated building. The room contained a large electric fan which circulated the air through several baffles. Temperature was controlled by a 25 amp. electric heating element.

All estimations of temperature and of relative

humidity were made on an electrically driven Asmann aspirating hygrometer.

The humidity of the room atmosphere was varied by pumping water into it through a spray atomizer of the 'Flit gun' type. When required, the humidity was lowered by exposing large amounts of anhydrous calcium chloride to the air in the room. Although these methods could not provide absolute saturation or complete dryness of the air, the range of humidity could be varied from 30% to 90%.

While cutting the sections, care was taken not to breathe on the blocks in order to avoid any local rise in temperature or humidity. Sections were cut at 10μ, 7μ, and 4μ and it was noted on each occasion whether they would (a) cut and (b) ribbon. The least sign of cutting as single sections, or of ribboning in strips of four or more sections, was accepted as cutting or ribboning. In other words a 'perfect' section or ribbon was not necessarily required to demonstrate cutting ability under these conditions. Attempts were made to section the tissues at five separate levels of temperature, 17-18°C., 19-20°C., 20.5-21.5°C., 22-23°C., and 24-25°C. At each separate level of temperature the relative humidity was made to vary from 35% to 90%. There were thus three variables, and at least four attempts, usually more, were made at each thickness for each level of temperature at suitable intervals throughout the range of humidity.

If a number of blocks were made beforehand in preparation for cutting in the specially prepared room these had to be stored in a desiccator. If the blocks were stored in the usual small cardboard boxes they were often covered with a film of water which prevented cutting for some depth into the block. It was found, moreover, that blocks not stored in a desiccator gained weight. If these blocks were then put in a desiccator for some time and then reweighed it was found that they had lost the weight which they had gained. Blocks stored in a desiccator did not gain weight. If the blocks are covered with a film of paraffin wax by dipping them into the molten wax on a thread, they will be protected from this absorption of water.

(f) Results.

Table 3 contains the entire list of temperatures and relative humidities at which cutting experiments were carried out. After each temperature and humidity range a plus indicates cutting and ribboning, a plus-minus cutting only, and a minus neither cutting nor ribboning. The variation at different thicknesses are also shown in this table. Fig. 9 contains a graphical representation of the results. There is a curve for each thickness at which cutting was attempted i.e. 4μ, 7μ, and 10μ. The curves are drawn through the means of the upper and lower

points represented by the tips of the vertical arrows in the graph. At any one temperature, at relative humidities less than the values indicated by the tip of the lower arrow, ribboning and sectioning were both satisfactory. The tip of the upper arrow indicates the degree of humidity above which both ribboning and sectioning inevitably failed. At intermediate values, indicated by the interval between the two arrow heads, results were erratic, but as the humidity increased there was progressive deterioration. Ribboning first became doubtful and ultimately ceased, and then sectioning failed altogether.

If we consider the curve for sections cut at 7μ it will be seen that in the 24-25°C. range, the sections first ceased to ribbon at a relative humidity of 40% and that no sections were obtained at or above 56% relative humidity. At 19-20°C. there was a maximum permissible relative humidity of 55% for successful ribboning. In the 17-18°C. range it will be seen that there are no points for the 10μ curve. This is so because the blocks continued to section and ribbon even at the highest humidity obtained. In all cases it will be seen that, as the temperature drops, the maximum permissible relative humidity for obtaining ribbons and sections rises. It will also be seen that, as the thickness of the sections diminishes, the highest permissible relative humidity for a given temperature drops.

(g) Conclusions.

This area of the country has a temperate climate and high relative humidity. Thus not infrequently, conditions are present which prevent the use of polyethylene glycol waxes. This will be realised if it is remembered that even when sections and ribbons were obtained, they were, for the purposes of the experiment, not necessarily of the highest quality. To be sure of good histological results, the relative humidity should be well below the values indicated by the tips of the lower arrows. A convenient way of eliminating the influence of relative humidity is to work in a room with a temperature of 18°C. or less when the humidity can be allowed to reach a reasonably high value before impairing the quality of the sections.

Incidentally, the trick, beloved of technicians, of breathing on the block and knife while cutting tissue embedded in paraffin, is absolutely disastrous with this wax. The double action of raising the temperature and the relative humidity causes the sections to dissolve into a greasy sludge.

The deleterious effect of increased temperature, when the relative humidity is stable, may be partially offset by using a higher percentage of hard wax. There are limits to which this can be carried as the addition of even

a small amount of hard wax to the mixture makes the block too brittle for sectioning.

Preliminary checking of the temperature and relative humidity will help to avoid a lot of futile effort and offhand condemnation of polyethylene glycol wax as an embedding medium.

IV

USE OF FREEZE-DRYING TO ELIMINATE ARTEFACTS IN THE IDENTIFICATION OF PERIODIC ACID-SCHIFF POSITIVE SUBSTANCES.

(a) Disadvantages of using conventional fixation in the study of tissue chemistry.

Fixation is an attempt to preserve the tissue in as close a condition as possible to the living state, and present it for microscopic examination. As has been stated in chapter one numerous fixatives have been used in histology in the past because it was found that different structures were presented in better or worse condition depending on the fixative used. Gross morphological distortions, are produced in the tissues by all fixatives and it is some of the factors governing these changes that are now going to be discussed.

The main disadvantages in conventional fixation are

(i) Loss of materials.

(ii) Chemical alteration of materials.

(iii) Alteration of physical relationships.

These will now be considered in detail.

(i) It has been known for many years that certain fixatives will retain certain structures and substances in a tissue in a better state of preservation than others. For example glycogen has for some time been considered very soluble in water and may be dissolved out by aqueous fixatives. Consequently an acetic acid-formalin-alcohol mixture has always been recommended for glycogen fixation. Similarly alcoholic fixatives will remove lipids and certain watery

fixatives will remove certain mucins.

In this respect it has been stated that certain fixatives which do not appear to fix certain minute structures may not be guilty of this fault but merely may not have distorted an invisible living area so that it is visible as a dead area. i.e. producing an artefact that is accepted as a structure. This will be discussed later under 'alteration in physical relationships'.

The actual loss of materials from tissues during fixation has been studied quantitatively and Sylven (1952) has shown that there is a very high percentage loss of substances from tissue blocks during fixation and these extracted substances can be isolated from the fixative used. He states that during conventional processes of fixation, embedding, mordanting and staining there may be a total loss of mass of 40-60% of the tissue being prepared. Engstrom and Glick (1950) using their X-ray absorption technique have shown that there is a similar loss of mass of 10-30% from gastric mucosa cells during formalin fixation as compared with freeze-drying.

Qualitative observations on the loss of material from conventionally prepared tissues as compared with frozen-dried ones may be found in the publications of Bensley and Gersh (1933, a, b and c) Gersh, (1932), Hoerr, (1936), Julen, Snellman, and Sylven (1950), and Sylven (1950, 1951).

(ii) That fixation produces chemical alterations in the tissue has been known for many years (Mann, 1902.). The complexity of these alterations is so great however that very few details of the mode of alteration, in relation to the type of fixative used and the chemicals present, are known. The investigations of Tellyesniczky (1898, 1902, 1905), Hardy (1899), Fischer (1899), Mann (1902), and Berg (1903), showed that this was so and in later years Strangeways and Corti (1927), Gersh (1932), Bensley and Gersh (1933 a, b and c), Hoerr (1936), and Pischinger (1937) reinvestigated these problems and produced more definite information.

Knowledge of these effects is very incomplete but some useful guides to possible reactions taking place between tissue and fixative are available in the work of Cain (1949), Caspersonn (1950), Wolman and Greco (1952), and Pearse (1953).

(iii) One of the alterations in physical relationship which occurs during fixation is shrinkage and it is usually accepted as inevitable unless certain swelling agents e.g. acetic acid. are added to counteract the effect, but even these are not very effective. Berg (1908), Berthold (1882), Sjobring (1900), Sjovall (1906), Carleton (1922), Patten and and Philpott (1922), Tarkhan (1931) and Young (1935) have all shown that shrinkage occurs depending to a certain extent on the fixative and tissue used. The shrinkage produced by

fixation must not be confused with that produced by dehydration and embedding which has already been discussed.

Diffusion of substances from one point to another within a tissue during fixation and subsequent techniques is an artefact which has become increasingly important in connection with the use of specific methods in studying dynamic processes. Tellyesniczky (1936), Underhill (1932), Medawar (1941), Danielli (1953), Gomori (1952) and Pearse (1953) warn against it and suggest means of reducing the effect. It is of little use identifying a substance in a tissue if it is not in the position that it normally occupies in the living animal.

Frey-Wyssling (1948), has shown in a very lucid manner that some microscopic morphology may be pure fixation artefact and some may be due merely to an exaggeration, on a microscopic level, of an amicroscopic structure. e.g. the striation of collagen fibres.

To decide what is true morphology and what is artefact can really only be accomplished by examining tissues or cells in the living state and this is now possible using the newer forms of microscopy. Thus an object can be examined alive and then again after fixation and the morphological states compared.

It is inevitable however that, no matter how perfect the fixation and excellent the specimen appears to be microscopically, there will be alterations of molecular or

micellar relationships that are synonomous with fixation and death of the tissue.

It was in attempting to reduce these various factors to a minimum that freeze-drying was introduced and, as the possible loss of hyaluronic acid and chondroitin sulphate from the tissues used in this investigation was important, it was decided to use freeze-drying to prepare the tissues for examination.

When I first started to build the apparatus about to be described the publications of Malmstrom (1951), Gersh (1952) and Pearse (1953) had not yet appeared and there was not available in concise form the wealth of theoretical data presented by them. I gleaned what information I could from the publications available, and from textbooks of kinetic theory, but my knowledge of the problems involved was not at all clear in my mind until I started corresponding with Dr. Knud Max Moller of the Carlsberg Laboratorium Denmark. His lucid explanation, in the course of this correspondence, of the basic theory involved helped me considerably in formulating my own ideas of the subject, and I would here like to express my appreciation to him.

(b) History of freeze drying.

Altmann (1890) was the first person to use freeze-drying for the preservation of tissues and his method, inefficient though it was, was used by Mann (1902) and perhaps by others.

It was not however until Gersh (1932) introduced his freeze-drying apparatus that the method became a practical proposition. His lead was followed by others (Scott, 1933; Goodspeed and Uber, 1934; Hoerr, 1936; Scott and Williams, 1936; Packer and Scott, 1942; Gersh, 1948; Wang and Grossman, 1949; Glick, 1949; Mendelow and Hamilton, 1950; Jennings, 1951; Stowell, 1951; Malmstrom and Glick, 1952; Pearse, 1953; Moller, 1953; and Andrew and Hale, 1953, 1954.)

These various designs differed in many ways and the details of them will be considered in a later chapter.

(c) Theory of freeze-drying.

Although the earlier types of apparatus produced good fixation and drying the theory of the subject remained confused in the minds of biologists for many years. Freeze-drying of intact tissues is not quite so simple as freeze-drying of solutions or suspensions as practised in biochemical and industrial bulk preservation. A tissue is a much more complex system than a solution or suspension and as such must be treated by much more complicated processes. Although

these earlier methods were succesful they were very complicated or deficient in some respect and the times required for drying were unnecessarily long. If the basic theoretical principles are understood a comparatively simple apparatus can be designed and built which will dry tissues in a very short period of time.

The object of freeze-drying is to fix the material as quickly as possible after the death of the animal and to keep it fixed until dehydrated, with the minimum of removal or alteration of its constituents.

(i) Principle. If a tissue, or any substance, is frozen in order to fix it, ice will vapourise off, at room temperature, until eventually all water is lost. In the process of losing ice by vapourisation the tissue will heat up, the ice will melt and the tissue will no longer be frozen. Thus the tissue must be kept frozen in order to keep it fixed. When the tissue is frozen the rate of water loss from vapourisation of ice, at atmospheric pressure, is negligible and in order to increase the rate of loss the pressure must be lowered. Most solutions when frozen and subjected to a vacuum will remain frozen because of their very high heat loss from rapid sublimation of the ice at that lowered pressure, as long as the water vapour produced is removed from that environmental vacuum. Thus once frozen and subjected to a vacuum, from which the water vapour is continuously removed, they will remain frozen until dry, when they will heat up to room

temperature. Tissues however have a very low rate of sublimation and a fairly high heat gain from their environment and thus if frozen and placed in a vacuum free from water vapour they will not stay frozen but will heat up to the environmental temperature. Thus they must be kept frozen by placing them in a very cold environment.

(ii) Temperature of the tissue. If the tissue is to be kept frozen until dried what then is the best temperature at which to keep it? Altmann (1890) used -20° to -30° C. and since then various figures have been suggested. This difference of opinion as to the best temperature for preservation while drying (Gersh, 1932; Scott, 1933; Packer and Scott, 1942) centres round the argument about the eutectic point of tissue mixtures.

When a mixture of two liquids is cooled one of the components will freeze out first and will, in so doing, concentrate the other component. When this happens the freezing point is lowered and if cooling is continued the temperature is eventually reached where both components freeze out simultaneously and the whole mass then solidifies without a further fall in temperature. This is the eutectic point. The presence of various salts in solution modifies ~~and~~ the eutectic point. As tissues consist of very complex salt solutions whose eutectic points cannot be accurately determined this discussion as to the exact temperature is rather irrelevant but it will be seen that the lower the temperature

the better chance there is of good preservation. The temperature of the tissue is also important in relation to the degree of vacuum used and will again be discussed under that heading. Theoretically according to the possible eutectic point of tissue it should be dried at -50°C but no harm seems to be produced by drying at -30°C. If the tissue was not kept at or below the eutectic point while drying then the separation of salts might cause a highly localised diffusion artefact within the cells.

When the tissues are kept frozen and under a vacuum they will only lose water if the water vapour pressure of their ice is greater than the water vapour pressure of their environment. i.e. if the vacuum is kept relatively free of water vapour. Now the water vapour in a vacuum can be reduced by two methods, (a) by chemical desiccants or (b) by condensation in a cold wall trap.

(iii) Water vapour removal. The two methods of desiccation mentioned above can be applied in two different ways and it is that that I shall discuss now.

If any vacuum system is used it must be continuously evacuated by a pump in order to remove all the gases, absorbed on the surfaces of the system and being produced by the tissue, which are freed and tend to destroy the vacuum. This continuous pumping and release of gases causes 'streaming' towards the pump and if water vapour is

to be removed by a chemical desiccant or a cold trap they can be introduced into the system at any point between the tissue and the pump. If this method of drying is to be used two criteria must be satisfied if the system is going to be at all efficient.

Firstly the swept volume, i.e. the volume of gas removed from a system in a given time, of the pump must be sufficiently high to remove all the gas produced in the system in that time. According to Packer and Scott (1942) 1 sq.cm. of ice at a temperature of -60° C. and at a pressure of 10^{-6}mm of mercury will give off 1,600 litres per second. This figure is for ice and that for tissue will be lower, according to Gersh (1952), but even though it is the volume of gas to be cleared in a given time will be very large. To cope with this very large volume a mercury or oil diffusion pump backed by a mechanical pump will have to be used thereby introducing a costly and complicated apparatus.

Secondly there must be sufficient chemical desiccant or sufficient area of cold trap available to absorb all the water produced by the system.

If both of these criteria are satisfied then a 'streaming' system of water vapour removal will be quite efficient.

If the chemical desiccant or the cold wall trap is placed close to the tissue being dried then the efficiency

of the system does not depend on the swept volume of the pump but will depend on the mean free path of the water molecules leaving the ice of the tissue.

The mean free path of any gas is an estimate of the average distance that two or more molecules of that gas will travel, at a particular pressure, before they will collide and thus be arrested in their movement. If the mean free path is greater than the distance from the tissue to the desiccator then the rate of movement of water from tissue to desiccator will be high. The mean free path is dependant on the vacuum: The lower the pressure the greater the mean free path. The approximate mean free path of water molecules at a pressure of 10^{-2}mm Hg. is 5 mm, at 10^{-3} mm Hg., 50 mm and at 10^{-6} mm Hg., 800cm. Thus depending on the degree of vacuum used the tissue can be closer or further from the desiccator as long as the distance from the tissue to the desiccator is less than the mean free path available.

If there is sufficient desiccant available and it lies close enough to the tissue then this provides a very efficient and rapid method of removing water from the environment of the tissue.

(iv) Desiccator requirements. The efficiency of any desiccant depends on the water vapour pressure at its surface.

The water vapour pressure of ice at -30°C. is 0.3 mm Hg. At -80°C it is around 4×10^{-4} mm Hg. and

at -195°C it is around 3×10^{-24} mm Hg. Thus we see that as the temperature of an ice surface drops so also does the water vapour pressure at its surface. If the tissue is held at a temperature higher than that of the wall of a cold trap it will have a higher vapour pressure and water will move out of the tissue environment and will condense on the wall of the cold trap. This will reduce the surface pressure at the tissue and more water will then move out, and the process will be repeated.

Previous considerations have shown that the tissue may be held at any temperature between -30°C. and -80° C. or lower without it coming to any harm. It is obvious, from the above argument, that the higher the temperature of the tissue the greater will be the water vapour pressure at its surface. Thus if the tissue is held at around -30°C. and the cold wall of the vapour trap is kept at -80°C. or less then there will be an appreciable gradient of vapour pressure from the tissue to the cold wall and water will move out. There is no great advantage in using a temperature below -80° C. for cooling the vapour trap as the relative differences in vapour pressure between -80° C. and -195° C. is only the difference between 0.0004 and 0.000000000000000000000003 mm Hg. which is really an infinitesimal amount. In addition the use of a very cold refrigerant, such as liquid nitrogen, to produce a temperature of -195° anywhere

near the tissue, as required in an efficient system, means that a far greater amount of energy must be put into the tissue to keep it around -30°C. where it will have an appreciable vapour pressure, than if the environmental temperature was only -78°C. Malmstrom (1951) considers the reduction of partial pressure offered by liquid nitrogen worthwhile but Moller (1952) agrees with the above statement.

When phosphorus pentoxide is used as a desiccant it is relatively efficient when dry because of its low vapour pressure (10^{-5}mm Hg.), but it rapidly becomes saturated with water and loses its efficiency and must be replaced.

(v) Vacuum requirements. If a dehydrating system is dependant on 'streaming' then the vacuum requirements of the pump are a high swept volume and a pressure low enough to permit rapid release of water from the ice in the tissue. If dehydration is dependant on the mean free path then only a sufficiently low pressure, independant of swept volume, is required.

The degree of pressure required to permit rapid movement of water out of a tissue is governed by the practical possibilities more than by the theoretical ones but it is very convenient that these two do not differ greatly.

The most efficient pressure is governed by the fact that the rate of loss of water vapour from a surface

into a perfect vacuum, according to kinetic theory is given by

$$m = \alpha P \left(\frac{M}{2\pi RT}\right)^{\frac{1}{2}} \text{ grams/cm}^2\text{/sec.} \quad \ldots\ldots ①$$

where m is the mass in grams, M the gram molecular weight, R the gas constant, T the absolute temperature, P the saturated vapour pressure in dynes per cm. and α a constant. If vapour is present above an ice surface at a pressure of P_v the resultant loss of ice is given by

$$m = 14.63\,\alpha\,\frac{P_s - P_v}{T^{\frac{1}{2}}} \text{ gram/cm}^2\text{/min.} \quad \ldots\ldots ②$$

where α represents the fraction of vapour molecules which recondense on the ice surface and both P_v and the saturated vapour pressure P_s are measured in mm of mercury. For a water surface α is approximately 0.01 but its value for ice is unknown. If P_s is 292 microns (its value at -30°C.) then nothing significant is gained by having P_v less than one micron, for their differences would not be appreciably increased even though P_v were equal to zero. At this pressure ($1\mu : 10^{-3}$ mm Hg.) which can be obtained with an ordinary rotary pump the mean free path of a water molecule is over 5 cm and the swept volume is dependant on the pump capacity.

Thus irrespective of the type of dehydrating system used, as long as it is designed to conform with the requirements stated previously, a pressure of less than 10^{-3}mm Hg. is not required if P_v is kept lower than P_s. Malmstrom (1951) sets this minimum practical pressure P_v at 10^{-5} mm Hg. and in discussing equation (2) shows that the rate of evaporation rapidly increases with rising temperature (T) and that a rapid rise occurs above -40°C. Thus the tissue should be held slightly above this temperature, which has previously been shown to be suitable in other respects.

Theoretically there is a slight improvement in efficiency if a very high vacuum (10^{-6}mm Hg.) system is used but there is no practical advantage. This is so because the main resistance to rapid dehydration is produced by the tissue itself. According to Gersh (1952) when tissue is frozen, ice separates out. In so doing, the cytoplasm is deformed and peculiar ice-crystal compartments are formed which give a honeycomb effect. The wall of the compartments is not continuous but is interrupted by a series of sub-microscopic apertures. The net transfer of water vapour through the apertures depends on the difference in its density on both sides of each compartment wall. As there is no net transfer in directions parallel to the ice-vapour interface, one must consider that drying takes place through a series of long, tortous tubes, perpendicular to the ice-vapour interface

and separated into compartments of porous membranes. This series of long microtubes should offer a considerable resistance to the diffusion of water vapour, a resistance which in fact exceeds that of the rest of the vacuum line under ordinary conditions.

Dr.Gersh presents the expression

$$r_o X + \frac{r_x X^2}{2} = p_s (t_1 - t_2) \qquad \ldots\ldots (3)$$

where r_o is the resistance to diffusion due to the apparatus, X is the thickness of the dry shell of the tissue, r_x is the resistance to diffusion due to the tissue, p_s is the pressure of saturated vapour at the temperature of the interface and $t_1 - t_2$ is the time of drying. By actual experiments he showed that r_x was approximately 4.5 x 10 mm Hg./hr/gm/cm. That is the resistance to diffusion caused by a shell of dried tissue 1 cm thick was about 100,000 times that of the rest of the apparatus. A shell of dried tissue 1 mm thick would have a resistance to diffusion of about 10,000 times that of the rest of the apparatus. These estimates applied when a vacuum of about 10^{-4}mm Hg. was being used. With a lower vacuum, the relative resistance to diffusion of the rest of the apparatus will increase. For example at a customary pressure of 10^{-3}mm Hg. the resistance of the shell of dried tissue to diffusion would be approximately 4 times that of the rest of the apparatus.

Thus there appears to be no advantage in using a vacuum lower than 10^{-3}mm Hg.

Malmstrom (1951) in considering Knudsen's equation(1)

$$m = \frac{4}{3}\left(\frac{2\pi M}{RT}\right)^{\frac{1}{2}} \frac{r^3}{L}(p_1 - p_2) \qquad \ldots\ldots (4)$$

where r is the radius of the tubing through which the gas flows L is the length of path from sample to trap, p_1 is the actual pressure in the system, p_2 is the partial pressure at the trap and when r is small compared to the mean free path available, for example 3 cm, states that if p_1 is assumed to be so small that it can be neglected, as considered above, and L is smaller than r, then the diameter of the tubing (2r) used is a very important factor in governing the drying time of the tissue. He states that it is an undoubted advantage to have as large a radius as possible as long as the pumping speed is good enough to produce the required vacuum at this radius, and this degree of vacuum makes L smaller than r.

(d) Previous designs.

Altmann introduced freeze-drying in 1890 and the

(1) International critical tables. Vol III p.385.

essence of his technique was that he froze the tissue and kept it at -20° C. in vacuo over sulphuric acid until it was dry. This method was advocated by Mann (1902) who dried the tissue at -30°C.

The method was re-investigated by Gersh in 1932 and put on a more practical basis. His apparatus depended on the production of a very high vacuum and large swept volume, produced by a diffusion and a mechanical pump, and dehydration was carried out by streaming over a phosphorus pentoxide trap placed some considerable distance from the tissue. The tissue was held in a tube immersed in a refrigerant at -40°C. The complicated glass tubing system was long and narrow, thus increasing the resistance to diffusion, and the high vacuum used was obviously inseparable from the high swept volume required in such a system. The drying time was very long.

The apparatus used by Scott (1933) for microcinceration studies was essentially that of Gersh and no further details were given. Goodspeed and Uber (1934) improved on this type of apparatus by producing a much simpler and inexpensive type which depended on dehydration by absorption onto the phosphorus pentoxide covered walls of the tube in which the tissue was lying. The tissue tube was immersed in a refrigerator, at between -32 and -22°C. The tubing was short and the vacuum (10^{-3} mm Hg.) was produced by a mechanical pump only. The distance from tissue to desiccant was within the mean free path available in the system as the authors stated.

The drying time was said to be 1 - 2 weeks. This appeared to be a reasonably efficient system.

Hoerr (1936) produced a rather confused argument about various details of freeze-drying and mentioned only certain points about his own apparatus. It required one mechanical and three diffusion pumps to produce a vacuum of 10^{-6} mm Hg. in a tubing system of unknown dimensions. He used phosphorus pentoxide, placed some distance from the tissue, as a desiccant and stated that it required to be changed several times during a run. He also used a cold wall trap some distance from the tissue and stated that solid carbon dioxide was a poor desiccant in the trap but liquid air was good. He held the tissue at -33°C. and took 2-3 days to dry it. He makes no mention of a temperature regulator or the type of refrigerant used.

Scott and Williams (1936) produced the apparatus which has become the model for many other unpublished types. It was basically simple and depended on the production of a vacuum of 10^{-3} mm Hg. by a mechanical pump. The tissue was held in the centre of a heater so that the distance from the tissue to the wall of the tube in which it was lying was less than the mean free path available. Thus the tissue dried by sublimation to the wall of the tube which was cooled to -78°C. by a mixture of solid carbon dioxide and alcohol. There was however no provision for

preventing water, not trapped by the cold wall from diffusing into, and then perhaps back from the general tubing system. This would cause increased drying time because of inefficient vapour trapping and unless a phosphorus pentoxide trap was placed between the tissue holder and the pump might cause damage to the pump. The temperature of the tissue depended on the energy production in the heater which was regulated by an alcohol-mercuty thermostat. No means of measuring the vacuum was found necessary or desirable. This appeared to be a well considered practical attempt to design an efficient system.

Packer and Scott (1942) produced a very complicated apparatus which required a mechanical and two diffusion pumps to produce a vacuum of 10^{-6} mm Hg. The temperature regulating mechanism, which was a gas expansion chamber lying around the tube in which the tissue lay, was complicated and drying depended on streaming over phosphorus pentoxide placed some distance from the tissue. It was open to the same objections as that of Gersh. They made an attempt to determine when the tissue was dry by measuring the pressure gradient along the tubing while water vapour was still coming off the tissue. This was done by using two ionization guages inserted at different ends of the tubing. These gave different readings until water vapour stopped coming off when the readings equalised. This device was complicated, expensive and of doubtful efficiency. This apparatus

is also described by Hoerr and Scott (1947).

Gersh produced a new apparatus in 1948. It required a mechanical and a vapour pump to produce a vacuum of 10^{-6} mm Hg. in a very long narrow tubing system. The tissue tube was immersed in a refrigerator at the required temperature and drying depended on streaming over phosphorus pentoxide which lay at some distance from the tissue. The pressure was measured with a McLeod guage. This appears to be merely a slight modification of his original design.

In 1949 Wang and Grossman produced a very simple apparatus that was a combination of the Goodspeed and Uber and the Hoerr types. It required a mechanical and diffusion pump to provide a vacuum of 10^{-6} mm Hg. The tubing was short and drying was dependant partially on sublimation to the chemical desiccant lying very close to the tissue and partly on streaming to a cold trap was placed some distance away. The temperature of the tissue was controlled by changing the refrigerant in which the tissue tube was immersed. It was normally held at $-31^{\circ}C$. The vacuum was measured by a high frequency spark coil. No means of embedding the tissue within the apparatus was provided.

Mendelow and Hamilton (1950) produced the first 'cold finger' type of apparatus which consisted of a large flask with the tissue in it, immersed in a freezing bath

at around -40°C. This was subjected to a vacuum of 0.2 x 10^{-4}mm Hg. with a mechanical and a diffusion pump. A smaller flask (cold finger), containing liquid nitrogen at -195°C., projected into this larger flask, close to the tissue and the water sublimed from the tissue to this cold wall. This it did rapidly because the distance from cold wall to tissue was much less than the mean free path available. The apparatus had no means of estimating temperature or vacuum but was very simple, cheap and efficient and utilised the authors' knowledge of the principles involved. Jennings (1951) has described an apparatus which is very similar to that of Wang and Grossman despite his reference to the more efficient one of Mendelow and Hamilton.

The method of Stowell (1951) utilised very short wide bore tubing with the tissue suspended on a heater hanging in the dehydrating tube. This tube was immersed in a refrigerant at -78°C. or less. The water vapour from the tissue, held by the heater at around -40°C., was prevented from escaping into the rest of the tubing by an overlapping vane deflector. The vacuum of 10^{-6} mm Hg. was produced by a mechanical and a diffusion pump. The tissue was dehydrated by sublimation to the cold wall of the tissue tube, which was a shorter distance from the tissue than the mean free path available. The temperature of the heater, and thus the tissue, was varied by a rheostat. There was no

provision for vacuum testing. The drying time was very short and embedding could be carried out in situ. It was an excellent design and the author obviously understood the principles involved.

Glick (1949) refers to the apparatus of Scott and Williams and gives no further details.

Malmstrom and Glick (1952) have constructed a freeze-drying apparatus based on the theoretical considerations presented by Malmstrom (1951). In the first model that they describe the vapour trap is situated in a tube other than that in which the tissue lies but it is still within the mean free path available. Their second model is very similar to those of Pearse (1953) which are based on the 'cold finger' type of apparatus, and their third model is a combination of the first two. The tissue in all three models is held in a tube immersed in a refrigerant at the desired temperature, -60° C. in this case. The apparatus operates at a vacuum of 10^{-5} mm Hg. produced by an oil diffusion and a mechanical pump. An ionization guage is used to estimate the degree of vacuum. The drying times quoted are very short and it is obvious that the authors have used their very full theoretical knowledge to produce a very efficient type of apparatus.

Pearse (1953) considers the principles of freeze-drying and reviews several types including one commercial model based on the design of Scott and Williams. He also

includes some details of his own modifications of the 'cold finger' type of apparatus. These designs use the principle introduced by Mendelow and Hamilton but are simpler to build and operate and have much smaller dimensions.

I have myself used a further modification of the 'cold finger' type, that was made available to me by Dr. L.G.E. Bell, King's College, London. It was extremely simple and consisted of a large flask, with the tissue lying in the bottom, which was subjected to a vacuum of 10^{-3} mm Hg. with a mechanical pump. A slightly smaller flask projects into it, as a 'cold finger', and lies very close to the tissue where it presents a fluted surface to the vapour to make available as large a condensing area as possible. (fig.27). The tissue can be warmed to room temperature or higher, as in any freeze-drying system where the tissue lies on the outside wall of a flask, by removing the refrigerant and heating the flask. The tissue can be embedded by melting the wax in the side tube shown and tilting it so that the wax runs down over the tissue. The apparatus appears to work quite well although no extended investigations were carried out with it.

Moller (1953) has constructed an apparatus very similar to that of Stowell and appears to be well pleased with the results produced by it.

Summary of the requirements for tissue freeze-drying.

The tissue must be kept frozen and its temperature must be below that of its possible euctectic point to prevent artefact formation, but higher than -40 °C. in order to give a high rate of vapour loss. The desiccant used should have a vapour pressure lower than that of the tissue at the temperature used and must be closer to the tissue than the mean free path, of water molecules, available so that water vapour will rapidly sublime from the tissue to the desiccant. The vacuum must be sufficiently high to present as large a mean free path as possible but need not be higher than 10^{-3} mm Hg. as the resistance to diffusion of water through frozen tissue at higher vacua is much greater than the relative resistance in any part of the apparatus. The vacuum tubing should be as short and as wide as possible and the diameter used only restricted to the mean free path requirements in the region of the tissue.

It should be possible to heat the tissue to room temperature or to embed it without altering the vacuum characteristics of its environment. A means of estimating the degree of vacuum in the apparatus should be provided.

(e) Present design.

(i) Aims. When the system about to be described was designed certain facts were borne in mind. The first of these was cost. Although some of the previous designs were efficient they were expensive to build and to run. In the present design I feel that both of these objections have been overcome. The entire apparatus cost well under one hundred pounds and the only running costs are the price of the small amounts of alcohol, solid carbon dioxide and electricity used.

If a very high vacuum is required a mercury or oil diffusion pump, and the concomitant connections to them, must be used. This produces a complicated and costly apparatus and moreover it has been shown above that there is no practical advantage in using a pressure lower than 10^{-3} mm Hg. as long as the vacuum system is designed to conform to that pressure. Thus it was decided that only a mechanical pump would be used.

As has been mentioned in the last section various devices have been used in the past as regulators controlling the temperature of the tissue. These, which will be discussed in detail under the design of the present regulator, are all open to objections thus a new system of temperature control using a 'thermistor', was introduced.

If results in tissue preservation are to be easily reproduced then the drying process must be easily and accurately

regulated, so that any temperature or time factor can be easily reproduced. The regulator provided should be completely automatic thus dispensing with any need to supervise its operation. This I hope has been achieved in this design.

Above all, the apparatus should be simple to build and to use. The simpler an apparatus is to build the easier it is to maintain and repair and thus the cheaper it is to run.

(ii) Design of vacuum system. As a mechanical pump producing a vacuum of 10^{-3} mm Hg. was being used the tubing system and vapour trap had to be designed to make full use of that pressure.

The apparatus is very similar to that of Stowell (1951) but varies from it in the following ways. The pump is an Edwards Speedivac 2S20 two stage rotary pump which has a swept volume of 20 Litres/min. and produces the vacuum required.

The pump is connected to what was originally an all pyrex glass system. The tubing is built in four parts. (fig.10.) The first part connects the pump to a Dreschel's bottle containing phosphorus pentoxide. This is a moisture trap to prevent contamination of the oil of the pump with water vapour when it is running open to the atmosphere

during testing, and starting and stopping, procedures. The third part connects this bottle to the chamber containing the tissue holder and vapour trap. All of the joints in this original system are B.24. ground glass joints. The pump connection is also of this type although being glass to metal.

This type of four part system with standard interchangeable joints was chosen so that initial costs would be low and any broken part could be easily and cheaply replaced. It also facilitates leak finding as will be described later. Before use all joints are ground in with jewellers rouge and then put together after having been thoroughly washed and thinly smeared with silicons grease.

The tubing system was kept as short and as wide as was compatible with the use of B.24 joints. If bigger joints had been used the dimensions of the first vapour trap designed would have been too large for the mean free path available. The diameter was only rigidly controlled in the vapour trap as will be described later. The overall length of the tubing is 60 cm. and the narrowest diameter is 3 cm.

An ordinary ground glass stopcock, on a short narrow sidearm of the third part of the tubing, was provided as an air inlet in the original system.

In the later system the third part was replaced by a metal section which was connected to the Dreschel's

bottle by a B.24. ground glass-metal conical joint and to the vapour trap by a flat surfaced glass-metal joint with a rubber seal. (fig.10.) The details of this metal section will be considered under the vapour trap.

The pump is rubber mounted in the base of the machine and the tubing system is held in place by a 'Lablox' arrangement of stands. When subjected to a vacuum the clamps are loosened slightly to let the joints take up and then are retightened. This system has been found to completely eliminate any possibility of fractures due to vibrations which tend to occur in a relatively rigid glass tubing.

Glass was chosen for tubing as it is easily worked and it permits leaks to be traced and the vacuum to be tested by a very simple means (see notes on operation). The ability to see if the phosphorus pentoxide is being contaminated is also at times an advantage.

(iii) Design of vapour trap. As a mechanical pump producing a vacuum for 10^{-3} mm Hg. was being used the vapour trap was designed with a shape and dimensions to take full advantage of this.

A cold wall vapour trap similar to that of Stowell (1951 was chosen. It has the advantages that

(a) The rate of condensation on a cold wall trap gives a very much higher rate of water removal than that

produced by a chemical desiccant as has been discussed before.

(b) A trap lying very close to the tissue permits a very much greater rate of water removal than one lying some distance from it as has been shown above.

(c) It is more efficient than the cold finger type of cold wall trap because the condensing surface lies all around the tissue instead of just above it.

(d) The coolant used for the vapour trap is also used as the refrigerant for the tissue at both functions take place in the same tube.

(e) It is simple to construct and handle. Chemical desiccants are messy to handle and often require replacing during a run.

The dimensions of the first trap designed were governed by the size of the B.24. joints used and were 4 cm. x 15 cm. The vane deflector fitted into the neck of the conical joint (fig.11). This deflector consists of three superimposed overlapping metal plates each shaped as a quadrant of a circle. Any gas which passes up the tubing from the vapour trap must follow a relatively tortous path between these plates and, as they are very cold when the machine is running, any water molecules will condense on their surface. Thus all water is kept in the vapour trap and yet the efficiency of the vacuum system is unimpaired

by throttling. The vane trap is mounted on a hollow metal stem which hangs in the centre of the tubing. The tissue holder with underlying heater is held at the bottom of this stem 5 cm. above the bottom of the vapour trap. The thermistor lies in the centre of the hollow stem with its sensitive tip projecting from the bottom in order to contact the tissue. The wire connections to the thermistor and heater also lie in the stem.

A short side arm of the tubing system arises just above the vapour trap. This is filled by a removable vulcanised rubber plug pierced by the connections to the heater and thermistor. (fig.11).

This vapour trap produced reasonably satisfactory results but the narrowness of the conical joint at its lower end limited the size of heater and tissue holder which could be lowered into the vapour trap and so an alternative system was designed. It was here that the advantage of using interchangeable glass joints became obvious.

This newer system is shown in fig. 10. It consists of a metal section carrying the plug, with the heater and thermistor connections, and an air inlet. It replaces the third and fourth glass sections of the original system and is connected to the Dreschel's bottle by a ground glass-metal joint. It is connected to the vapour trap by a flat machined metal surface with a lip at the edge, which is

separated from the flat machined glass lip of the upper end of the vapour trap by a very thin, non-porous, pliable, but stiff, rubber gasket. The vapour trap is held tight on this surface by atmospheric pressure as soon as the vacuum is formed. The success of an air tight seal at this point depends on perfect machining of both the glass and metal surfaces and a very fastidious choice, noting the points mentioned above, of rubber gasket. The vapour trap is a straight tube 20 cm. long and 4 cm. diameter.

Some considerable trouble was encountered in both designs in finding a vacuum tight method of introducing electrical connections into the vacuum system. A glass 'pinch' is the obvious choice but its permanency is a drawback if a change of parts is required. After experimenting with several devices the following type was found foolproof.

A vulcanite plug is machined to fit exactly a conical joint of either glass or metal, which is introduced into the wall of the tubing near the vapour trap. Four holes are bored in this plug and tapped to take four long 4 B.A. brass screws. The internal connections are fastened to these screws and then the plug is fitted. This is best done by heating the conical hole with a small bunsen, running a film of molten beeswax and resin around the plug, and then pushing the plug into the cone while the rest of

the vacuum system is closed and the pump is running. While the plug is being pushed home the receiving cone should be heated to keep the resin and beeswax soft. Some more of this mixture should then be run over the upper surface of the plug around the projecting ends of the screws. This will give an absolutely vacuum tight seal. It is very important that neither the vulcanite or the resin and beeswax be overheated. If they are they will be carbonised and will become conductors and will short circuit the electrical system.

The dimensions of both traps described produce a distance between the centre of the tissue holder and the cold wall of the trap which is smaller than the mean free path (5 cm.) produced by the vacuum used.

(iv) Temperature regulator. Various types of temperature regulator have been employed in previous designs and they are all open to certain objections.

If a tissue is put in a constant temperature chamber (Gersh, 1932; Scott, 1933; Goodspeed and Uber, 1934; Hoerr, 1936; Packer and Scott, 1942; Gersh, 1948; Wang and Grossman, 1949; Mendelow and Hamilton 1950; Jennings, 1951; Malmstrom and Glick, 1952; Pearse, 1953.) its temperature stabilises at that level in a very short time and remains there. Its surface may be cooled to a certain degree by the water loss, and thus slow the drying process, but it is

certain that all of the tissue is at least as cold as the chamber in which it lies.

If the tissue is kept at a certain temperature level by a heater, lying in a much colder environment, (Scott and Williams, 1936; Stowell, 1951; Moller, 1953; Andrew and Hale, 1953,1954.) then there is the danger that if the heat is applied to only part of the tissue as is always the case, then that part may be heated above the critical level of diffusion artefact formation while the free surface of the tissue is very much colder. In the design of Scott and Williams the heater surrounds the tissue and partially prevents this. One way of preventing this artefact formation is to make the temperature regulator react to the temperature of the heater but then the surface of the tissue lying away from the heater will be much colder than the heated surface and water loss will be very slow.

If the tissue temperature is held above that of its immediate environment by a heater then it can be controlled by one of the following devices.

If a bimetallic strip is used as a thermostat it is not particularly efficient because of its large size, its liability to contamination with ice formation or carelessly handled embedding wax, and the fact that it must be fastened to the tissue holder some distance away from the tissue. It thus reacts to the temperature of the holder and not to that of the tissue. The bimetallic strip is usually coupled

directly to the heater and is fully automatic but operates at only one temperature. This method is used in the Edwards commercial tissue freeze-dryer which appears to be based on the design of Scott and Williams.

Another method of temperature regulation utilises a thermocouple which estimates the temperature of the tissue environment. This temperature can then be varied by altering the input to the heater. (Stowell, 1951; Moller, 1953) Again as the thermocouple is fastened to the holder, it does not really react to the temperature of the tissue. An additional objection to it is that it requires a lot of supervision, as there are temperature changes within the system as the tissue dries, and the mechanism is not automatic. It can however be controlled over a wide range.

If an alcohol-mercury expension thermostat is used (Scott and Williams, 1936) the reaction is again to the holder and not to the tissue and in addition, although fully automatic, it only operates for one temperature without complicated adjustments which cannot be made during a run.

None of these mechanisms reacts to the temperature of the tissue itself but only to that of the immediate environment. In this design I hope that I have overcome this difficulty.

The ideal system would be to have the tissue heated uniformly throughout and have a regulator reacting to the temperature at the surface and, by rule of thumb investigation,

find at what maximum surface temperature the system would operate without artefact formation occuring in the centre.

If the tissue could be heated by short radio waves and if the temperature regulator could be of a sufficiently different dielectric constant from the tissue so as not to react directly to the frequency used, but to react to the temperature of the tissue then we would have an ideal system. This would be especially so if the heating plates, focussed on the tissue, lay on the outside wall of the cold tube and thus there was no confining apparatus lying around the tissue slowing its rate of water loss. (Fig.12.)

In this present design a thermistor is used which comes into direct contact with the tissues and reacts to their temperature. The other advantages that it possesses as a temperature regulator in a freeze-drying apparatus are

(a) It is small (external dimensions of glass are 4 mm. by 70 mm)

(b) It is very sensitive. (Andrew and Hale, 1954)

(c) It is robust.

(d) The temperature sensitive tip is sealed in glass and is thus protected from contamination by ice, wax or tissue which might adhere to it and alter its sensitivity.

(e) It can be handled easily and is readily brought into contact with the tissue.

(f) It provides a fully automatic means of regulating the temperature.

(g) It produces, in a suitable circuit, a much greater change in output voltage for a given temperature change than would a thermocouple.

(h) Its tip has a very low thermal capacity and thus does not store heat which might raise the tissue temperature.

It has the disadvantage that some power must be dissipated in it, with consequent raising of its temperature above that of its surroundings, but this power can be very small.

A thermistor is an electrical resistor having a high negative temperature coefficient of resistance. The resistance R decreases with increasing (absolute) temperature T according to the relationship

$$R = ae \times p\,(b/T) \qquad \ldots \textcircled{5}$$

where a and b are constants. In a suitable circuit it becomes increasingly sensitive to temperature changes as the operating temperature is reduced. This is so partly because the temperature coefficient ($-b/T^2$) becomes numerically greater as the temperature falls. Another reason is that, since the thermistor resistance increases with decreasing temperature, the permissible applied voltage for a given power dissipation also increases, and provided the output of

the thermistor circuit is applied to a high-impedance load such as a tube grid, the variation in output voltage for a given change in temperature are greater at lower levels. (Andrew and Hale, 1954).

Since constructing the apparatus, Mr.A.Andrew, who designed the electronic circuit for this regulator, and myself have been informed by the makers of the thermistors that the thermistor type F 1512/300 is not considered to be the most suitable for use at sub-zero temperatures. For use in this range they have developed type F.2200, which has a much lower resistance than type F 1512/300 and also a smaller temperature-coefficient, at any given temperature. Because of these properties use of the type F 2200 thermistor simplifies the design of a circuit for use over a wide range of temperature, and we could have devised a circuit with less complex switching than that shown here in our final circuit (fig. 14.) had we used a type F 2200. On the other hand the sensitivity, in output-voltage change per degree centrigade, would have been much lower, and more amplification would have been required.

Fig. 13. shows a practical circuit giving regulation to a temperature in the range -45° C. to -28° C., depending on the setting of potentiometer P_1. It has been found that after subjecting tissues to temperatures in the range of -45°C to -28° C., we frequently required to raise their

temperature to -15 ° C., the rapidity of the change being unimportant, and then to raise it gradually from -15° C. to 15° C. Figure 14 shows the complete circuit of the regulator, which is designed to facilitate this procedure. A 12 position rotary switch is incorporated. In one position of the switch the circuit is effectively that of fig. 13. and the temperature may be regulated to any value between -45° C. and -28° C. by adjustment of P_1. This position is referred to as position 0. The other positions are numbered from 1-11. In position 1 the regulator controls the temperature to -15° C. and in position 11 to 15° C. The intermediate positions give approximately 3 ° temperature steps within this range. (For further details in the design of the circuit see Andrew and Hale, 1954),

(v) General layout. The entire mechanism is built into a box-shaped 'Dexion' framework trolley covered in on all sides except one. (figs. 15 and 16.) The pump and the wooden base for the clamp system are rubber mounted and the tubing is supported by a 'Lablox' system of rods and clamps. The Dewar flask containing the freezing mixture slides up and down, around the vapour trap, on the 'Lablox' rods and can be held in any position by two small clamps. An ordinary domestic wide mouthed 'Thermos' is used. The temperature regulator mechanism is housed in the top left hand corner of the trolley (fig.17.) and immediately below it is the switch for the Tesla

coil which hangs beside the tubing. The transformer for the heater circuit is mounted just below this immediately beside the junction box for the division of the power supply. Slots at the top of the box frame and the open bottom permit adequate cooling of the pump when it is running for a long time, thereby preventing undue consumption of the coolant in the Dewar flask. A perspex panel slides across the front of the box when required.

(f) Description of use.

Freeze-drying of tissues for histochemical examination is an exacting technique and demands constant attention to detail if a good result is to be obtained. The following detailed scheme of operation appears complicated at first sight but repitition of it soon resolves any difficulties. It is wise to plan and adhere to a definite technique so that any results obtained are easily reproducible. Reproducibility is the essence of success in any technique.

The liquid nitrogen, for freezing the tissue, must be ordered at least a day before it is required as it takes some considerable time to fill the double thermos in which it is transported.

Before starting to use the apparatus see that it is switched off at the mains.

(i) Fill the vapour trap thermos half-full with methyl

alcohol and add one or two lumps of solid carbon dioxide and leave the mixture until it reaches equilibrium at about -78° C. This may take an hour. The bottom of the thermos must not be obstructed by lumps of solid carbon dioxide or else the vapour trap cannot be properly immersed. Over-filling of the thermos will cause spilling when it is raised around the vapour trap. Place the glass tube of the vapour trap in this mixture until it reaches equilibrium temperature.

(ii) While this is happening renew the phosphorus pentoxide, or other desiccant, in the Dreschel's bottle and grease any joints that may be dry. (Ordinary grease will vapourise at high vacua and must not be used. Silicone grease is the most suitable for this purpose.)

(iii) Chill the isopentane container and tissue agitator (fig.18.) in the other thermos, filled to the brim with liquid nitrogen. (This substance is safe to work with. Tip it out of its container rapidly. Wear thick gloves to prevent ice burns from the spluttering liquid.) After the isopentane container is properly chilled fill it with isopentane. (This substance is highly inflammable and under NO circumstances must it be used with liquid air with which it forms a highly explosive mixture.) Isopentane is used because it only freezes at extremely low temperatures and has a very rapid heat loss. When tissue are plunged into it it does not bubble and produce a gas envelope around the warm tissue, which might slow their cooling. Leave the

container in the liquid nitrogen until the isopentane starts to solidify.

(iv) Lift the container out of the thermos just before the animal is killed so that the isopentane warms until it is liquid again. Its temperature will still be below -150° C.

(v) Kill the animal and rapidly remove the required tissues cutting them into slices up to 5 mm thick.

(vi) Plunge the slices into the container in the special agitator and swirl it round rapidly. Replace the container in the liquid nitrogen until the isopentane solidifies.

(vii) Remove the container and chill the entire tissue holder of the apparatus with liquid nitrogen by lifting the thermos up to it.

(viii) Rapidly transfer the tissues from the, by now, liquid isopentane to the chilled tissue holder and see that the top of the thermistor is lying against the tissues. All of this manipulation must be carried out with chilled forceps, to prevent undue heating of the tissue.

(ix) Put on the meains switch and start the pump.

(x) Connect the chilled tube of the vapour trap to the rest of the system and close the air inlet. Slide up the thermos containing the freezing mixture until the cold trap touches the bottom.

(xi) Add solid carbon dioxide to the thermos until the vapour trap is completely immersed.

(xii) Check the vacuum with the tesla coil and if necessary tighten up any joints.

(xiii) Turn the temperature control to its lowest reading and switch to controlled.

(xiv) Put on the electronic circuit switch when the mains pilot will light and a few minutes later the heater pilot will go on IF the tissue is at a low enough temperature. The lag is caused by the heating of the valves in the circuit. If the heater pilot does not go on at this time but only if the temperature regulator is turned to a higher level then it means that the tissue has heated to that level and must be rejected and the whole process repeated from the beginning.

(xv) Slacken all clamps on the glassware so that the joints draw up tight.

(xvi) The machine on an average requires to be run for 48 hours, if it contains 3 blocks of size 5 x 15 x 15 mm., in order to completely dry the tissue. During this time the thermos must be kept topped up with solid carbon dioxide. The level in this thermos will drop less quickly if the ratio of alcohol to CO_2 is kept high.

At the end of 24 hours the temperature control knob No.1. (fig.17.) should be gradually turned up to the upper limit. This should be done over a period of eight hours. It should be left overnight in that position. Next morning switch control knob No.2 to the first position and

and leave it there for an hour. Thereafter turn this knob one place every twenty minutes until it reaches the highest position i.e. at - 15° C. Leave it there for twenty minutes or more.

If embedding in vacuo is required either of two procedures are possible. First the tissue can, in the initial stages, be placed on the top of some solid paraffin wax in the embedding pan. This wax must previously have been degassed by melting it under a high vacuum or else when it is melted during embedding the gas coming out of it will bubble violently and throw the tissue out and ruin the entire process. With the second method a small blob of wax can be pressed onto a part of the side of the tissue holder slightly above the embedding pan in which the tissue is lying. When this melts during embedding it runs down into the pan in a very thin film and loses its gas from this layer, without any violent bubbling.

To embed by either of these methods turn the heater switch to free until the tissue is seen to be covered with wax.

(xvii) Tighten all clamps on the glassware, and switch off the electronic circuit when the mains and heater pilots will go out.

(xviii) Remove the thermos filled with the freezing mixture then open the air inlet and remove the vapour trap,

carefully, wearing thick gloves. Delay in removing the tissue after the thermos has been lowered away will cause water to move from the now warm vapour trap to the tissues.

(xix) Remove the tissues and switch off at the mains.

It is imperative that no time should be wasted at any stage in the initial preparation or parts may not remain cold enough for efficient working.

Vacuum testing, and tracing leaks, is carried out with a tesla coil which is a device producing a high voltage spark when earthed. At low degrees of pressure this spark produces fluoresence in the vacuum due to the ionisation of the gases present. The colour of this fluoresence changes from purple, through pink, to a very pale grey-blue as the pressure drops. At 10^{-3} mm Hg. or lower the fluoresence ceases. This provides a cheap and easy method of vacuum testing within the range 1 mm. - 10^{-3} mm Hg.

If the vacuum fails to reach the usual level it is due either to poor pumping or to a leak in the system.

The easiest way to find a small leak in the system is to dismantle all the parts of the tubing except the first part and to occlude the open end of the tube with a piece of thick flat rubber, start the pump and test with the tesla coil. If the part is air tight then the correct fluoresence sequence is seen. This procedure is repeated after replacing the various parts successively (fig.19.) until the part which

introduces a leak is found. When this part is found the leak can easily be detected by running a few drops of alcohol over the surfaces of that part while using the tesla coil. Where the alcohol vapour is sucked in through a leak it gives a white streaming cloud.

(g) Conclusion.

The apparatus has proved entirely satisfactory in operation and is being used for preparation of specimens for histochemical examination in this depart. Once the technique is learned the machine can be handled very easily and will produce good results even if left in the hands of a comparatively untrained person. It is extremely robust and in two years of running has only required the replacement of one very small part.

The histological preservation produced by it is very good and ice crystals artefact and cracking, of the type shown in fig. 22 is not commonly encountered. Figs. 20 and 21 show tissue prepared in the machine and it will be seen from these photographs that morphologically they leave nothing to be desired.

If very small pieces of tissue (3mm. x 5mm. x 5mm.) are individually placed in the machine they can be dried in about eight or ten hours depending on the tissue used, but normally several slightly larger pieces are inserted at

the one time and the machine is run for 48 hours. There is very little difference in cost between running for short and long periods as most of the money involved is in the liquid nitrogen used for the initial freezing and in the alcohol in the thermos.

V

INVESTIGATION INTO THE POSSIBILITY OF DEVELOPING A NEW HISTOCHEMICAL TECHNIQUE FOR HYALURONIC ACID.

Hyaluronic acid has already been shown to be one of the constituents of ground substance of connective tissue. As such it is often considered to be one of the class of substances loosely described as 'mucins'. In this case it is usually referred to as a connective tissue mucin. Other examples of these substances are the ground substance of cartilage, sarcolemma, basement membranes, colloid of endocrine glands, and the mucuc of epithelia. Histochemically they are related and the basis of this relationship is the presence of a carbohydrate found either as a separate entity or in combination with protein or lipid.

Many different histochemical techniques have been introduced for the identification of the varied members of this group of substances. Some of them are specific and some are not: At the present moment it is not possible to differentiate all the members of the group histochemically.

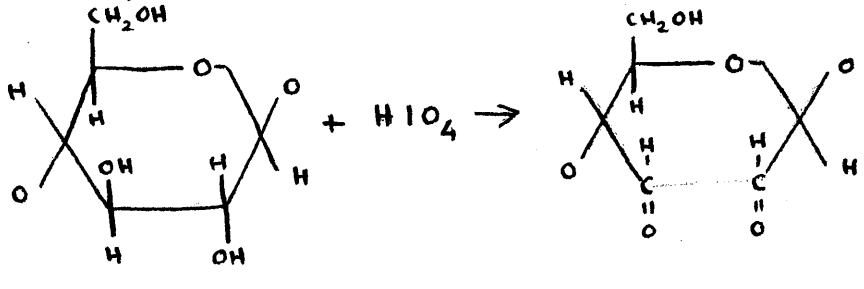
I shall now consider these methods in detail to show in which respect they are deficient.

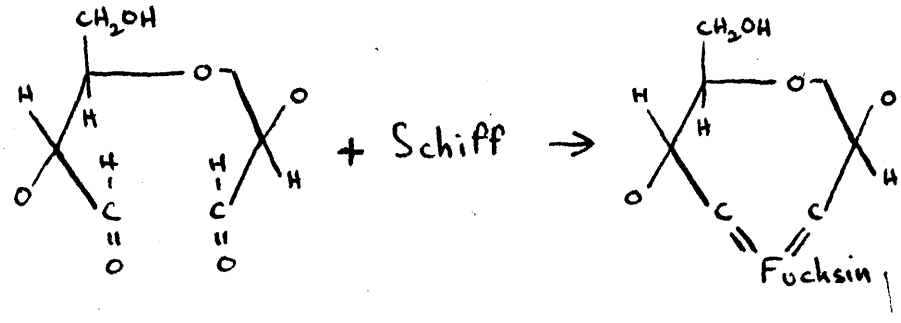
(a) <u>Lack of specificity of histochemical techniques for identifying polysaccharides containing substances</u>.

(i) The periodic acid-Schiff (P.A.S.) technique. This method was introduced as a morphological method for epithelial mucuc by McManus (1946). Lillie (1947) and Hotchkiss (1948), whose unpublished work on the technique antedated that of

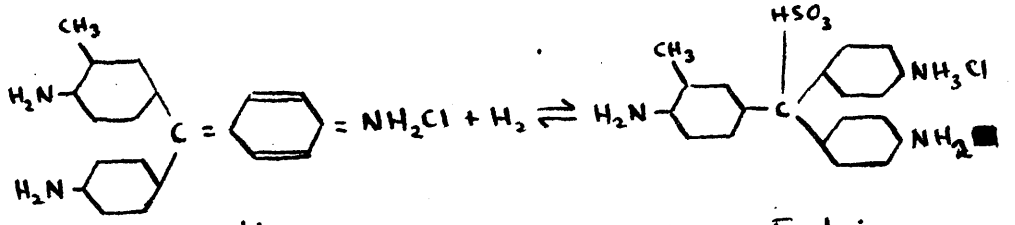
McManus, realised its histochemical significance and applied it to identifying a number of polysaccharide containing substances in the tissues. McManus (1948a) continued to use it as a histological method for some time but later stated that it could be used in histochemistry. (1948b).

It is really a Malaprade reaction (Malaprade, 1934) and identifies .CHOH.CHOH., .CHOH.$CHNH_2$., and .CHOH.CHNHR. groups (Nicolet and Shinn, 1939; Lillie, 1950) in polysaccharides, mucopolysaccharides, mucoprotein, glycoprotein, glycolipid, and certain other substances, by production of aldehydes, through periodic acid oxidation, which colourize the Schiff aldehyde reagent. Pearse (1953) states that 'compounds in which the glycol groups are substituted do not' give the reaction and Gomori (1952) states that if either the OH or NH_2 groups are substituted no reaction will occur. For the sake of completeness, I have accepted the possibility that substituted groups might react. These five substances mentioned by name all contain a hexose sugar of some type which is split by the periodic acid to produce aldehydes in the following manner

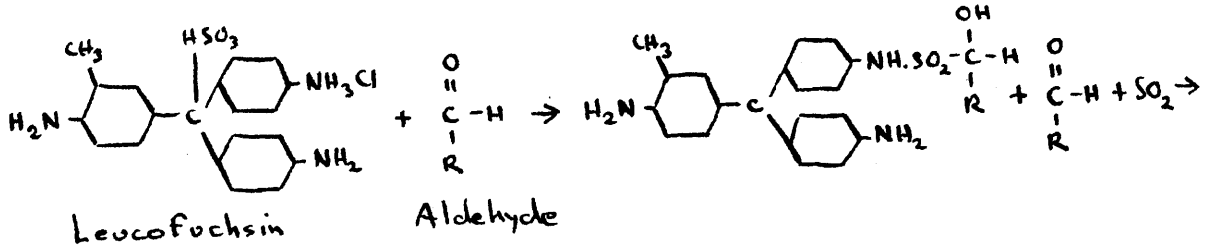


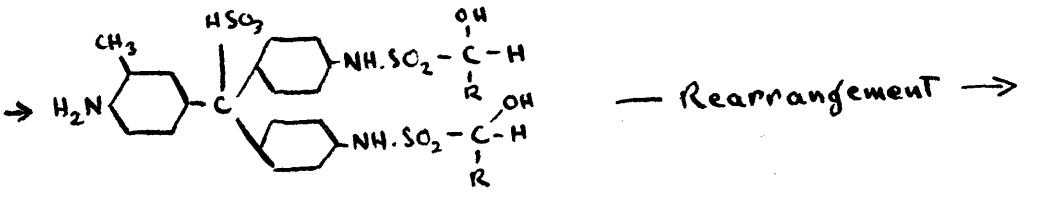


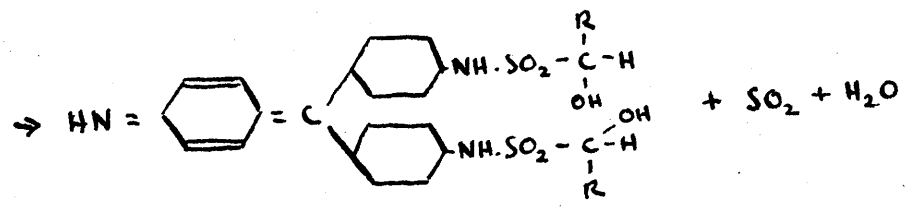
The mode of action of the colour production in leucofuchsin is not all clear but it may be of the following type. The dye used in preparing leucofuchsin is basic fuchsin which is a mixture of rosanilin, pararosanilin and magenta II. All of these are slight variations on the tri-amino tri-phenyl methane structure common to the rosanilin series of dyes. These dyes, in common with all chromophores, have unsatisfied affinities for hydrogen and are thus easily reducible and can be made to lose their colour. The double bond in the quinoid structure, found in the above mentioned dyes, may be broken and hydrogen atoms become attached to valencies thus freed and thus as the quinoid structure is los the dye becomes a colourless compound called leucofuchsin.



In preparing the reagent the basic fuchsin is reduced through the action of sulphite. It is not known exactly how this occurs but it is thought that the sulphite radical in some way enters into the composition of the leucofuchsin. Aldehydes produce colour in this compound by what is thought to be an addition followed by a condensation (Wieland and Scheuing, 1921)







Since the quinoid structure is restored to the compound it becomes coloured. In early preparations of leucofuchsin it was found that a brownish colour remained (Scanlin and Melin; 1937.) but Coleman (1938) showed that this could be removed by agitating the solution with carbon. Although most batches of basic fuchsin are now free from the contaminant which appeared to be producing this brown colour it is important to ascertain that only the best dye preparations are used.

As it is not known in what way the sulphite plays a part in letting the aldehyde colourise the leucofuchsin it is not safe to attempt to draw any conclusions from the different tints of recolourisation produced under different conditions. (Lison, 1932; Gomori, 1952; and Pearse, 1953.)

It has been suggested (Wieland and Scheuing, 1921; Lison, 1932 and 1936; Stedman and Stedman, 1943 a and b, 1944) that many substances other than aldehydes can produce colour in Schiff's solution but Glegg, Clermont and Leblond (1952) have shown that in relation to the P.A.S. technique, this is not so.

The coloured substance produced is a new compound and not just re-oxidised fuchsin.

The great value of periodic acid as an oxidant for these hexose sugars is that the aldehydes which it produces are not further oxidised by it and thus are all available

for colour production. This is not true of other reagents which are sometimes used to oxidise C-C bonds e.g. potassium permanganate, chromic acid, and hydrogen peroxide. Carbonyl groups are said to be oxidised to carboxyl groups and thus will not give a colour reaction with the Schiff's solution (Pearse, 1953). It has been claimed by Lohtka (1952) that depending on the time of exposure to the acid used it may be possible to differentiate between cis and trans glycols, but this has not been shown in histological sections. Danielli (1953) has suggested that these cis and trans glycols may be differentiated by blocking of the former by acetonyl or borate compounds.

Hotchkiss (1948) claims that positive results are only produced if the following criteria are satisfied:-

(a) The substance must contain one of the groupings listed above.

(b) It must not have diffused during fixation.

(c) It must not produce an oxidation product which is diffusable.

(d) It must be present in sufficient concentration to give a detectable final colour.

These last three criteria are common to any specific technique but it is useful to remind oneself of the necessity to observe them in this one.

The main representatives of the hexose sugar

containing substances in mammals are (Meyer, 1945; Pearse, 1953) :-

Polysaccharides	- glycogen
Mucopolysaccharides	- neutral - part of gastric mucin, blood group substances.
	- acid (simple) - hyaluronic acid.
	(complex) - mucin, heparin, chondroitin sulphate.
Mucoproteins	- submaxillary mucoid, serum mucoid, seroglycoid, pituitary gonadotrophins, chorionic gonadotrophins, fractions of pituitary secretion.
Glycoproteins	- serum albumin and globulin.
Glycolipids	- cerobrosides (kerasin and phrenasin).

Although it is accepted that these substances are the ones most likely to produce a positive reaction in tissues certain other theoretically possible positives must be borne in mind. Periodic acid will give a positive reaction in vitro with amino substituted alcohols such as those of serine, threonine, and hydroxylysine (Nicolet and Shinn, 1939, 1941; McManus, 1948B.) but they will not do so when the glycol groups are substituted, as when these amino acids are combined

in the polypeptide chains of proteins. (Pearse, 1953).

It has been suggested that certain unsaturated lipids and phospho-lipids will give a positive reaction with periodic acid technique. Hack (1949) has shown that kerasin, phrenasin, phosphatidylethanol-amine (kephalin) and phosphatidylcholine (lecithin), acetal and inositol phosphatides and ganglioside will give a positive reaction in vitro. According to Malaprade (1934) periodic acid will oxidise polyalcohols at the terminal .CHOH. groups in the chain to form aldehydes which will react with the leucofuchsin of the Schiff's solution. Inositol derivatives might react but inositol itself has no terminal group of this type and is converted into formic acid (Pearse, 1953). Wolman (1950) using preparations of cerebrosides, sphingomyelin, lecithin-kephalin mixture and saturated and unsaturated fatty acids, decided that unsaturated lipids stained and so also did sphingomyelin whether they contained carbohydrate or not.

It has been suggested (Jeanloz, 1950) that the presence of unsubstituted 1,2.glycol groups did not necessarily infer a positive reaction since substances such as celloboise gave a negative reaction, but this has been contradicted by McManus and Hoch-Ligeti (1952).

A distinct possibility is that pentose sugars with unsubstituted glycol groupings might give a positive reaction. It is known (Pearse, 1953) that adenosine-5-phosphoric acid

and adenosine triphosphate, in which the 1,2.glycol of D(-) ribose is free, give a positive result in vitro and in the position where they would be expected to be found in sections.

Thus we see that it is a possibility that a large number of widely differing substances might give a positive reaction with the P.A.S. technique, as long as they contain vicinal -OH or -OH and $-NH_2$ groups, and perhaps -OH and -NHR groups.

(ii) Metachromasia. A metachromatic reaction is seen when there is staining of a tissue component so that the absorption spectrum of the resulting tissue-dye complex differs sufficiently from that of the original dye, and from its ordinary tissue complexes, to give a marked contrast in colours.

It may be produced by a wide variety of dyes but those of the thiazin series are usually employed. The reaction was first observed by Cornil in 1875 and since then it has been used extensively in morphological studies. In more recent years the method has been used extensively in histochemical studies.

The first suggestion regarding its interpretation was that of Michaelis (1902) who thought that the different colour reaction with different substances might be due to formation of tautomers of the dye with these substances. Each tautomer possessing a different absorption spectrum

(Holmes, 1926). More recent work suggests that it might be due to polymer formation in the presence of certain chemical groups. (Kelly and Miller, 1935; Bank and Bungenberg de Jong, 1939; Hempelmann, 1940; Michaelis and Granick, 1945; Michaelis, 1947; Landswmeer, 1951; Massart, Coussens and Silver, 1951.)

The substances which produce this polymerisation, at least as far as they are found in histological sections, are thought to be polysaccharides containing uronic acids in addition to sulphate groups (Lison, 1935), or polymeric metaphosphate groups (Bank and Bungenberg de Jong, 1939; Michaelis and Granick, 1945; Michaelis, 1947).

According to the technique used the degree of metachromasia varies (Bensley, 1934; Feyrter, 1936; Bienwald, 1939; Herland, 1943; Hess and Hollander, 1947), and one must be guarded in drawing conclusions as to specificity when alcoholic dehydration of the section is used prior to examination (Michaelis, 1947). Sylven (1950) goes as far as to say that it is only safe to draw conclusions regarding positive and negative reactions when the section is examined in water. When examined in water gamma (red) metachromasia is probably due to sulphate esters; these are relatively alcohol resistant. Less alcohol resistant metachromasia and beta (violet) metachromasia can be caused by highly polymerized carbohydrate or phosphate containing

compounds, i.e. gamma metachromasia is thought to identify acid mucopolysaccharides and beta metachromasia is taken to indicate polymerized compounds containing carboxyl or phosphoryl groupings (Pearse, 1953). Sylven and Malmgren (1952) state that metachromasia 'cannot be used to distinguish between different polyelectrolytes.'

Nucleic acids according to some authors (Wislocki, Bunting and Dempsey, 1947; Pearse, 1953.) will stain metachromatically in paraffin sections but according to Michaelis (1947) they will only stain in an undehydrated state. The relationship between alcoholic dehydration and metachromasia remains a very confused question that has not yet been answered satisfactorily. It is considered in some detail by Pearse (1953).

The relationship between metachromasia and the P.A.S. technique is an interesting one and it will later be discussed in detail.

(iii) Dialysed iron method. This method which was introduced by C.W.Hale in 1946 depends on the combination of dialysed iron with the sulphate groups of mucopolysaccharides or with the uronic groups of hyaluronic acid where this occurs in its non-sulphated form, after suitable fixation. (This question of sulphated and non-sulphated forms of hyaluronic acid has already been considered in section one of this thesis.) The combined iron is demonstrated after

thorough washing of the tissues, by the Prussian blue reaction. Its successful use has been reported by Hudack, Blunt, Higbee and Kearin, (1949) and by Ritter and Oleson (1950), but its specificity is doubtful. (Lillie and Mowry, 1949; Pearse, 1953.) A modification of it introduced by Rinehart and Abul-Haj (1951b) does not seem to be any more specific.

(iv) Lipid methods. As has already been pointed out certain substances, glycolipids, unsaturated lipids and phospholipids, may give a positive P.A.S. reaction and if their presence is suspected at any site one of the following methods can be used to identify them.

(a) Comparison of sections from two blocks of tissue one of which has been subjected to a lipid extraction. (Baker, 1946; Roberts and Jarret, 1947).

(b) Staining of control sections, either paraffin or frozen, with Sudan black.

(c) Staining by the performic acid-Schiff method (Lillie, 1952; Pearse, 1953) which identifies unsaturated lipids.

(v) Hyaluronidase extraction. The relationship between hyaluronidase and the ground substance of connective tissue has already been discussed and will not be repeated here. This method has been used in an attempt to increase the

specificity of the P.A.S. and metachromatic reactions. It usually consists of incubating sections in a solution of the enzyme for a certain time at a certain temperature and comparing the subsequently stained section with a control. Where any positive colour reaction is removed then the site of that removal may be considered the site of a hyaluronidase sensitive material. The specificity of the method for removal of hyaluronic acid is open to doubt, depending on the technique used, and a very full consideration of its use is given by Pearse (1953). He considers that metachromatic material, in fixed tissues embedded in paraffin, whose metachromasia is reversible by a three hours incubation with purified testicular extract, is (a) of a mucopolysaccharide nature and (b) is either chondroitin sulphate or hyaluronic acid, or a mixture of these.

(vi) Chromic acid, potassium permanganate, lead tetra-acetate, and sodium bismuthate techniques. The method of Bauer (1933) utilised the hydrolysis and oxidation of glycogen and mucopolysaccharide etc., with 4% chromic acid and identification of the aldehydes produced by Schiff's reagent.

The Casella reaction (Casella, 1942) used potassium permanganate for the same purpose and gives a very similar result (Lillie, 1951b). Both of these techniques identify the same groups as do the P.A.S. method. They are open to

the objection that the polyaldehyde produced by them may be further oxidised to carboxyl groups and thus a potentially positive substance may give a negative result. Their potential specificity is the same as that of periodic acid but their practical application is not so reliable or so easy.

The lead tetra-acetate reaction (Crippa, 1951; Jordan and McManus, 1952; Shimizu and Kumamato, 1952; Lhotka, 1952a) identifies all groups detected by periodic acid but it will only produce aldehydes from hydroxylysine and not from serine and threonine, and it will only produce half as many aldehydes from a given number of .CHOH.$CHNH_2$. groups as will the periodic acid. In addition it will oxidise.CHOH. COOH. groups to produce aldehydes. (Fuson, 1950; Glegg, Clermont and Leblond, 1952). These different specificities produce weaker staining in the protein fibre background of sections and in certain structures, and stronger staining in certain epithelial mucins, when compared with the P.A.S. technique. The weaker background staining may be due to the fact that only bound hydroxylysine in the protein fibres may be reacting but bound serine, threonine and hydroxylyxine are not supposed to react with periodic acid in the tissue in any case (Pearse, 1953). The stronger reaction with periodic acid in kidney brush border for instance might be due to more aldehydes being produced from .CHOH. $CHNH_2$ groups present and the stronger reaction with

lead tetra-acetate in certain rectal goblet cells may be due to the presence of .CHOH.COOH. groups. A similar results is obtained with sodium bismuthate (Rigby, 1950; Lhotka, 1952b) The aldehydes produced in both these techniques do not appear to be further oxidised.

(vii) Glycogen methods. There are several ways of identifying glycogen in tissues.

The iodine method first used by Claude Bernard (1877) and subsequently utilised by others (Langhans, 1890; Driessen, 1905; Gage, 1917; Carleton, 1938; Mancini, 1944;) can be successfully used, despite its non-specificity for colour reaction, if a diastase control is employed.

The ammoniacal carmine method of Best (1906) is the best known and most widely used despite the fact that its mode of action is not understood. Other methods are the silver nitrate techniques of Mitchell and Wislocki (1944), Gomori (1946), Arzac (1948) and Arzac and Flores (1949) and the P.A.S. and other oxidant techniques mentioned above.

None of these methods is particularly specific for glycogen, except that of Best, and in all of them control sections, exposed to diastase must be used. Some authors (Bauer, 1933; Bensley, 1939) use salivary diastase others (Lillie and Greco, 1947; Bunting and White, 1950.) recommend malt diastase but both are equally good.

It has long been considered that glycogen is very soluble in water and many investigators (Arnold, 1908; Mullen, 1944; Lillie and Greco, 1947.) use celloidin covered sections to prevent its loss during staining, while others limit its exposure to aqueous solutions. (Langhan, 1890; Driessen, 1902; Gage, 1917; Carleton, 1938; Hotchkiss, 1948.) If celloidin is used in conjunction with the P.A.S. or associated techniques it will give a colour reaction due to its hexose nature, which will completely invalidate any observations.

(viii) Methylene blue extinction. This method, which was introduced by Pischinger (1926, 1927.) has been used by Dempsey and Singer (1946), Dempsey Bunting, Singer and Wislocki, (1947) and Dempsey, Singer and Wislocki (1950) as a quantitative spectrophotometric method of estimating the degree of basophilia of tissue compounds. It entails the use of extremely dilute solutions of methylene blue at various pH levels for 24 hours and comparison of the sections so prepared. Pearse (1949, 1950) has found the method particularly useful for differentiating between acid mucopolysaccharides and mucoproteins, and he states (1953) that if nucleic acids can be excluded the capacity to bind methylene value below pH 4 almost certainly indicates sulphate groups and thus acid mucopolysaccharides, except for certain lipid complexes.

By using any of the techniques listed above (ii-viii) a further differentiation of the groups of substances giving a positive result with the P.A.S. technique can be made.

Metachromasia ought to identify simple and complex acid mucopolysaccharides in dehydrated sections. The dialysed iron method has been stated to have identified the simple acid mucopolysaccharide hyaluronic acid. The lipid methods will identify glycolipids and other lipid containing substances. Hyaluronidase extraction might permit the identification of the hyaluronic acid-chondroitin sulphate group. Diastase will eliminate glycogen and the methylene blue extinction method will permit the identification of complex acid mucopolysaccharides with a reasonable degree of accuracy.

Thus glycogen, the glycolipids and other possible lipid contaminants can be definitely, and sulpher containing (complex) acid mucopolysaccharides possibly, eliminated from the classification of P.A.S. positive substances. This means however that some difficulty will be found in differentiating such substances as hyaluronic acid and chondroitin (simple and complex acid mucopolysaccharides of connective tissue origin which were the substances of initial interest in this investigation) and it will not be possible to differentiate between complex acid mucopolysaccharides such as epithelial mucin and similar secretion products in other

areas, and the mucoproteins and glycoproteins. Although the site of reaction often gives information as to which classification the substance belongs this is not a reliable method of differentiating between them.

It was thus felt that it would be desirable if some method of doing this could be found.

(b) Attempts to develop a new histochemical method for polysaccharides.

While considering the oxidising agents available for production of aldehydes from 1,2. glycols in polysaccharides it was brought to my notice that Munro (1951) had used an alkaline solution of nickel ferricyanide for this purpose. He found that this solution will react, at a rate depending on the temperature with certain polyhydric alcohols and their analogues, oxidising cis or trans glycols. It was not known whether substituted glycols were affected and whether or not the reaction stopped at the aldehyde stage. Of the many substances which give the reaction those of interest in the present discussion are serine, threonine and glucosamine.

It was felt that if the production of aldehydes by this reaction was sufficient to convert leucofuchsin to the coloured compound the technique might prove a useful

supplement to other methods for investigating carbohydrates in tissues.

Sections were deparaffinized taken to water and incubated in a mixture of one part $NiSO_4$ (10mg./ml.): five parts alkaline ferricyanide (0.2N NaOH as solvent) for times varying from 15 minutes to 48 hours at room temperature (approximately 19° C.) and 15 minutes to 4 hours at 37° C. Incubation for periods longer than these caused removal of all sections from the slides. After incubation in the alkaline nickel ferricyanide mixture the sections were washed and placed in Schiff's solution at room temperature for times varying from 15 minutes to 1 hour, and then were washed dehydrated and mounted. No colour reaction was produced and it was thus concluded that, either no reactive substances were present in sufficient amount, or insufficient aldehydes were produced, or any aldehydes which were being produced were being rapidly oxidised.

It was then supposed that the alkaline ferricyanide solution might act upon 1,2 glycols in certain substances and oxidise them, with destruction of the aldehydes, so that these particular glycols would not be available for subsequent oxidation by periodic acid. Thus the degree of reactivity with the P.A.S. technique might be reduced and the method made more specific.

Thus sections of a selection of tissues were treated

with alkaline nickel ferricyanide at the above times and temperatures, carried through the P.A.S. technique, dehydrated, cleared and mounted. The hoped for colour reduction was not seen but there was an increase in the intensity of the colour reaction of the mucus of the Brunner's glands of the rabbit duodenum and of the goblet cells of human rectum. Further investigation showed that the maximum change of colour could be produced by incubation of the sections in the solution at room temperature for 15 minutes.

The nickel is present in the solution as a catalyst and can be left out and the reactions observed by Munro can still be obtained but at a slower rate. It thus might be possible that a slower reaction might produce a different effect, especially with regard to the production and subsequent oxidation of aldehydes. Thus the above mentioned tests were repeated using the alkaline ferricyanide solution alone in a 5:1 mixture with water. The sections were carried through as before and the same observations were made.

As the ferricyanide and nickel ferricyanide solutions split 1,2-glycols and tend to oxidise the aldehydes produced they would be expected to reduce the intensity of any colour reaction not to increase it as has occurred. Realisation of this suggested that the colour change may have been due to the alkaline nature of the solutions.

Consequently the entire procedure was repeated using a 0.2$\bar{N}$ solution of NaOH in place of the ferricyanide solutions. This produced the same result as before. Because of this finding it was decided to investigate the effect of this procedure on numerous tissues before continuing to study the changes in P.A.S. positive material in healing wounds.

(c) Investigation into the effect of alkali on subsequent staining with the P.A.S. reaction.

(1) Materials and methods. As interpretation of the results depended on intensity of the colour reaction which developed, care was taken to subject all sections to identical procedures.

The periodic acid solution was prepared as described by Hotchkiss (1948) and was used in preference to an aqueous preparation because of the question of solubility in water of the substances involved. A time of 15 minutes for oxidation was used and was found to produce the same intensity of reaction as any other time at the temperature used. All oxidations were carried out at room temperature i.e. approximately 19^{o} C. The Schiff solution was prepared as suggested by Lillie (1951a) and modified to conform with findings of Longley (1952) and Atkinson (1952) in order to make the results more easily

reproducible. (Appendix III)

The reducing rinse suggested by Hotchkiss (1948) to remove periodate or iodate, which became attached to the tissues during periodic acid oxidation, and which in themselves produce colour in the Schiff solution, was not used. McManus (1948b) and Lillie (1951b) have shown that it decreases the amount of aldehydes available for colour production and my own findings, in agreement with those of McManus and Hock-Ligeti (1952) and contrary to those of Pearse (1953), are that it can safely be replaced by washing in water.

The sulphite bath used by Hotchkiss (1948), McManus (1946), and Lillie (1947,1948) can, I find, be safely replaced by washing.

Acetylation blocks .CHOH.CHOH., .CHOH.$CHNH_2$., and .CHOH. CHNHR. groups (Gersh. 1949; McManus and Cason, 1950) and prevents the production of aldehydes by subsequent periodic acid oxidation. De-acetylation returns the groups so blocked to their original potential reactivity. Acetylation of sections was carried out as described by Lillie (1951b). It was found that deacetylation in 75% ethanol and ammonia was more efficient than that in absolute ethanol and ammonia as used by Lillie. (Appendiz III).

Immersion of sections in hydroxylamine hydrochloride (Danielli, 1949), to block all aldehydes, was used to

demonstrate the specificity of the Schiff's solution and to identify the nature of the reaction product. (Appendix III).

The method for the Feulgen reaction, in so far as it could be carried out in paraffin sections, followed the instructions of Danielli (1949).

In all cases, batches of sections containing one slide from each tissue were carried through each procedure. Batches were placed in glass racks and times were accurately controlled. All sections, irrespective of the fixative used, were taken through iodine and thiosulphate when being brought down to water before staining. All solutions were renewed from standard stock bottles each day. Washing was carried out in running tap water. Glasgow tap water is very pure and constant in quality and no harm appears to be caused by washing sections in it (Garven and Gairns, 1952). Before and after the periodic acid solution the sections were washed in 70% ethanol. All sections were dehydrated in 90% ethanol and two changes of absolute ethanol, cleared in xylene, and mounted in D.P.X. Counterstains were not used. The tissues used were known to have a weakly positive P.A.S. reaction in at least a part of their substance. They were:-

Human Tissues fixed in 10% neutral formalin; heart (Lipofuscin), cervix, uteri, prostate, rectum, pancreas, trachea, submaxillary gland.

fixed in mercuric formalin: rectum, amyloid spleen, aorta, umbilical cord, amyloid liver, myxoma, heart valve.

fixed in Bouin's fluid: lung (pneumonic exudate), umbilical cord, small intestine, stomach.

Rabbit tissue — fixed in 10% neutral formalin: duodenum, kidney.

fixed in Bouin's fluid: bone marrow.

Rat tissues — fixed in 10% neutral formalin: eye, mesentery.

Representative batches of sections were carried through by each of the following procedures after having been brought to water. Room temperature (approximately 19° C.) was used except where otherwise stated.

(i) Periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(ii) Schiff soln. 30 minutes; wash.

(iii) 0.2$\bar{N}$ NaOH 15 minutes at 22° C; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(iv) 0.2$\bar{N}$ NaOH 15 minutes at 22° C; wash; Schiff soln. 30 minutes; wash.

(v) Acetylation; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(vi) Acetylation; wash; deacetylation; eash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(vii) 0.2$\bar{N}$ NaOH 15 minutes at 22° C.; wash; acetylation; wash; periodic acid 15 minutes; wash; Schiff sol. 30 minutes; wash.

(viii) Acetylation; wash; 0.2$\bar{N}$ NaOH 15 minutes at 22° C.; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(ix) Deacetylation 15 minutes; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(x) Deacetylation 24 hours; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(xi) Diastase (salivary) 30 minutes; wash; periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash.

(xii) 0.5% toliudin blue 30 min; wash.

(xiii) 0.2$\bar{N}$ NaOH 15 minutes at 22° C.; wash; 0.5% toliudin blue 30 minutes; wash.

(xiv) 0.2$\bar{N}$ NaOH 15 minutes at 22° C.; wash; periodic acid 15 minutes; wash; hydroxylamine 60 minutes; wash; Schiff soln. 30 minutes; wash.

(xv) Periodic acid 15 minutes; wash; hydroxylamine 60 minutes; wash; Schiff soln. 30 minutes; wash.

(xvi) Periodic acid 15 minutes; wash; hydroxylamine 24 hours; wash; Schiff soln. 30 minutes; wash.

(xvii) Hydroxylamine 60 minutes; wash; 0.1$\bar{N}$ HCl. 15 minutes; wash; Schiff soln. 30 minutes; wash.

(xviii) Periodic acid 15 minutes; wash; Schiff soln. 30 minutes; wash; in three changes of sulphite soln., 5 minutes in each change.

(xix) 0.2$\bar{N}$ NaOH 15 minutes at 22° C.; wash; periodic acid 15 minutes; wash; Schiff solution 30 minutes; wash in three changes of sulphite soln., 5 minutes in each change.

(2) Significance of methods and Results.

(i) This is the standard technique used to identify .CHOH.CHOH., .CHOHCHNH$_2$., and .CHOH.CHNHR. groups by production of aldehydes by periodic acid oxidation and identification of the aldehydes by recolourization of Schiff's solution.

(ii) Immersion directly in Schiff's solution, which gives no reaction, shows that none of the colour produced in (i) is caused by the presence of 'free' aldehydes in the sections or by spontaneous recolouration of the Schiff's solution. By 'free' aldehydes is meant substances of the nature of plasmal aldehyde. A full consideration of the identification of these substances is given by Cain (1949) and Pearse (1953).

(iii) This is the method which increases the intensity of the staining reaction of certain substances which otherwise give only a pale colour with procedure (i). The maximum amount of change from the standard appearance occurred in the first few minutes of incubation of sections in 0.2$\bar{N}$ NaOH at 22° C. Times of temperatures in excess of this produced no further change. When compared with the results from (i) the sections of human rectum and rabbit duodenum showed the most striking changes. The Brunner's glands of the rabbit duodenum changed from a faint pink colour with the standard technique to a deep magenta colour after treatment with the NaOH solution (fig.23 and 24.) The goblet cells of the human rectum changed from a pink to a very deep magenta colour (fig.25 and 26.) The fibrin clot of the pneumonic exudate of the lung increased slightly its depth of colour after treatment. In all the tissues there was slight increases in intensity of staining of reticulin, in certain irregular areas of collagen and sarcolemma, and in the amount of diffuse pink connective tissue background (ground substance).

(iv) Incubation of sections in NaOH, without subsequent periodic acid oxidation, followed by immersion in Schiff's solution was done to find out if aldehydes were being produced. No colour appeared.

(v) The acetylation blocks the periodic acid reactive

groups. Colour was not produced.

(vi) This is a check on the specificity of (v) by removing the blocking agent, and it produced the same colour reaction as (i).

(vii) The acetylation after incubation in NaOH was introduced to find whether the alkali was releasing groups, other than those enumerated above, which were subsequently being oxidised to produce aldehydes. No colour was produced. This showed that any groups released by the NaOH were of the above type.

(viii) It was possible that the action of the NaOH might only be a deacetylation, so this step was introduced. An excellent deacetylation, identical to that produced by the ethanol-ammonia mixture, was brought about, but in addition, increased staining of rectal goblet cell and Brunner's gland mucus was seen.

(ix) As the NaOH can deacetylate this step was carried out to show that the cause of the increase in intensity was not of this nature. This procedure, giving the same reaction as (vi) and (i), did not produce any increase in intensity of the staining reactions as enumerated in (iii). Therefore the increased colour in (iii) is not due to deacetylation.

(x) As in (ix).

(xi) The diastase digestion was introduced to show that

none of the increased colour produced in (iii) was associated with the presence of glycogen. The glycogen reactions of the squamous epithelium of the cervix and rectum, of the walls of the blood-vessels of the umbilical cord, of the macrophages of the pneumonic exudate, and of the chondriocytes of trachea, were removed.

(xii) This is the standard procedure used for producing metachromasia.

(xiii) It was thus shown that the NaOH did not remove those groups responsible for the production of metachromasia, as the appropriate metachromatic reactions of all tissues were obtained.

(xiv, xv and xvi) These procedures were carried out to show that this colouring of the Schiff's solution was due only to the presence of aldehydes, whether procedure (i) or (iii) was used. In all three procedures a pale reaction persisted in what were normally strongly staining areas.

(xvii) Blocking of 'free' aldehydes and production of a Feulgen reaction were carried out for comparison with the sites of colour production in the procedures. A faint positive reaction was produced in the nuclei of the rectal epithelial cells, the retinal cells, and the pancreatic acinar cells. There was also a persistent positive reaction in the cartilage of the trachea and throughout the connective tissues and all nuclei of the rat

mesentry.

(xviii and xiv) Rinsing sections in sulphite solution after leaving the Schiff's reagent prevented the formation of non-specific colour formed by atmospheric oxidation of absorbed Schiff's reagent. These sections showed no difference as compared with those of (i) and (iii) respectively.

(3) Discussion.

The substances which react positively with the P.A.S. test and those which stain metachromatically have previously been enumerated and discussed and their relationship to each other will now be considered. Hotchkiss (1948) and Jorpes, Werner and Aberg (1948) stated that hyaluronic acid gave a positive spot test with the P.A.S. technique and a positive reaction in certain tissues has been taken as indicative of its presence (Gersh, 1947; McManus, 1948; Jorpes, Werner and Aberg, 1948; Glick, 1949; Arzac, 1950; Staughton and Wells, 1950; Pearse, 1951; Cavallero and Braccini, 1951; Friberg, Graf and Aberg, 1951; Schrader and Leuchtenberger, 1951.) Davies (1952) states that hyaluronic acid, considered to be the main constituent of the 'ground substance' in connective tissues (see second part of this thesis), will only stain very faintly with the P.A.S.

technique according to the formula of Meyer and Fellig (1950). He himself found, as Glegg, Clermont and Leblond (1952) did also, that it neither stains metachromatically nor with periodic acid if it is properly purified.

My own findings with completely and incompletely purified hyaluronic acid preparations confirm this. (Appendix VII). Sylven and Malmgren (1952) state that although hyaluronic acid shows a low degree of metachromasia when concentrated it will not do so in histological sections, and Meyer and Fellig (1950) and Jeanloz and Forchielli (1951) have shown that hyaluronic acid consumes little periodic acid.

With regard to chondroitin sulphuric acid, a complex (i.e. sulphur containing) acid mucopolysaccharide, Davies points out that according to the formula given by Haworth (1946) the reaction with P.A.S. should be strong; but according to that of Meyer, Odier and Siegrist (1948), the reaction would be very faint. Jorpes, Werner and Aberg (1948) and Wolfrom, Madison and Cron (1952) found that a very small amount of periodic acid is consumed by chondroitin sulphuric acid after prolonged oxidation. Short oxidations will produce negative reactions with chondroitin sulphuric acid (Meyer et al, 1948; Jorpes, Werner and Aberg, 1948; Dziewiatcwski,1951;

Glegg, Clermont and Leblond, 1952.) Thus this substance might give a positive reaction with the P.A.S. technique.

Lillie (1949) reports that the mucin (? hyaluronic acid) of the connective tissue of the umbilical cord stains metachromatically but gives no colour reaction with periodic acid. Sylven (1941, 1945, and 1950), Davies (1943) Wislocki, Bunting and Dempsey (1947), Grishman (1948), Campani and Reggiani (1950), Mancini (1950), and Wislocki and Sognnaes (1950) report the presence of metachromatic ground substance in granulating tissue and other actively growing connective tissues. It appears from the discussion in the second part of this thesis and from the above remarks that the metachromatic ground substance stained by the numerous investigators is likely to be free chondroitin sulphate which is present in actively multiplying connective tissue. Thus as hyaluronic acid is P.A.S. negative and not metachromatic it does not come into the initial classification of P.A.S. positive substances.

If the strongly P.A.S. reacting substances are left out in Meyer's classification it is now necessary to differentiate between those mucopolysaccharides which react weakly and the persistently weakly reacting mucoproteins and glycoproteins.

Chondroitin sulphates, which are bound in

collagen to form a mucoprotein, and to globulin as in amyloid to form a glycoprotein (Krakow, 1897; Lillie, 1950), show a varying degree of metachromasia and of reactivity with the P.A.S. method. Free chondroitin sulphate and that bound in cartilage show a strong metachromasia and a strong reaction with P.A.S.

Human rectal mucus and the mucus of Brunner's glands in the rabbit duodenum are consistently strongly metachromatic and weakly positive with periodic acid. Rabbit Brunner's glands are characteristic in this respect (Chapman, 1952). Lillie (1951b) has pointed out, in discussing intestinal mucus and its staining reaction with the Bauer and Casella techniques, that there appears to be an inverse relationship between strongly P.A.S. positive substances and metachromatically positive ones; for example, gastric mucus stains very strongly with periodic acid but is only very weakly, if at all, metachromatic. Rectal mucus gives the reverse reaction. He suggests that a mucus weakly P.A.S. positive but strongly metachromatic has fewer groups available for aldehyde production with the P.A.S. technique because of a longer chain structure.

Mucoproteins such as sub-maxillary mucoid are consistently weakly positive.

Procedure (iii) causes an increase in the P.A.S. staining reaction of human rectal mucus and of the mucus of

the rabbit Brunner's glands. The first is a complex acid mucopolysaccharide. The exact nature of the second is not known. An indreased depth in staining also occurs in reticulin and on the surface of collagen, both of which are considered to be mucoprotein (Meyer, 1952). The change in reaction occurs partially in the clot of pneumonic exudate, which is probably a mixture of serum mucoprotein and glycoprotein. It does not take place in sub-maxillary mucoid, which is a mucoprotein. It does not take place in amyloid, which is a glycoprotein. Gersh (1949b) intimates that 0.00001N NaOH, acting for one hour at an unspecified temperature, weakens the P.A.S. staining reaction of the 'glycoprotein' of the Golgi apparatus. He does not report the effect on the surrounding tissues.

Meyer and Rapport (1951), in discussing the nature of the bonds between the mucopolysaccharide and the protein in the mucoproteins of connective tissue, intimate that they extract the mucopolysaccharides with a 0.33$\overline{N}$ solution of NaOH at 0° C. Meyer (1952) suggests that mucoproteins of connective tissue are mucopolysaccharides bound to protein by strong electrovalent forces. Therefore the staining reaction change which I have obtained may be due to the breaking of weaker bonds in certain mucoproteins.

As the change in reaction appears more clearly in mucus, it may be that it is occuring more selectively

in mucoitin sulphates as distinct from chondroitin sulphates. It is thus a possibility that the variation in reaction may be due to the different hexosamine present; acetylglucosamine in mucoitin sulphates and acetylgalactosamine in chondroitin sulphates. Morgan and Elson (1934) have shown that alkaline hydrolysis of acetylglucosamine and acetylgalactosamine (acetylchondrosamine) can be used to give quantitative colorimetric estimations of their products with paradimethyl-aminobenzaldehyde. Aminoff, Morgan and Watkins (1952) show that by this method acetylchondrosamine gives only 23% of the degree of colour reaction of acetylglucosamine, and the blood group mucoids give only 7-10%. The exact mode of reaction is not known but it is suggested that the alkali forms an oxazoline or a pyrazine with the hexosamine. It is not known whether these products are P.A.S. positive or not. It has been stated (Mann, 1902; Dempsey, Bunting, Singen and Wislocki, 1947) that fixation of tissues in formaldehyde will prevent this colour reaction. Frey-Wyssling (1948) states, in discussing the chemical nature of bytoplasm, that formaldehyde is thought to fix tissues by forming bridges between neighbouring polypeptide chains. So it may be that the difference in the type of hexosamine present might influence the P.A.S. reaction after alkaline hydrolysis, though the effect of the formalin fixatives must be borne in mind.

Mucopolysaccharides, according to Meyer's definition (1945) are polysaccharides which contain hexosamine as one component, whether they occur free or bound to substances of higher molecular weight. Mucoproteins are those substances which contain a mucopolysaccharide in firm chemical union with a peptide, where the hexosamine content is greater than 4%. Proteins which contain less than 4% hexosamine are classified as glycoproteins. Thus the selectivity of the change in reaction may be due to the relative amount of hexosamine present.

If all the preceding arguments are reconsidered it appears that the selectivity of the reaction may be due to one or more of the following causes:

(a) The number of reacting molecules available, perhaps depending on the chain length of the substance.

(b) The degree of chemical union with protein.

(c) The type of hexosamine present.

(d) The relative amount of hexosamine present.

All that can be said regarding the nature of the extra groups being made available, by the caustic solution, for aldehyde production by periodic acid is that:-

(a) No aldehydes are being produced by the alkali as shown by procedure (iv) where no colour is produced.

(b) The groups which are being made available are .CHOH.CHOH., .CHOH.$CHNH_2$ or .CHOH.CHNHR. groups,

as shown by procedure (vii), in which all colour reaction is blocked by acetylation.

(c) The action of the alkali is not just a deacetylation as suggested by procedure (viii), in which the full colour reaction is produced, because procedures (ix) and (x) restore the normal colour reactions to the tissues but do not intensify the mucus-reactions.

(d) The sulphate, metaphosphate or other groups thought to be responsible for metachromasia are not affected; this is shown by procedure (xiii), where the metachromasia remains after alkaline treatment.

(e) None of the increased colour intensity is due to absorbed leucofuchsin which has been subsequently oxidized in the atmosphere, as shown by procedures (xviii) and (xix).

(f) An attempt to show by procedures (xiv), (xv) and (xvi) that the increased colour reaction was due to the eventual production of aldehydes was prevented by the retention of a slight degree of colouration in normally strongly reacting areas in all three procedures. It is known that the condensation of hydroxylamine with aldehydes is dependant to a certain extent on the pH of the reaction; as the pH rises, within certain limits, so does the rate and degree of condensation (Olander, 1927). It may be that these areas, which are known to be of acidic nature (Dempsey et al, 1947) prevent complete condensation and

thus leave a certain amount of aldehyde available to recolourize the Schiff solution.

(d) Conclusions.

It has been shown that certain substances that are weakly positive to the periodic acid-Schiff reaction can be made to stain more strongly by first exposing them to a solution of sodium hydroxide.

It is suggested that this may provide a method of differentiating between weakly staining long chain mucopolysaccharides, mucoproteins, and glycoproteins. The first deepen markedly in colour, the second show an inconstant small increase in depth of colour, and the last remain unchanged.

T he relationship of the strength of the colour reaction to the varying chemical structure is not definite; the colour may depend on the chain length, the degree of conjugation with protein, or possibly on the type or amount of hexosamine present.

From the notes on methods and results it can be shown that the difference in staining reaction is produced by making available more .CHOH.CHOH., CHOH.$CHNH_2$., or CHOH.CHNHR. groups for aldehyde production by periodic acid without affecting those sulphate or metaphosphate

groups which give certain tissues their metachromatic affinities. Whether the requisite groups for aldehyde production are made available by protein extraction or by some other reaction is not yet known. The process does not appear to be a deacetylation.

The investigation to this date was not completely conclusive. It did however provide some information about polysaccharide containing substances which was not previously available, and showed that there was still a definite need for the development of a histochemical test for hyaluronic acid.

The last section of this thesis outlines a method that may prove useful in identifying mucoitin and chondroitin sulphates and contains further information on the above alkaline reaction.

VI

INVESTIGATION INTO THE EFFECT OF ALKALI ON PERIODIC ACID-SCHIFF STAINING.

(a) The effect of alkali on glucosamine.

In discussing the effect of alkali on polysaccharides, in the previous section of this thesis, the observations of Mann (1902), on the effect of formaldehyde on the paradimethylaminobenzaldehyde reaction of mucus were mentioned.

This reaction was first noted by Ehrlich (1901) who discovered that urine turns a carmine-red colour on the addition of a few drops of a yellow solution of paradimethylaminobenzaldehyde in normal hydrochloric acid. Proescher (1901) showed that the reacting substance was glucosamine and Ehrlich (1901) stated that formalin fixed cartilage gave a reddish-violet colour in the perichondrium and in some elastic fibres around the blood vessels. Other tissues described gave no reaction. He stated that unfixed mucin did not give the result but fixation might be producing a formyl or an acetyl glucosamine which might be giving the reaction.

Muller (1901) confirmed the statement that 'pure' (unfixed) substances will not react but showed that they would if warmed with a little alkali before being put in the reagent. Mann (1902) stated that good histological results can be obtained by fixing tissues in 0.5% KOH in 90% alcohol for 48 hours, or longer at 30-40° C., and then transferring the tissues to a 2.5% solution of the dye in

1% hydrochloric acid. This reaction, in acid solution, is the basis for the identification of indoles, pyrolles and aromatic amines. (Lison, 1936).

Morgan and Elson (1934) have investigated this reaction and they consider that N-acetylglucosamine in an alkaline solution reacts in the enolic form and passes over with loss of water into the oxazole derivative, 2-methyl-tetrahydrox-n-butyloxazole, and they consider that it is the condensation of this substance with paradimethylaminobenzaldehyde in acid solution which gives rise to the intense reddish purple colouration. Aminoff, Morgan and Watkins (1952) show that this reaction can be used to determine whether N-acetylglucosamine or N-acetylgalactosamine is present, if accurately controlled.

Dempsey and Wislocki (1946) have reported without giving details, that with gentle acid or alkaline treatment mucoproteins can be made to give the reactions characteristic of glucosamine in histological sections.

I have attempted to modify this reaction to produce accurate localisation of hexosamine in frozen-dried material where the effects of fixation on the reaction could be easily studied. It was hoped, by rigid controls of times and temperatures according to the directions of Aminoff, Morgan, and Watkins, that some method of differentiating glucosamine from galactosamine might be

found. This would thus permit identification and differentiation of hyaluronic acid and chondroitin sulphuric acid.

Slight modifications of the techniques of Mann (1902) and Aminoff, Morgan and Watkins (1952) were tried and in frozen-dried material a slight purple colour was obtained in sites of epithelial mucin. The colour is transient and is often missed, which is not surprising considering the observations on the difficulty of reproducibility of results made by Ogston and Stanier (1950), Johnston, Ogston and Stanier (1951) and Schloss (1951). There are also certain substances (Sideris, Young and Krauss, 1938; Lutwak-Mann, 1941; Horowitz, Ikawa and Fling, 1950; Immers and Vasseur, 1950.) which might interfere with the reaction and produce false positives.

Another reaction of this types which might be adapted to histo-chemical needs is that of Dische and Borenfreund (1950) which produces deamination of the sugar and then identification of it by either indole and hydrochloric acid or orcinol. This method is supposed to be highly specific for glucosamine. I have tried one or two preliminary experiments with no results.

The last method whinh might be adapted is that of Tracey (1951, 1952) which utilises Nessler's reagent to identify ammonia produced by heating glucosamine containing

substances with alkali.

This investigation was only of a preliminary nature and is at present being pursued. I feel that one of these methods might make a possible colorimetric differentiation of glucosamine and galactosamine which is applicable to histology.

(b) The use of Freeze-dried material.

In the previous section it has been said that formalin may perhaps be influencing the staining reaction of certain tissue constituents. It was in order to investigate this effect that tissue were prepared for histochemical examination in the apparatus described in part four of this thesis.

It was decided to investigate one easily obtainable tissue that had already been described as giving this reaction (Hale, 1953 a and b), and to apply to it the procedures enumerated below. Rabbit duodenum, which shows the reaction well, eas chosen.

A trial experiment was carried out using the following methods.

(i) A section of frozen-dried tissue was deparaffinized and placed immediately in alcoholic periodic acid for sufficient time for the sensitive groups to be oxidised. It was then carried through Schiff's solution and mounted

and examined.

(ii) Another section from the same block was placed in 10% neutral formalin, after being deparaffinized, for 24 hours, and was then washed and carried through as in (i).

(iii) Another section was carried through as in (ii) but was exposed to 0.2$\overline{N}$ NaOH before being carried through the periodic acid etc.

The result of this pilot experiment was that section (i) gave a strong colour reaction in the mucus of the Brunner's glands, section (ii) gave a very weak reaction in that area, and section (iii) gave a strong reaction.

It thus appeared that formalin was having some effect on the mucus and this was reducing the strength of colour reaction which it gave with the P.A.S. technique.

In order to investigate this the following procedures were carried out:

The tissue was frozen-dried and vacuum embedded in paraffin wax for 10 minutes. Sections were cut at 6u, dry mounted on a cold slide, and used immediately. Sections from different blocks obtained by the same method at different times from different rabbits were carried through each of the following procedures. The tissues were all carried through exactly the same freeze-drying technique because of the accurate method of control available in

the apparatus used. The sections were deparaffinized by immersing them in xylol for 2 minutes. They were rinsed in absolute ethanol and then placed in the appropriate reagent, which is the first mentioned in any method. Room temperature was used throughout. All washing was carried out in tap water unless otherwise stated. After the sections were carried through their respective techniques they were dehydrated in two changes of absolute ethanol, cleared in xylol and mounted in D.P.X. The periodic acid and Schiff solution were prepared as before and all solutions were replaced each day from the stock bottles available.

The pH extraction technique is essentially that of Gersh (1949a) and Catchpole (1949). In it frozen-dried sections are exposed to a graded series of buffer solutions before being stained. Any water soluble material will be dissolved out in all the buffer solutions except those with a pH around the iso-electric point of the substance in question. The iso-electric point being the pH at which that substance is least ionizable. In this case the buffer substrate used was that of Ostling and Virtama (1946) which provides a pH range of 2.0 - 11.8 without a qualitative change in the solutions used.

(c) Methods and their significance.

(1) (i) Periodic acid. 15 minutes; wash 5 minutes;
Schiff soln. 30 minutes; wash 10 minutes.

(ii) 10% neutral formalin 24 hours; wash 10 minutes; then as in (i).

(iii) 10% neutral formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ NaOH 15 minutes at 22°C; wash 5 minutes; then as in (i).

(iv) 10% neutral formalin 24 hours; wash 10 minutes; Schiff soln. 30 minutes; wash 10 minutes.

(v) 10% neutral formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ NaOH 15 minutes at 22°C; wash 5 minutes; Schiff soln. 30 minutes; wash 10 minutes.

(vi) 10% neutral formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ NaOH 15 minutes at 22° C; acetylate (as in section five of thesis) 24 hours; wash 15 minutes; then as in (i).

(vii) 10% neutral formalin 24 hours; wash 10 minutes; diastase 30 minutes; wash 5 minutes; then as in (i).

(viii) Absolute ethanol 18 hours; then as in (i).

To determine what effect formalin fixation has on the P.A.S. staining reaction of the tissue.

(2) (i) 10% neutral formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ KOH 5, 15 and 60 minutes; wash 5 minutes; then as in (1, (i).

(ii) as above using 0.2$\bar{N}$ NaOH.

(iii) as above using 0.1$\bar{N}$ Na_3PO_4.

(iv) as above using 0.1$\bar{N}$ Na_2CO_3.

To study the relative effect of different alkaline solutions on subsequent P.A.S. staining of tissues fixed in formalin.

(3) Procedures (1) i, ii, iii and viii were repeated using four different formalin solutions buffered at pH 4, 6.3, 7, and 8. instead of 10% neutral formalin. To study the relationship between the formalin effect of (i) and the pH of the formalin.

(4) Procedures (1) i, ii, and iii were carried out using 10% and 50% buffered (pH 7) formalin for 1-5 days in place of 10% neutral formalin for 24 hours.
To study the effect of time of fixation and strength of solution on the formalin effect.

(5) This was the same as procedure (4) but the tissues were washed in running water for 1-3 days between fixation and the subsequent steps.
To study the effect of washing of fixed tissues on the formalin effect.

(6) (i) Acetylate 24 hours; wash 15 minutes; then as in (1, i).

(ii) Acetylate 24 hours; wash 15 minutes; deacetylate

24 hours; wash 15 minutes; then as in (1, i).

(iii) 10% buffered (pH 7.) formalin 24 hours; wash 10 minutes; then as in (6, i).

(iv) 10% buffered (pH 7.) formalin 24 hours; wash 10 minutes; then as in (6, ii).

(v) Acetylate 24 hours; wash 15 minutes; 10% buffered formalin 24 hours; wash 10 minutes; deacetylate 12 hours; wash 15 minutes; then as in (1, i).

(vi) Acetylate 24 hours; wash 15 minutes; 10% buffered formalin 24 hours; wash 10 minutes; deacetylate 12 hours; wash 15 minutes; 0.2$\bar{N}$ NaOH 15 minutes; wash 5 minutes; then as in (1, i).

(vii) 10% buffered formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ NaOH 15 minutes; wash 5 minutes; then as in (1,i).

(viii) 10% buffered formalin 24 hours; wash 10 minutes; 0.2$\bar{N}$ NaOH 15 minutes; wash 5 minutes; acetylate 24 hours; wash 15 minutes; deacetylate 24 hours; wash 15 minutes; deacetylate 12 hours; then as in (1,i).

To determine whether formalin effects the staining reaction by acetal formation at OH groups or by polymerisation which masks the OH groups.

(7) Deparaffinised sections were placed in solutions of buffer ranging in pH from 2.8 to 11.1 in 0.3 - 0.6 unit

steps for 30 mintes then they were washed for 15 minutes and carried through as in (1,i). See Appendix VI for more details of the method.

To determine the approximate iso-electric point of Brunner's gland mucus.

(d) Results.

Procedures (1) i etc. The colour produced in the Brunner's glands by methods (i), (iii), (vii) and (viii) was strong and identical in distribution in all sections. Method (ii) shows reduced colour in the Brunner's glands. Method (iv) and (v) give no colour. Method (vi) shows some persistence of colour in the goblet cells.

Thus it appears that formalin reduces the amount of colour which can be produced in the Brunner's glands by the P.A.S. technique and this effect can be removed by exposure to NaOH (iii). The colour enhancement with NaOH appears to be due to an effect on .CHOH.CHOH., CHOH. $CHNH_2$., or .CHOH.CHNHR. groups since acetylation (vi) can block it. None of the colour produced by the NaOH is due to release of aldehydes (v), The colour produced is not due to the presence of glycogen (vii) and does not seem to be affected by alcoholic fixation (viii).

Procedure (2). The use of different alkalis for varying times shows that NaOH is best in producing colour restoration in certain formalin fixed tissues since the colour is strongest in (2, ii). Fifteen minutes appears to be sufficient time for the development of the colour restoration. See Appendix VIII for further details.

Procedure (3). Colour was suppressed in (ii) and the suppression appeared to be greatest with formalin at pH 4 and least with pH 8. The colour was restored, to the strength of (i) and (vii), by exposure to NaOH (iii).

Procedure (4). Fixation in 50% buffered neutral formalin produces the same colour result as fixation in 10% buffered neutral formalin. Both produce a result identical to that produced in (1, i, ii, and iii).

Procedure (5). Washing for varying periods showed that these sections fixed for 1 or 2 days lose their mucus more readily on washing than do those fixed for longer periods. Use of 50% formalin did not appear to improve the efficiency of fixation. Washing appeared to have no effect on the staining of the mucus other than this removal effect.

Procedure (6). Methods (i) and (ii) showed respectively

more colour suppression and more colour restoration in the Brunner's glands than did (iii) and (iv). Thus it appears more difficult to acetylate and deacetylate after formalin fixation. Method (v) gives normal formalin fixed colour whereas (vi) gives the full strength colour reaction. Methods (vii) and (viii) are mere controls similar to (1, ii and iii) and show the same reaction. Method (ix) shows hardly any colour at all; i.e. it is easily acetylated. (x) shows a strong colour reaction showing that it is well deacetylated.

Procedure (7). The mucus was removed from the acini of the Brunner's glands at all pH values except those lying between pH 2.8 and 4.6. This is a rather severe technique to which the tissues are exposed and in some cases there is swelling and slight tearing of the connective tissues, but the epithetial detail, both in the glands and the mucosa, appears quite normal.

(e) Discussion.

It has been shown in the previous section of this thesis that certain conventionally fixed tissues can be made to stain more intensely with the P.A.S. technique if they are exposed to NaOH before oxidation. It was suggested

that this ability to alter their reactivity to the P.A.S. technique might be due to a number of factors concerned with the chemical composition of these substances and it was remarked (Hale, 1953b) that the possibility of a formalin effect on the tissue should be borne in mind.

The present investigation shows that, in fixing frozen-dried sections, formalin alters the chemical configuration of certain P.A.S. positive substances in such a way as to reduce the strength of their reactivity with this technique. The reduction of colour reaction seems to be slightly greater in acid formalin solutions but does not appear to be influenced by their strength. The loss of colour reactivity can be made up by exposing the sections to alkali before periodic acid oxidation. Of the several alkaline solutions used NaOH appears to be the most efficacious in returning the potential reactivity of the tissues, to the P.A.S. technique, to their original state.

Prolonged washing is known to remove a large amount of the formalin bound in tissues during fixation (Nitschmann and Hadorn, 1943; Pearse, 1953.), but in this case the only effect that is produced is removal of mucus which has not been completely fixed. This removal of

mucus by prolonged washing is reduced by increased time of fixation.

Formalin is known to fix tissues by polymerisation (Frey-Wyssling, 1948; French and Edsall, 1945.) The most frequently encountered reaction of formalin is its addition to a compound containing a reactive hydrogen atom with the formation of a hydroxymethyl compound

$$RH + CH_2O \rightleftharpoons R.CH_2(OH)$$

The compound may condense with another H atom to form a methylene bridge.

$$R.CH_2(OH) + HR' \rightleftharpoons R - CH_2 - R' + H_2O$$

These methylene bridges are readily ruptured by hydrolysis.

Formalin also forms hemi-acetals

$$R.OH + CH_2O \rightleftharpoons R.O.CH_2OH.$$

and acetals

$$2\,R.OH + CH_2O \rightleftharpoons R.O.CH_2.OR.$$

with hydroxyl groups (French and Edsall, 1945.)

Thus the action of formalin on certain potentially P.A.S. positive materials may be either a polymerisation which would mask OH groups by the configuration produced, or by hemi-acetal or acetal formation which would block the OH groups. Thus by masking or blocking the OH groups they would not be available for oxidation with periodic acid

and the reactivity of that affected substance for the P.A.S. technique would be diminished.

As has been stated above the methylene bridges formed by the formalin could be destroyed by hydrolysis. So exposure of formalin fixed sections to NaOH would hydrolyse these bridges, depolymerise the substance and thus by unmasking glycols increase the potential reactivity of that substance with the P.A.S. Method.

Procedure (6, i - iv) has shown that it is difficult to acetylate and deacetylate sections after formalin fixation. If the formalin effect was acetal formation then acetylation should be easier since there would be fewer OH groups left after acetal formation, to be blocked by the acetylation. If the effect was one of polymerization then the alteration of configuration would mask OH groups but they probably would still be available to a certain degree for acetylation for periodic acid oxidation, although both of the methods would be reduced in efficacy. This appears to be the case.

Methods (6, v - vi) producing colour enhancement in the latter, show that NaOH is either improving the deacetylation produced already by the ammonia-alcohol mixture, or else it is depolymerising the mucus, irrespective of the acetylation-deacetylation techniques, so that more OH groups are available for oxidation. As the acetylation

is carried out before fixation in formalin, if the formalin effect were due to acetal formation, then as the glycols are already blocked by acetylation, acetals would not be formed and no reduction in potential colour would be produced after they had been deacetylated (6, v). There is potential colour reduction thus it is probably not due to acetal formation and thus may be due to polymerization.

Procedures (6, ix, and x) show that after alkaline hydrolysis the mucus can be easily acetylated and deacetylated irrespective of whether acetals are formed or polymerization has occurred. i.e. the NaOH returns the glycol groups to potential reactivity.

It would appear then that formalin reduces the potential reactivity of certain substances with the P.A.S. technique. This effect increases with dropping pH of the formalin used but is independant, within the limits used, of the strength of the formalin solution and the time of fixation used. This effect is not reversed by prolonged washing in water but it can be removed by hydrolysing the affected tissue in an alkaline solution prior to the periodic acid oxidation. Of the alkaline solutions tried sodium hydroxide appears to be the most efficient in restoring the potential reactivity to normal. Its maximum effect can be obtained in 15 minutes at room temperature.

The formalin effect is probably a polymerisation

causing alteration in molecular configuration which protects the glycol groups from periodic acid oxidation.

Although this report is based on an investigation carried out with rabbit duodenum it has been repeatedly practically in toto with normal human colon and rectum which is known (Hale, 1953 a and b) to produce a similar effect on alkaline hydrolysis. The results with the human tissue exactly parallel those of the rabbit duodenum.

Lillie (1951) has shown that epithelial mucus of the intestinal tract varies in colour reaction, with the P.A.S. technique, depending on its position in the tract. That of the stomach stains strongly whereas that of the rectum stains weakly. He puts forward the explanation that this may be due to an increase of chain length of the mucus molecules with a descent in level in the tract, i.e. gastric mucuc has a short chain length and rectal mucus a long one. His observations were made on conventionally prepared tissues, fixed in formalin containing fluids.

It appears however, from the present investigation, that the fact may be that the rectal mucus is more susceptible to polymerization with formalin than is gastric mucus and thus gives a weaker staining with P.A.S. technique after conventional fixation. What the underlying cause of this

tendency to polymerization of only certain types, if this is the case, of mucus is not known.

Gomori (1953) points out that polysaccharides made up of long chain polymers linked at the C_4 position will have available OH groups at C_2 - C_3 positions and oxidation with periodic acid will yield aldehydes at these points throughout the entire chain. If the link occurs at the C_3 position as in chondroitin sulphate (Meyer, Odier and Siegrist, 1948) then only the terminal C_2- C_3 groups can be oxidised and thus the aldehyde yield for that substance will be small. Thus depending on the linkage in a substance its potential colour reactivity with the P.A.S. technique will vary. If formalin polymerizes, then substances of the C_3 - link type may give very weak or even negative reactions because of aggravation of this state, but if NaOH depolymerizes them then they may give a very strong reaction. The molecular structure of P.A.S. positive substances is incompletely understood and at present it is impossible to draw conclusions about the possibility of this being a factor influencing staining intensity.

Gersh and Catchpole (1949) using the P.A.S. technique to investigate the ground substance of connective tissue suggested that the variations in the strength of staining reaction obtained with this technique in the tissue

might be due to different degrees of polymerization of the substances present. They exposed frozen-dried sections to N/1000 NaOH and also 10% formic acid before oxidation and noted no change in the staining reactions in the areas studied. They also noted that exposure of frozen-dried sections to glacial acetic acid blocked the P.A.S. reactivity of these substances. Their potential colour reaction could be returned by treatment with dilute hydrochloric acid. They gave no details of times or temperatures used in the experiment and offered no explanation of the result. The lack of NaOH effect with frozen dried tissues in their experiment is to be expected, from the data presented in this thesis, but no explanation can as yet be offered for the reactions after formic and glacial acetic acid treatment. It may be that they are connected with the polymerization effect reported here but in a more significant manner than has yet been appreciated.

Glegg, Clermont and Leblond (1952) have shown that the goblet cells in the lower parts of the crypts of the colon of the rat stain weakly with the P.A.S. technique but strongly with lead tetracetate. It is known that lead tetracetate oxidises .CHOH.COOH. groups to produce aldehyde, which periodic acid does not do, and the authors suggest that the difference between the two reactions may be due to the presence of these groups at that site.

This may be so but the solvent for lead tetracetate is glacial acetic acid and its effect on P.A.S. positive frozen-dried tissues, reported by Gersh and Catchpole (1949), must be borne in mind in relation to the fixation (Orth's fluid containing formalin and bichromate) used. The differences in P.A.S. and lead tetracetate staining in the rectal goblet cells may be due to the presence of .CHOH.COOH. groups but their relationship to the formalin effect reported here will have to be investigated.

Apart from these formalin effects directly related to P.A.S. staining others are known which influence histochemical reactions. Hamperl (1951) and Pearse (1953) report that it is not possible to get certain histochemical reactions for argentaffin granules unless the tissue, either fresh or frozen-dried, is fixed in formalin.

Wolman and Greco (1952) have shown that formaldehyde combines with unsaturated lipids at the double bond. The resulting complex contains a free carbonyl group which probably originates from the formaldehyde. The reaction occurs over a wide pH range, and takes place in the absence of oxygen or moisture. The reaction product is visualised by Schiff's reagent. They claim that the reaction has the same significance as the pseudo-plasmal reaction, i.e. it denotes the presence of double bonds. This appears to be another formalin effect on tissue in no way connected

to the one described above but interesting in showing what can happen during fixation.

So it may be that the variations in intensity obtainable with the P.A.S. technique in different tissues may be related to their degree of polymerization and this variation may be accentuated by further polymerization brought about by formalin containing fixatives. These effects are of course independant of colour variations caused by variations in the amount of material present.

(f) Conclusion.

Although this investigation shows that formalin appears to have a definite effect on the reactivity of certain substances with the P.A.S. technique, this does not mean that observations on tissues fixed by that agent are useless. It does mean however that if the substance is weakly reacting it should be subjected to an hydrolysis before oxidation. This will ensure the identification of that weakly reacting substance if it is present in sufficient amount.

The histochemical significance of the reaction is not at all clear and further work will have to be carried out to discover why for instance, gastric mucus does not change in the same way as rectal mucus, after exposure to formalin.

The relationship between formalin fixation and lead tetracetate and periodic acid oxidation will also have to be investigated.

SUMMARY AND CONCLUSIONS.

The initial intention was to investigate the effect of hyaluronidase on growing connective tissue and to elucidate its relationship to the inhibition of connective tissue formation by corticosteroids. It has been shown that persistent application of the enzyme to granulating tissue causes rapid invasion of the surface exudate by fibroblasts and that the normal formation of bundles of reticular tissue is impaired. Investigations of the effect of a single application of hyaluronidase and the relationship of these effects to simultaneous corticosteroids application was not carried out as the histological techniques being used in the investigation were unsatisfactory.

Investigation of these techniques, the background of which is described, lead to the adoption of new ones. One of the techniques adopted was that of using water-soluble polyethylene glycol wax as an embedding medium. It permits identification of lipids in serial section and reduces losses and distortion of tissue constituents during embedding. While using this wax difficulties in sectioning were encountered and investigations showed that these were caused by high atmospheric relative humidities causing absorption of water by the very hygroscopic wax.

In order to eliminate chemical loss or alteration, or physical change, in the tissue, freeze-drying was adopted as a method of preparation. Due to inadequate knowledge at that time of the problems involved the theory of tissue

freeze-drying was entered into in some detail and a new apparatus, based on the theoretical findings, was designed and built.

Because of the lack of specificity of the histochemical techniques available for the identification of hyaluronic acids attempts were made to develop a new one. These attempts are described. The fact that exposure of conventionally prepared sections to sodium hydroxide, before periodic acid oxidate, greatly enhances the colour reaction obtained in certain tissues with the P.A.S. technique, was noted. The investigation of this effect is described and it is shown that formalin appears to affect certain substances, possibly by polymerising them, so that they only react weakly with the P.A.S. technique. The alkali reverses this formalin effect so that they react strongly to the P.A.S. technique again. The relationship between this formalin effect and the chemical structure of the substances involved is discussed.

The conclusion reached is that hyaluronidase has a definite effect on fibroblaastic infiltration and fibre formation in growing connective tissue but the relationship of this function to the action of adrenocorticosteroids is not clear. In a histochemical study of this type it is essential to ensure that the substances involved in the study, hyaluronic acid in this case, are not being lost during preparation for microscopic examination, and that there is available a technique for identifying them. As these two criteria were not satisfied initially in this case freeze-drying and the use of

polyethylene glycol waxes were adopted in order that the first would be. Investigation of the second has shown that although there is no histochemical method for identification of hyaluronic acid available it might be possible to develop one based on the alkaline decomposition of its hexosamine content. Then it might be possible to return to a more accurate study of the inter relationship of hyaluronidase and hyaluronic acid function in fibroblastic activity.

Tables, graphs, figures, appendices, references, and personal publications.

LIST OF PUBLICATIONS.

1. The minute structure of the liver: A review. (1951) Glasgow Medical Journal. 32, 283-301.
2. The effect of temperature and of relative humidity on sectioning of tissues embedded in polyethylene glycol wax. (1952) Stain Technology. 27, 189-192.
3. The effect of sodium hydroxide on the period acid-Schiff reaction. (1953) Stain Technology. 28, 160.
4. An apparatus for freeze-drying tissues for histochemical investigation. (In conjunction with A.M. Andrew). (1953) J. Physiol. 121, 36P.
5. Observations on substances that react weakly to the periodic acid-Schiff test. (1953) Quarterly Journal of Microscopical Science. 94, 303.
6. Tissues freeze-drying apparatus with an electronic temperature regulator. (1954) (In conjunction with A.M. Andrew). Laboratory Investigation. 3, 58.
7. Use of the interference microscope to determine dry weight in living cells as a quantitative cytological and morphological method. (1953) (In conjunction with Davies et al. in part of the investigation.) (In press).
8. The relationship between alkali, formalin and periodic acid-Schiff sensitivity. (In preparation).
9. The optical retardations of human red blood corpuscles: A quantitative study. (In preparation).

APPENDIX I.

Method sheet for process of softening tissue prior to embedding.

10% neutral formalin	-	4 days.
4% pheonol	-	72 hours.
50% methanol and phenol	-	8 hours.
75% methanol and phenol	-	16 hours.
96% methanol and phenol	-	4 hours.
Absolute ethanol	-	3 hours.
Methyl benzoate - 3 changes	-	36 hours.
Benzol	-	½ hour.
Paraffin wax - 3 changes	-	4 hours.(vacuum embedde

Sections cut at 8 u

APPENDIX 2.

Staining techniques used in wound healing experiments.

(1) HAEMALUM & EOSIN.

Bring section to water.
Haemalum 3-4 min.
Water 5 min.
Eosin (alcoholic) ½-1 min.
Rinse in 50% ethanol.

(2) MALLORY.

Bring section to water.
Acid fuchsin 1 min.
Rinse in water.
5% phosphotungstic acid 5-10 min.
Rinse in water.
Mallory stain (anilin blue and orange G.) 5 min.
Rinse in water.

(3) WEIGERT'S ELASTICA.

Bring section to 70% ethanol.
Place slide in cold Resorcin fuchsin solution and place it in an incubator at 52 deg. C. for 1 hour or longer. (This stain is prepared by dissolving 0.02 gm. Resorcin fuchsin in 100 cc. 70% ethanol which is boiled to dissolve all of the dry stain. Add 1 cc. nitric acid.

(4) GORDON AND SWEET'S RETICULUM.

Bring section to water.
Place in permanganate solution (47.5 cc. of 0.5% potassium permanganate and 2.5 cc. of 3% sulphuric acid.) for 5 min.
Wash well in water.
Bleach until white in 1% oxalic acid.
Wash well in distilled water.
Mordant in 2.5% aqueous iron alum for 20 min.
Wash well in distilled water.
Impregnate in diammine silver hydroxide for 1 min. (To 5 cc. of 10.2% silver nitrate add strong ammonia dropy by drop until the precipitate formed just dissolves. Then add 5 cc. of 3.1% sodium hydroxide and a precipitate will form. Redissolve this precipitate in strong ammonia and dilute to 50 cc. with distilled water.)
Wash well in distilled water.
Tone in 0.2% gold chloride for 3 min.
Wash in distilled water.
Fix in 5% sodium thiosulphate for 5 min.
Wash well in distilled water.
Counterstain in 1% neutral red.
Rinse in water.
Differentiate in aniline alcohol (1 cc.aniline oil in 1000 cc. 96% ethanol)
Wash in water.

APPENDIX 2.

(5) PERIODIC ACID-SCHIFF.

The method used was the same as that described in section V of this thesis.

(6) TOLUIDIN BLUE.

The method used was the same as that described in section V of this thesis.

TABLE 1.

Series 1. Weights of rats operated upon.

Rat No.	Weight at start in Gm.	Time killed (days after operation.)	Weight at death.
1	251	2	249
2	242	2	244
3	240	4	237
4	240	4	241
5	260	6	254
6	260	6	257
7	258	8	261
8	235	8	236
9	255	10	254
10	268	10	263

Even numbers are rats treated with active preparation of hyaluronidase. Odd numbers are those treated with control solution.

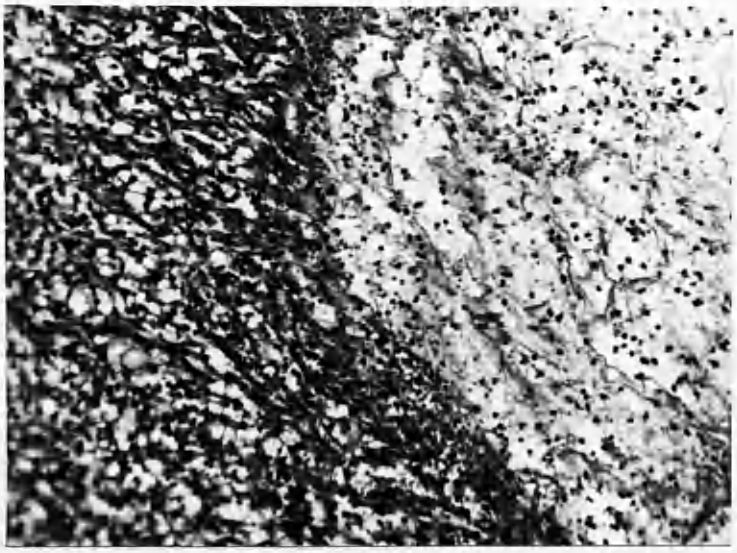
TABLE 2.

Series 2. Weights of rats operated upon

Rats 11-18 received active hyaluronidase. Rats 12-26 received inactive control solution.

Rat no.	Weight at start in Gm.	Time killed (days after operation)	Weight at death.
11	225	2	220
12	220	10	225
13	220	1	220
14	265	5	260
15	210	3	200
16	225	6	230
17	240	8	250
18	235	4	240
19	220	6	200
20	230	2	225
21	250	5	230
22	225	1	225
23	235	10	212
24	215	3	195
25	210	4	205
26	195	8	188

Fig.1.

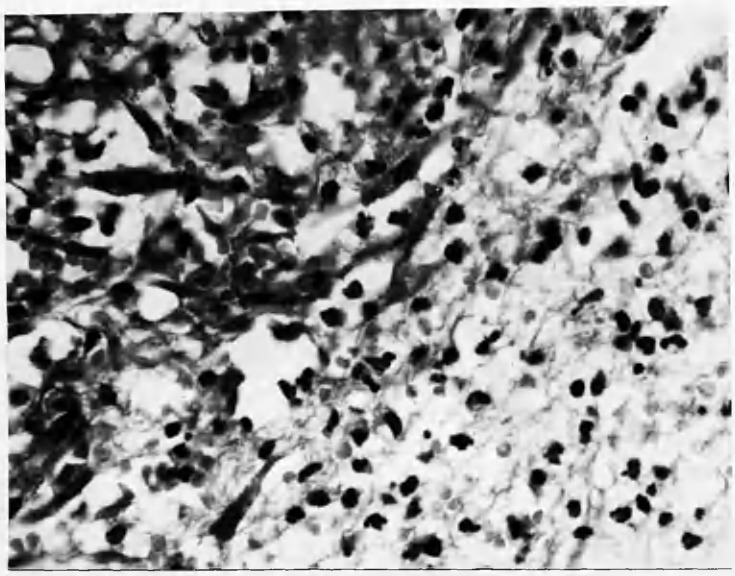


Granulating wound tissue stained with Haemalum and Eosin and showing the compact line of advancing fibroblasts. This woumd was treated with the control solution and is six days old. X 150.

Fig.2.

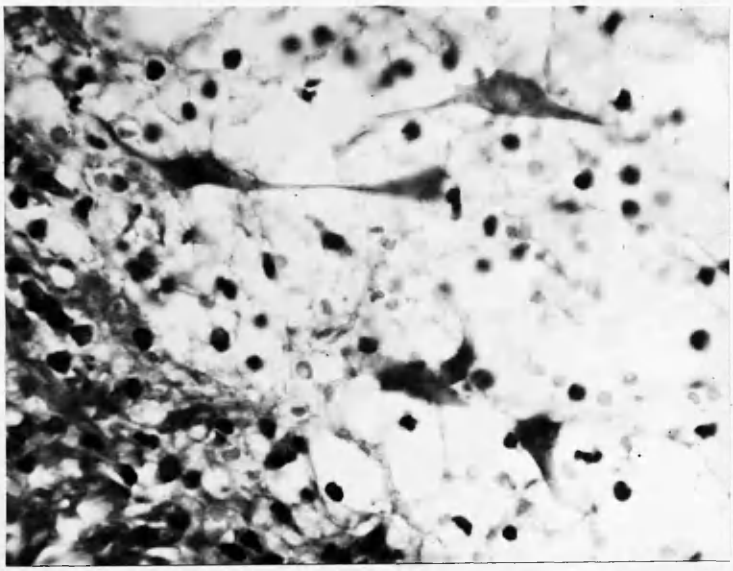
Granulating wound tissue stained with Haemulum and Eosin and showing the advancing fibroblasts infiltrating the serous exudate. They are not restricted to a compact line as in the previous figure. This wound was treated with the active preparation of hyaluronidase and is six days old. X 150.

Fig.3.



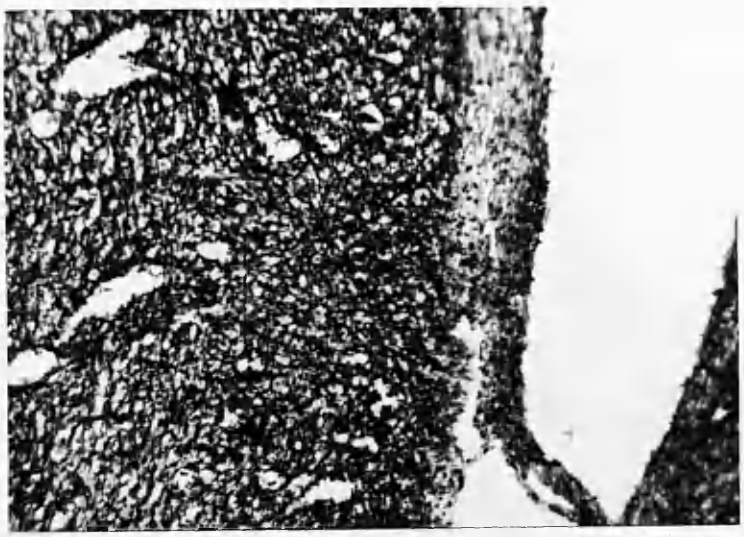
The same preparation as in Fig.1. The compact serous exudate lying to the left of the advancing line of fibroblasts is easily seen. X 500.

Fig.4.



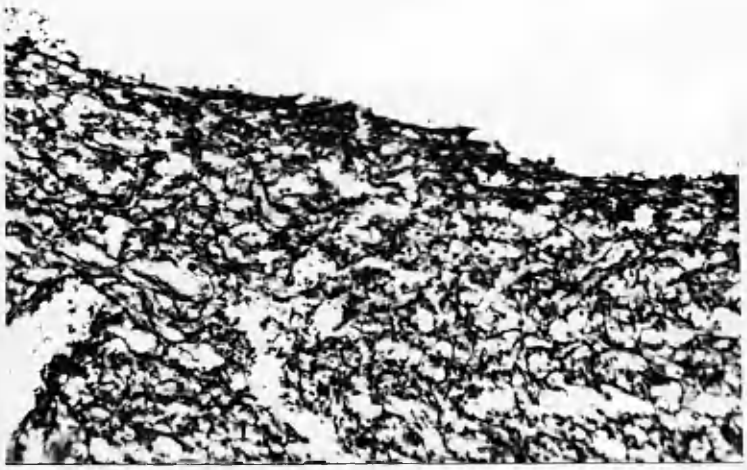
The same preparation as in Fig.2. The very loose serous exudate being penetrated by large widely separated fibroblasts is clearly seen. X 500.

Fig. 5.



An eight day old wound stained by Gordon and Sweet's method for reticulin. The reticulin has a very compact regular arrangement. This wound was treated with the control preparation of hyaluronidase. X 150.

Fig.6.

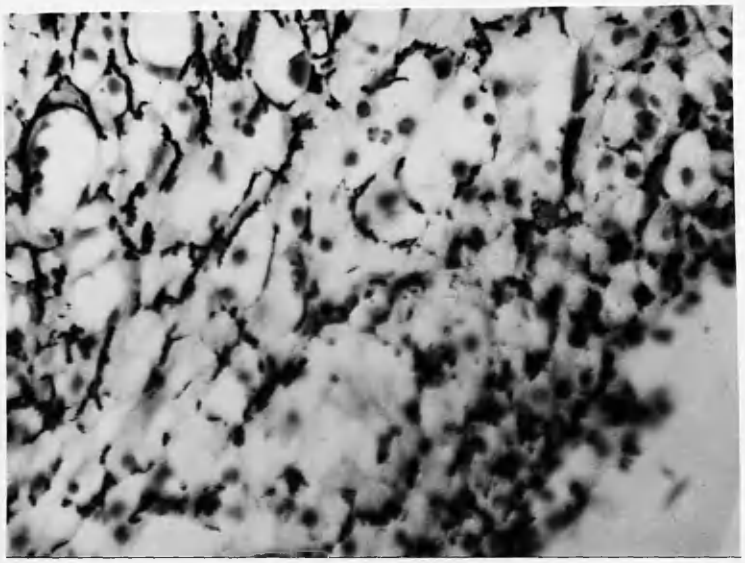


An eight day old wound stained by Gordon and Sweet's method for reticulin. The reticulin has a very ragged and widely separated appearance. This wound was treated with the active preparation of hyaluronidase. X 150.

Fig.7.

The same preparation as in Fig.5. The fine fibres of reticulin can be seen arranging themselves into bundles parallel to the surface of the wound. X 500.

Fig.8.



The same preparation as in Fig.6. The reticulin present consists of short fibres with a rather fragmented appearance. There is no evidence of the bundle arrangement seen in Fig.7. X 500.

Table 3.

S = sectioning R = ribboning.

Temperature group.	Relative humidity.	Thickness in u. 4 S	4 R	7 S	7 R	10 S	10 R
	89	−	−	±	−	+	+
	86	−	−	±	±	+	+
	82	−	−	+	+	+	+
	77	+	−	+	+	+	+
	72	+	+	+	+	+	+
	68	+	+	+	+	+	+
	64	+	+	+	+	+	+
17-18°C	62	+	+	+	+	+	+
	59	+	+	+	+	+	+
	57	+	+	+	+	+	+
	54	+	+	+	+	+	+
	49	+	+	+	+	+	+
	45	+	+	+	+	+	+
	41	+	+	+	+	+	+
	38	+	+	+	+	+	+
	35	+	+	+	+	+	+
	87	−	−	−	−	−	−
	84	−	−	−	−	−	−
	82	−	−	−	−	−	−
	80	−	−	−	−	−	−
	78	−	−	−	−	−	−
	74	−	−	−	−	+	±
19-20°C	70	−	−	+	+	+	+
	65	−	−	±	−	+	+
	63	±	−	±	±	+	+
	59	±	±	+	±	+	+
	55	+	+	+	+	+	+
	51	+	+	+	+	+	+
	48	+	+	+	+	+	+
	44	+	+	+	+	+	+
	41	+	+	+	+	+	+
	37	+	+	+	+	+	+

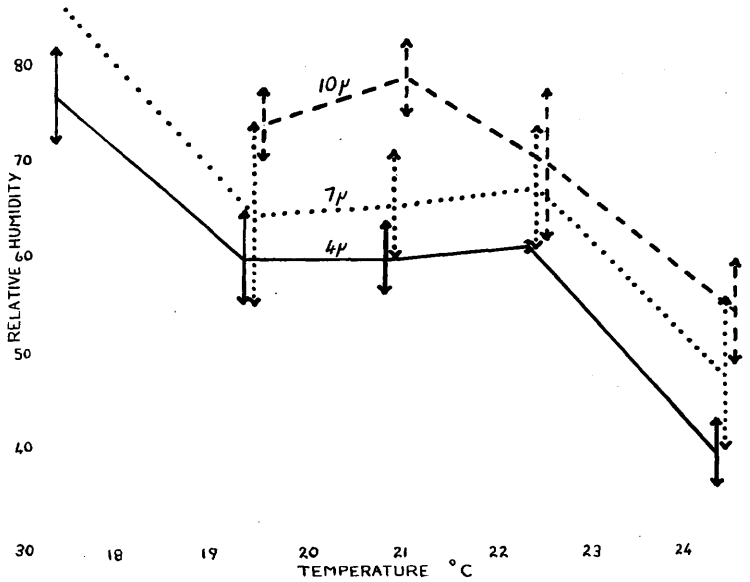
Table 3.

Temperature group	Relative humidity	Thickness in u. 4 S	4 R	7 S	7 R	10 S	10 R
20.5-21.5°C	87	−	−	−	−	−	−
	83	−	−	−	−	−	−
	79	−	−	−	−	±	−
	75	−	−	−	−	+	+
	71	−	−	−	−	+	+
	67	−	−	+	±	+	+
	64	−	−	+	+	+	+
	60	+	−	+	+	+	+
	58	−	−	+	+	+	+
	56	+	+	+	+	+	+
	49	+	+	+	+	+	+
	46	+	+	+	+	+	+
	42	+	+	+	+	+	+
	39	+	+	+	+	+	+
	36	+	+	+	+	+	+
22-23°C	88	−	−	−	−	−	−
	84	−	−	−	−	−	−
	78	−	−	−	−	−	−
	76	−	−	−	−	+	−
	72	−	−	±	−	+	−
	69	−	−	−	−	+	±
	68	−	−	+	−	+	+
	64	−	−	+	−	+	±
	62	−	−	+	−	+	+
	61	+	+	+	+	+	+
	58	+	+	+	+	+	+
	55	+	+	+	+	+	+
	51	+	+	+	+	+	+
	47	+	+	+	+	+	+
	44	+	+	+	+	+	+
	39	+	+	+	+	+	+

Table 3.

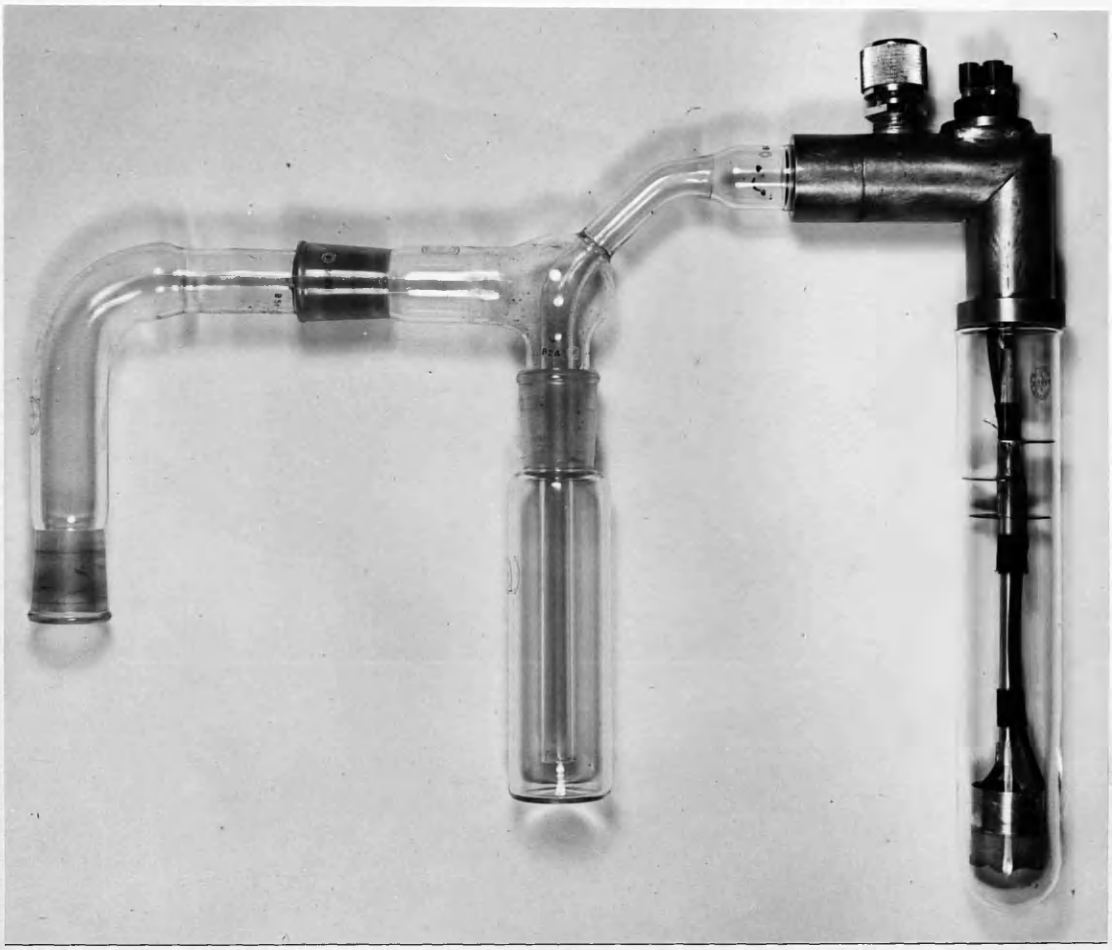
Temperature group	Relative humidity	Thickness in u. 4 S	4 R	7 S	7 R	10 S	10 R
24-25°C	84	-	-	-	-	-	-
	77	-	-	-	-	-	-
	73	-	-	-	-	-	-
	70	-	-	-	-	-	-
	69	-	-	-	-	-	-
	67	-	-	-	-	-	-
	66	-	-	-	-	-	-
	63	-	-	-	-	-	-
	60	-	-	-	-	-	-
	59	-	-	-	-	±	±
	56	-	-	-	-	+	±
	49	-	-	±	-	+	+
	46	-	-	+	-	+	+
	43	-	-	+	-	+	+
	40	+	-	+	+	+	+
	37	+	-	+	+	+	+
	36	+	+	+	+	+	+

Fig.9.



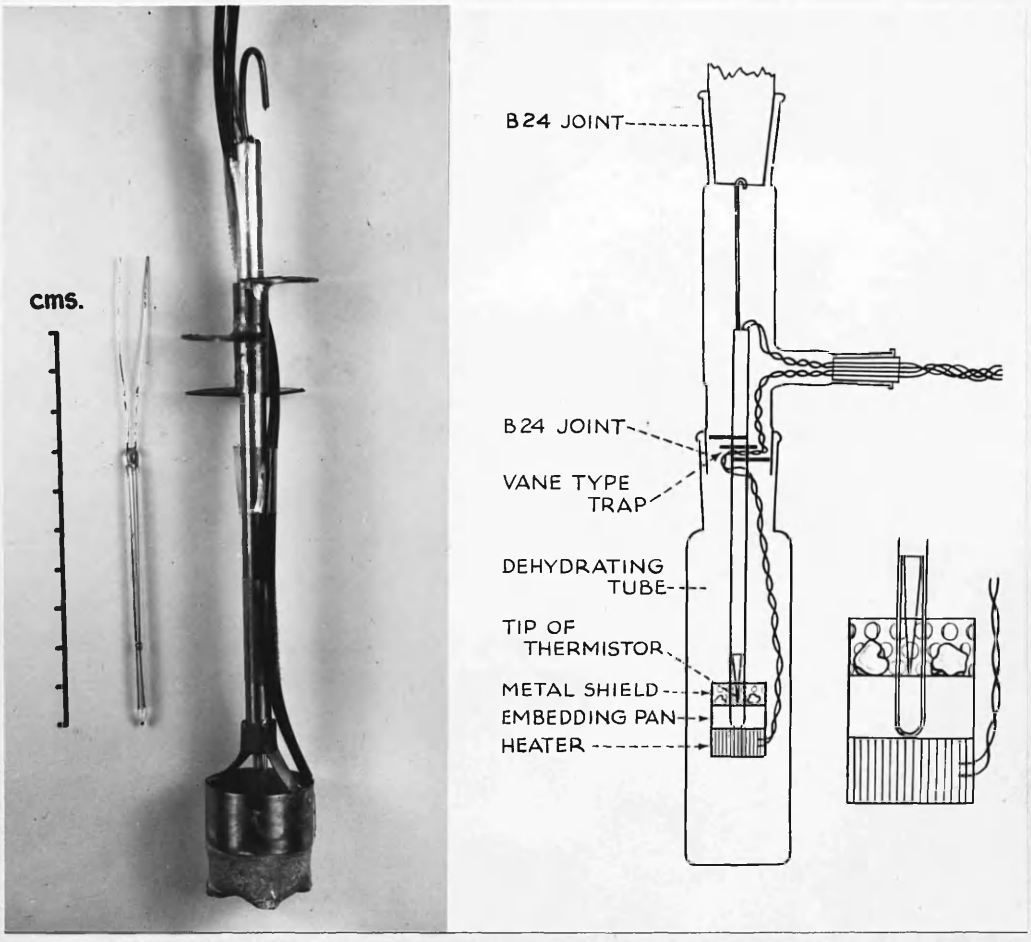
Graphic representation of the results shown in Table 3. The curves are drawn through the means of the upper and lower points represented by the tips of the vertical arrows in the graph. At any one temperature, at relative humidities less than the values indicated by the tip of the lower arrow, ribboning and sectioning were both satisfactory. The tip of the upper arrow indicates the degree of humidity above which both ribboning and sectioning inevitably failed. At intermediate values, indicated by the interval between the two arrow heads, results were erratic, but as the humidity increased there was progressive deterioration.

Fig10.



Glass tubing system of freeze-drying apparatus, showing ground glass joints, Dreschel bottle and the new type of vapour trap.

Fig.11.



The first type of vapour trap used showing the size of the thermistor and its relationship to the tissues and heater. Thin slices of tissue are placed on top of the heater and the tip of the thermistor is brought into contact with their surface.

Fig.12.

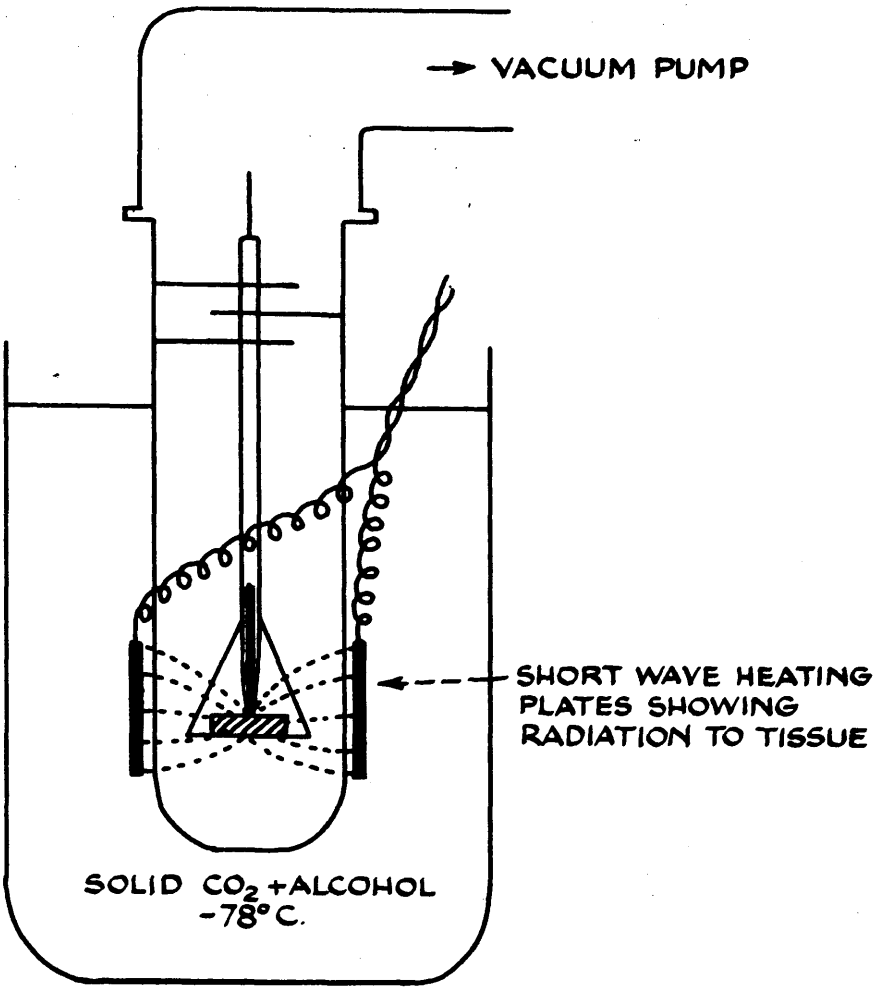
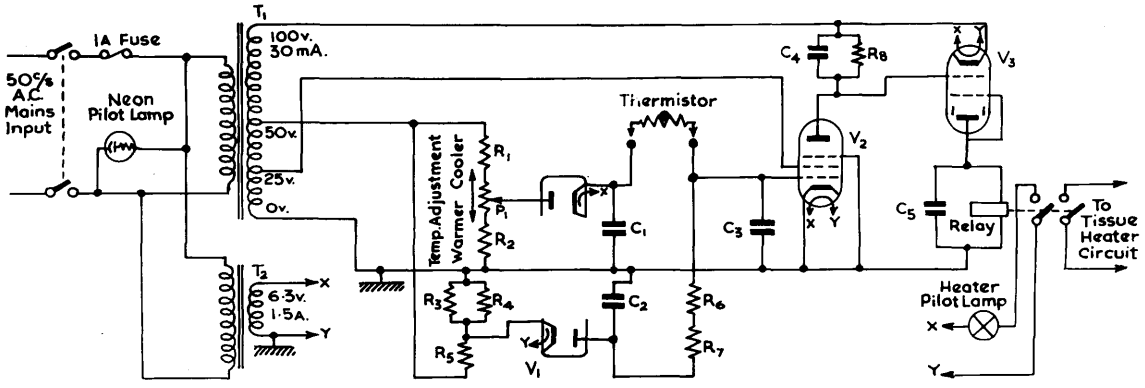


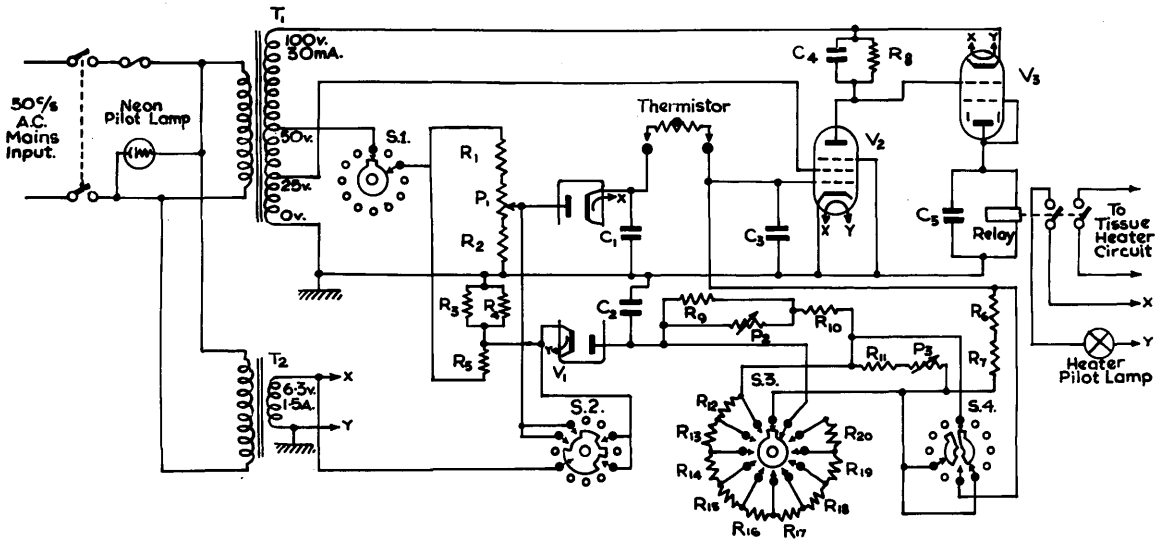
Diagram of freeze-drying apparatus with proposed method of short wave heating.

Fig.13.



Circuit of temperature regulator for range -45°C. to -28°C. Component values are as in table 4.

Fig.14.



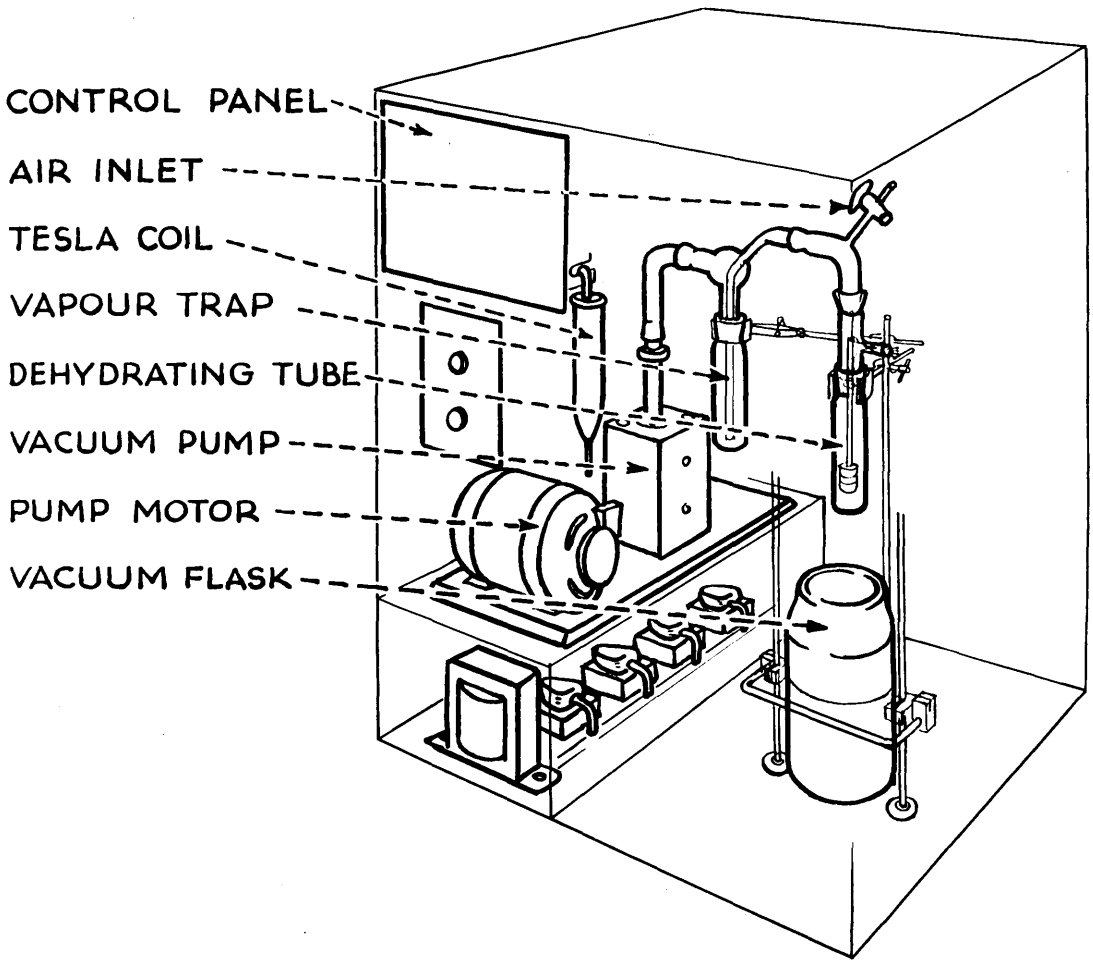
Complete circuit of temperature regulator. The switch sections S1 - S4 are ganged, and are drawn in position 0. Positions 1 - 11 are obtained in turn by clockwise rotation of the rotors from the position shown. Component values are as in Table 4.

Table 4.

Component values for thermistor circuits, shown in Figs. 13 and 14.

R_1 = 6.8 k ; R_2, 22 k ; R_3, 100 k ; R_4, 68 k ; R_5, 10 k ; R_6 & R_7 = 2.2 M , high stability; R_8, 2.2 M ; R_9, 470 k ; R_{10}, 270 k ; R_{11}, 47 k ; R_{12}, 10k ; R_{13}, 15 k ; R_{14}, 18 k ; R_{15}, 22 k ; R_{16}, 33 k ; R_{17}, 47 k ; R_{18}, 75 k ; R_{19}, 150 k ; R_{20}, 270 k ; P_1, 25 k ; P_2, 270 k ; P_3, 25 k ; $C_1 = C_2$ = 1 uF ; C_3, 0.1 uF, mica; C_4, 0.25 uF; C_5, 4 uF; V_1, 6H6; V_2, 6J7; V_3, 6V6. Relay, 2000 P.O. type.

Fig.15.

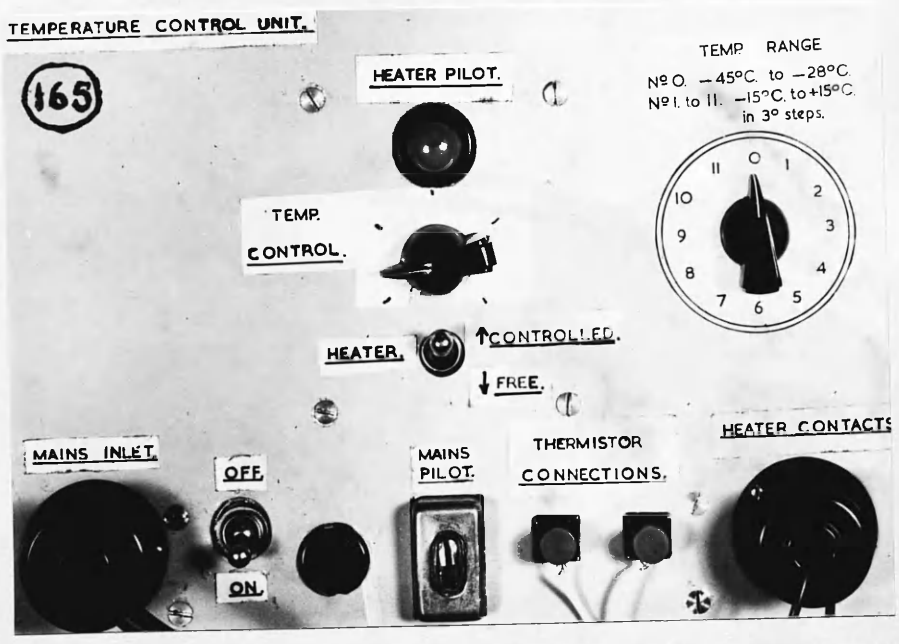


General layout of freeze-drying apparatus.

Fig.16.

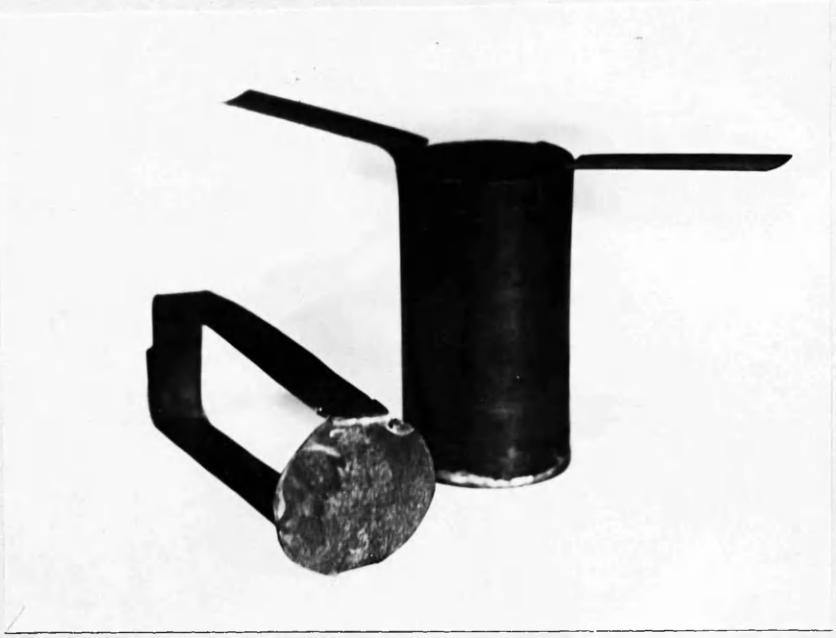
Photograph of general layout of freeze-drying apparatus.

Fig.17.



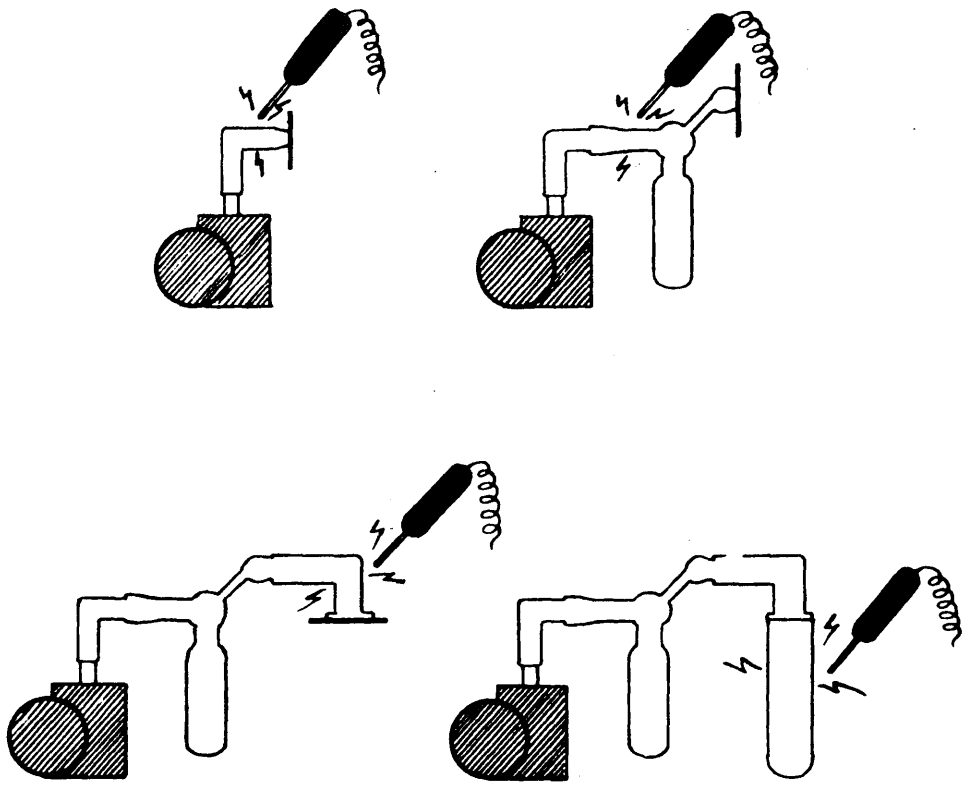
Photograph of control panel of freeze-drying apparatus.

Fig.18.



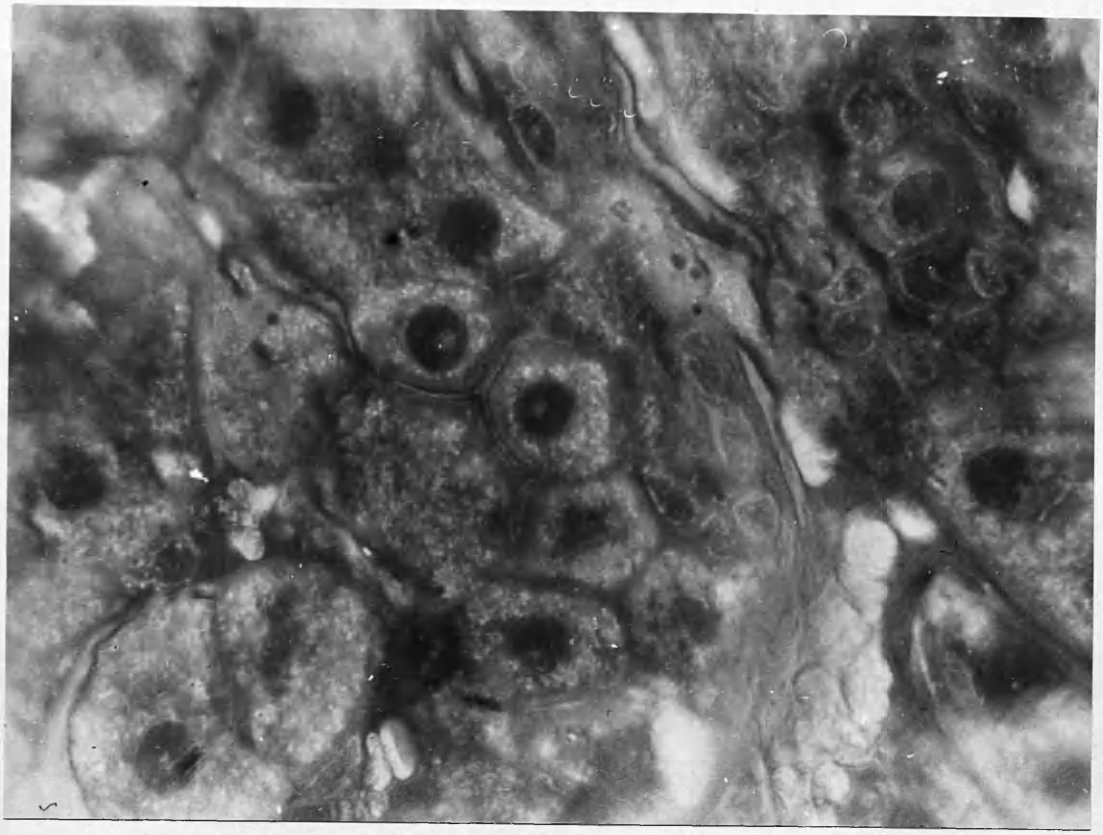
Isopentane container and tissue agitator which slides up and down inside it.

Fig.19.



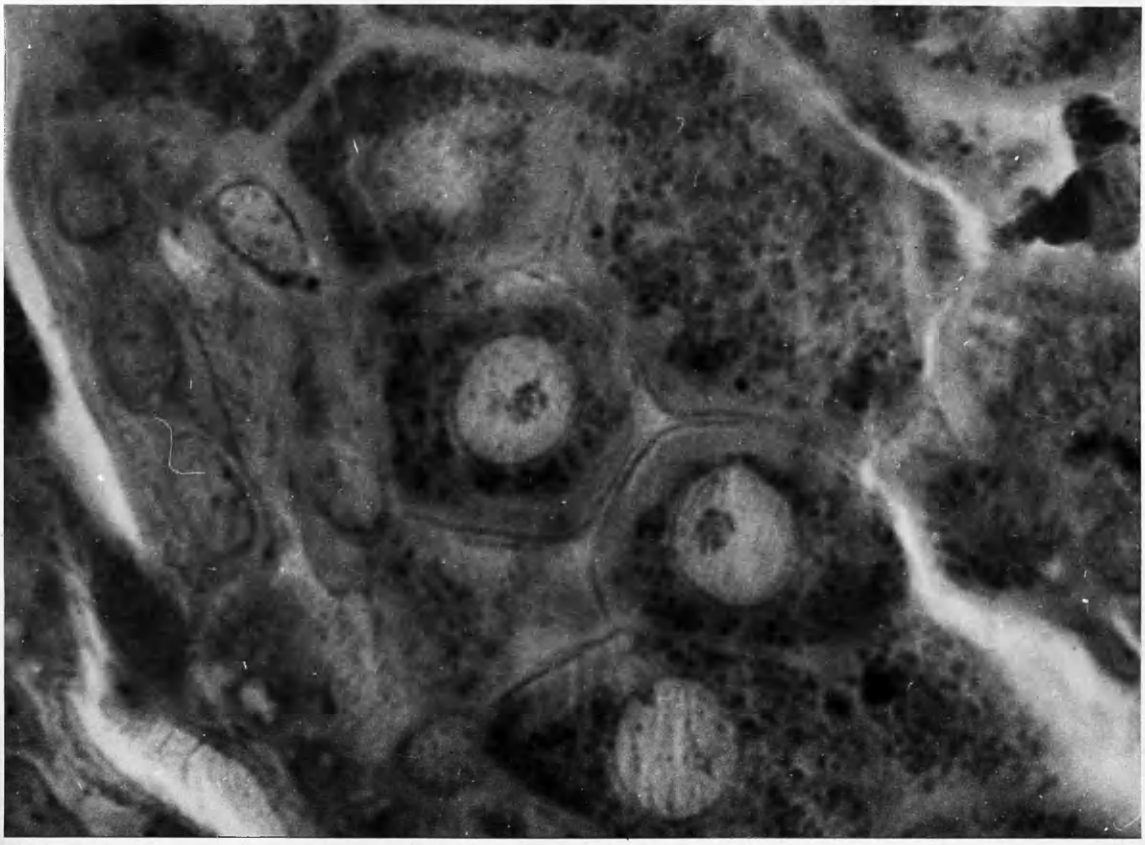
Method of detecting leaks in vacuum system by progressively replacing parts while the pump is running and testing the vacuum with the Tesla coil each time a part is replaced.

Fig.20.



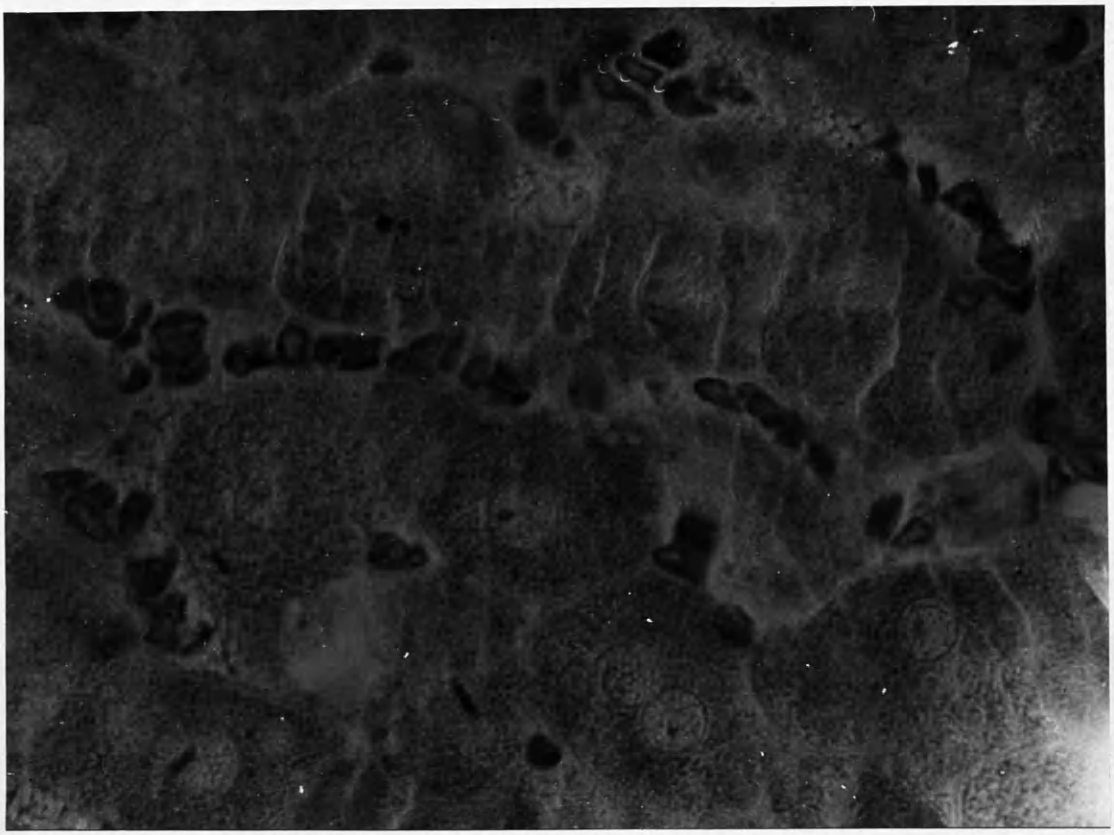
Section of frozen-dried rabbit liver cut at 6 u. Mounted in Nonane. Interference micrograph at infinite fringe separation. The nuclei are regular in shape and their contents are evenly dispersed. Nucleoli are clear cut. Inclusions are easily differentiated from the even dispersion in the cytoplasmic background. The cell membrane is well defined and there is no artificial separation of the cells. X 750.

Fig.21.



This is the same specimen as in Fig.20. but using a different phase change in the interference system.

Fig.22.



This section was prepared in the same way as that in Fig.20. but has not been well preserved. The nuclei are regular in outline but their contents are not evenly dispersed. They appear vacuolated because of gross ice crystal formation. The cell cytoplasm is distorted in a similar way and the inclusions cannot be differentiated from the uneven background. Cell outlines are vague and many cells are fragmented by contraction cracks. X 750.

APPENDIX III.

Method of preparation of solutions used in the periodic acid-Schiff technique.

Periodic acid - According to Hotchkiss (1948)

Dissolve 0.4 g. of periodic acid in 35 ml. of ethyl alcohol and add 5 ml. of M/5 sodium acetate and 10 ml. of distilled water. This solution is stored and used in a black painted bottle.

Schiff solution - According to Lillie (1951a) modified to conform with the findings of Longley (1952) and Atkinson (1952).

Dissolve 0.35 g. basic fuchsin and 1.9 g. sodium metabisulphite to 100 ml. 0.15 $\overline{N}$ HCl and shake the solution for 2 hrs. Let it stand overnight and then shake it up with fresh activated charcoal for one hour and then filter it. The solution should be water clear.

Acetylation mixture - The sections are placed in a solution of 40 ml. acetic anhydride and 60 ml. pyridine for 16 hours at room temperature. They are then removed and washed in alchol1

De-acetylation mixture. The Sections are placed in a mixture of 20 parts of 28% ammonia and 80 parts of 75% ethanol for 1-2 days at room temperature. They are then removed and washed in ethanol.

APPENDIX IV.

Effects of different alkali solutions increasing the colour reaction of formalin fixed frozen-dried rabbit duodenum. + indicates degree of colour produced, P means patchy colour enhancement.

Time of exposure in minutes at 22°C.	0.1$\bar{N}$ Na_2CO_3 pH 11.6	0.2$\bar{N}$ KOH 14.0	0.1$\bar{N}$ Na_3PO_4 12.0	0.2$\bar{N}$ NaOH 12.5
5	++	+++	+	++++
15	+	+++	++	++-
60	++-	++	++-	++-
5	+-	P+	+-	++++
15	+-	P+	P+	P++
60	+-	-	P++	+
5	++	+++	+	++++
15	+	+++	++	++-
60	++-	++	++-	++-

APPENDIX VI.

Method of buffer preparation and usage in relation to the determination of the approximate iso-electric point of the mucus of Brunner's glands.

Preparation according to Ostling and Virtama (1946)

Constant boiling HCl diluted to 0.1$\bar{N}$ (18.019g.-1000ml. at 760mm.) Saturated NaOH is prepared and centrifuged until all soluble carbonate settles. 58ml. or supernatant is diluted with CO free conductivity water to 1000ml. Determine strength by titration with 0.1$\bar{N}$ HCl. N.B. acid and base must correspond exactly.

Stock solution. In a 1000ml. flask place 8.903g. $Na_2HPO_4.2H_2O$ 7.00g. $C_6H_8O_7$ H_2O., 3.54g.H_3BO_3., 243.0ml. 1.$\bar{N}$ NaOH, and add CO_2 free water to 1000ml. Keep in CO_2 protected bottle.

To 20ml. stock add 0.1$\bar{N}$ HCl as in the following table, for the required pH.

APPENDIX VII.

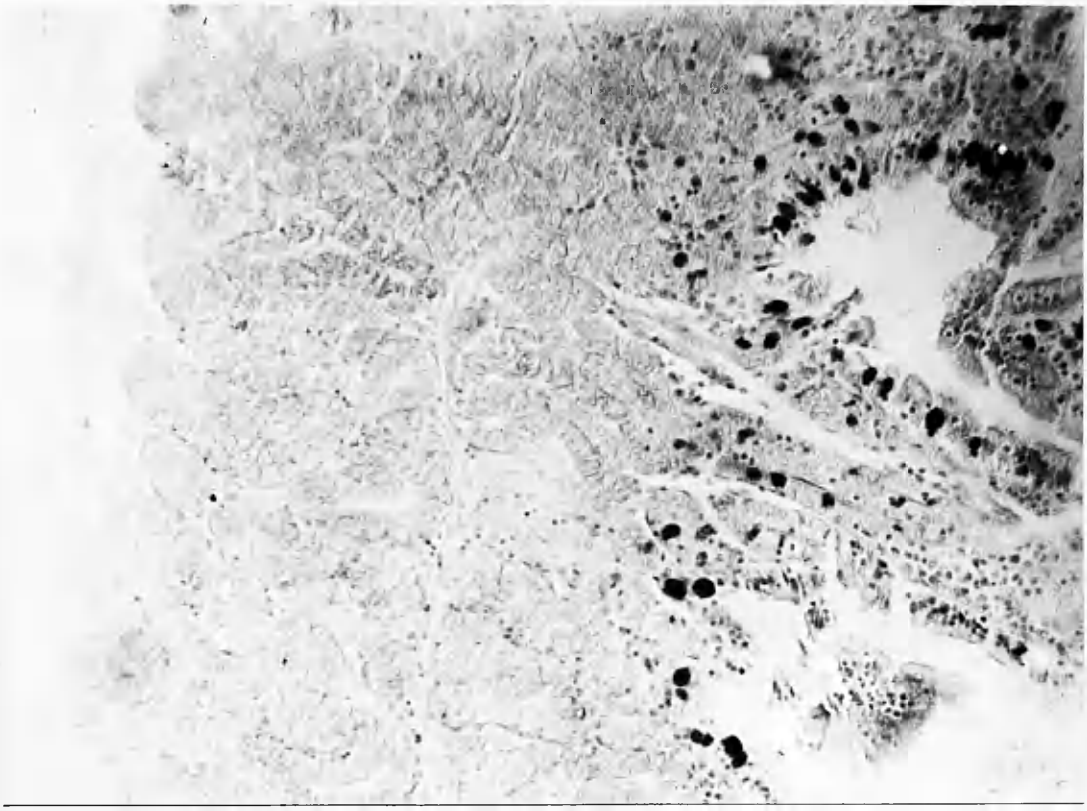
One solution used was considered to be a pure preparation of hyaluronic acid, the other was considered to be contaminated with a sulphur containing mucopolysaccharide.

Clean glass slides were covered with thin films of these substances and dried in air at room temperature. They were then carried through the periodic acid-Schiff and metachromatic techniques previously described.

The slide covered with pure hyaluronic acid gave no reaction with either technique. The slide covered with the sulphur containing preparation gave a very slight pink colour with the P.A.S. method and showed quite strong metachromasia.

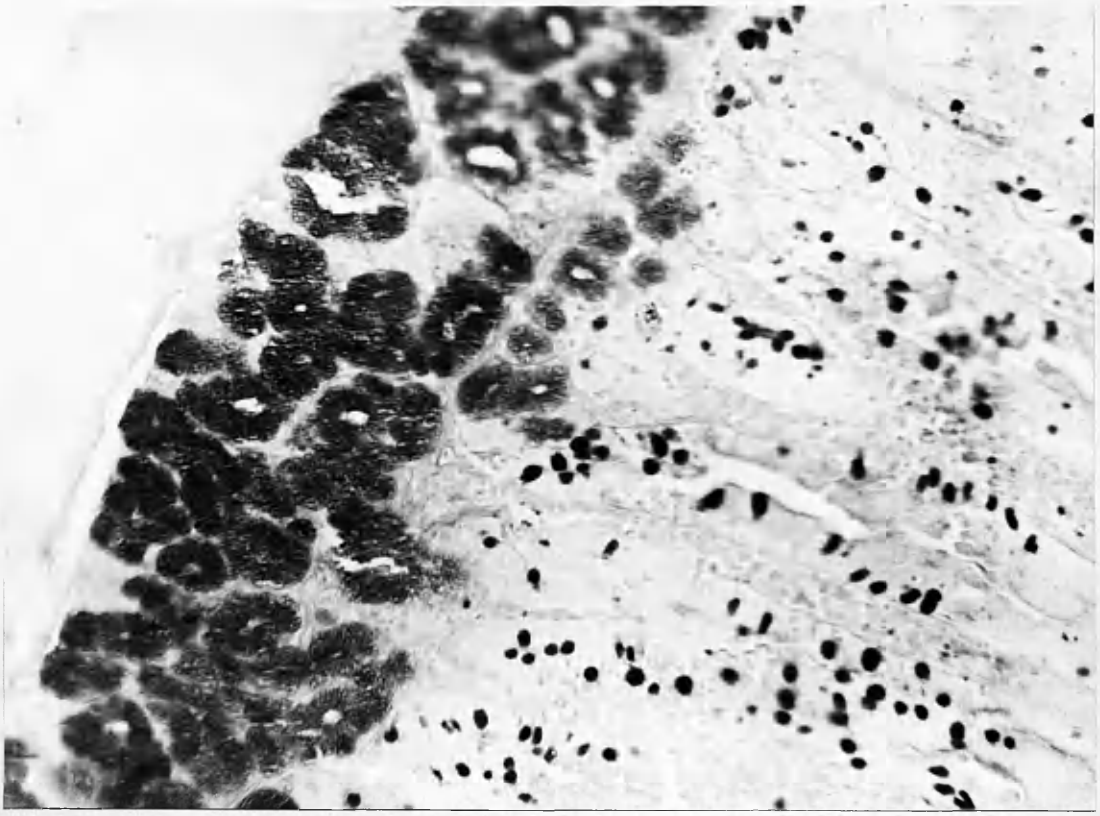
pH	.00	.10	.20	.30	.40	.50	.60	.70	.80	.90
2		72.1	69.25	66.87	64.90	63½25	61.77	60.48	59.29	58.29
3	57.49	56.76	56.05	55.42	54.83	54.28	53.72	53.17	52.61	52.07
4	51.52	51.00	50.46	49.92	49.40	48.88	48.35	47.81	47.28	46.72
5	46.18	45.64	45.10	44.54	43.99	43.40	42.77	42.15	41.55	40.89
6	40.28	39.66	39.02	38.31	37.54	36.73	36.02	35.36	34.72	34.13
7	33.51	32.97	32.46	31.90	31.36	30.82	30.33	29.88	29.45	29.06
8	28.70	28.44	28.20	27.91	27.56	27.20	26.83	26.34	25.77	25.12
9	24.48	23.82	23.21	22.60	21.95	21.32	20.71	20.13	19.60	19.60
10	18.65	18.24	17.84	17.51	17.20	16.92	16.68	16.35	15.98	15.56
11	15.09	14.59	13.92	13.08	12.09	10.75				

Fig.23.



Section of rabbit duodenum fixed in formalin and stained by the P.A.S. technique. X 225.

Fig.24.

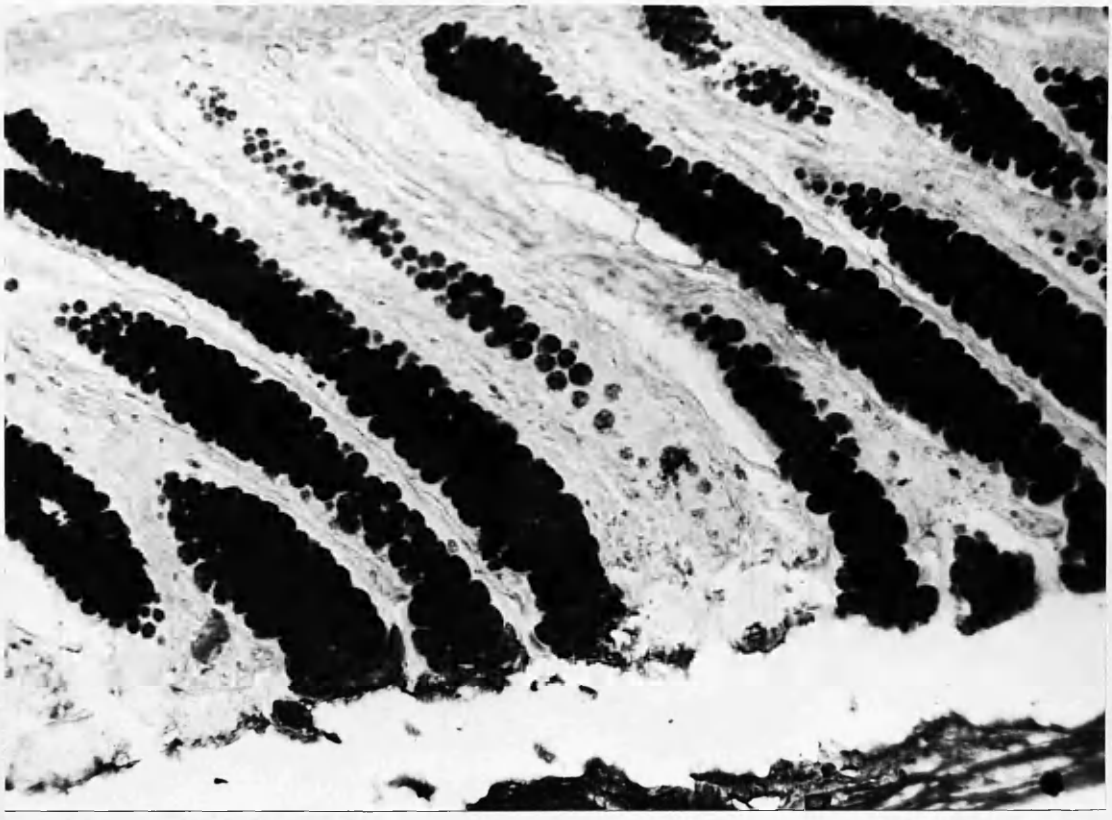


Section of rabbit duodenum fixed in formalin and stained by the P.A.S. technique after exposure to sodium hydroxide. X 225.

Fig.25.

Section of human rectum fixed in formalin and stained by the P.A.S. technique. X 225.

Fig.26.



Section of human rectum fixed in formalin and stained by the P.A.S. technique after exposure to sodium hydroxide. X 225.

Fig.27.

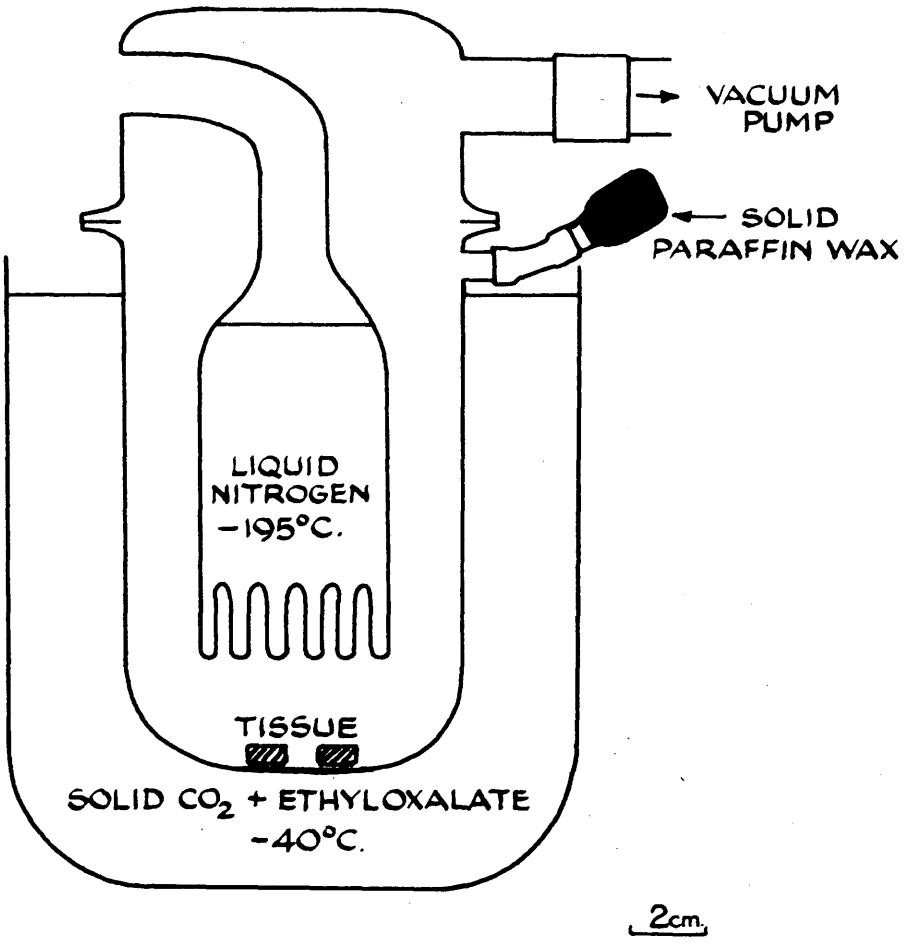


Diagram of freeze-drying apparatus made available by Dr.L.G.E.Bell.

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Altmann, R. 1890. "Die Elementarorganismen und ihre Beziehungen zur Zellen". Leipzig.

Aminoff, D., Morgan, W.T.J., and Watkins, W.M. 1952. Studies in immunochemistry. II The action of dilute alkali on the N-acetylhexosamine and the specific blood group substances. Biochem. J. 51, 379.

Andrew, A., and Hale, A.J. 1954. Tissue freeze-drying apparatus with an electronic temperature regulator. Lab. Invest. 3. 1.

Arnold, J. 1908. Zur morphologie der leberglykogens, und zur struktur der leberzelle. Arch. path. Anat., 193, 174.

Arzac, J.P. 1948. Acido peryodico-carbol fuchina para la demonstracion de carbohidratos en les Tejidos. Analecto Med. 9, 15.

Arzac, J.P. 1950. A simple histochemical reaction for aldehydes. Stain Tech. 25, 187.

Arzac, J.P. and Flores, L.G. 1949. Demonstration of Galgi zones by three techniques for carbohydrates. Stain Tech. 27, 9.

Atkinson, W.B. 1952. Studies on the preparation and recolourisation of Fuchsin sulphunous acid. Stain Tech., 27,153.

Baker, J.R. 1943. The discovery of the uses of colouring agents in biological technique. J. Queckett. Micro. Club. Ser. 4. Vol. 1, 256.

Baker, J.R. 1945. "Cytological Technique". 2nd Ed. Methven. London.

Baker, J.R. 1946. The histochemical recognition of lipine. Quart. J. micr.Sci, 87, 441.

Baker, B.L., Ingle, D.J., Li, C.H., Evans, H.M. 1948. Growth inhibition in the skin induced by parentral administration of Adrenocerticotropin. Anat. Rec. 102, 313.

Baker, B.L. and Whittaker, W.L. 1948. Growth inhibition in the skin following direct application of adrenal cortical preparations. Anat. Rec. 102, 333.

Baker, B.L. and Whitaker, W.L. 1950. Interference with wound healing by the local action of Adrenocortical steriods. Endocrinól. 46, 244.

Bank, O and Bungenberg de Jong, H.G. 1939. Untersuchungen uber metachromasie. Protoplasma. 32, 489.

Bayer, H. 1933. Mikroskopisch-chemischer nachweis von Glykogen und einigen auderen polysacchariden. Z.mikr. anat. Forsch. 33, 143.

Belchier, J. 1736. An account of the bones of animals being changed to a red colour by aliment only. Phil. Trans. Roy. Soc. London. 39, 287 and 299.

Bencke, A. 1862. Korrespbl. Ver. Gemeinsch. Arbeiten. 59, 980. quoted from Conn. H.J.

Benditt, E.P., Schiller, S., Wong, H., and Dorfman, A. 1950. Influence of ACTH and Cortisone upon alteration in capillary permeability induced by hyaluronidase in rats. Proc. Soc. exper. Biol. N.Y. 75, 782.

Bensley, C.M. 1939. Comparison of methods for demonstrating glycogen microscopically. Stain Tech. 14, 47.

Bensley, R.R. and Gersh, I. 1933a. Studies on cell structure by the freezing-drying method. I. Instroduction. Anat. Rec. 57, 205.

Bensley, R.R. and Gersh, I. 1933b. Studies on cell structure by the freezing-drying method. II The nature of the mitochondria in the hepatic cell of amblystoma. Anat. Rec. 57, 217.

Bensley, R.R. and Gersh, I. 1933c. Studies on cell structure by the freezing-drying method. III The distribution in cells of the basophil substances, in particular the Nissi substance of the nerve cell. Anat. Rec. 57, 369.

Bensley, R.R. and Hoerr, N.L. 1934. Studies on cell structure by the freezing-drying method. Anat. Rec. 60, 251.

Bensley, S.H. 1934. On the presence, properties and distribution of the intercellular ground substance of loose connective tissue. Anat. Rec. 60, 93.

Bensley, S.H. 1950. Histological studies of the reactions of cells and intercellular substances of loose connective tissue to the spreading factor of testicular extracts. Ann. N.Y. Acac. Science 52, 983.

Berg, W. 1903. Beitrage zur theorie der Fixation mit besonder berucksichtigung des zellkerns und seiner eiweisskorpen. Arch. mikr. Anat. 62, 367.

Berg, W. 1908. Quoted by Tellyesniczky (1926)

Berlin, R.B. and Brines, O.A. 1951. Detection of lymph node involvement in cancer. Amer. J. clin. Path. 21, 332.

Bernard, C. 1877. "Lecons sur le Diabete." Paris.

Benthold, G. 1882. Beitrage zur morphologie und physiologie der meersalgen. Jahrb. miss. Bot (Pringsh) 13, 569.

Best, F. 1906. Uber karminfarbung des glykogens der kerne. Z. miss. Mikr. 23, 319.

Bienwald, F. 1939. Zur histochemie der hirnanhanges. Virchows Arch. 303, 577.

Blank, H. 1949. Rapid Embedding technique for histologic sections employing water-soluble wax. J. invest.Derm, 12, 95.

Blank, H. and M'Carthy, P.L. 1950. A general method for preparing histologic sections with a water soluble wax. J. Lab. clin. Med. 36, 776.

Bolles-Lee, A. 1946. "The Microtomist's Vade Mecum". J.A. Churchill. London, 10th Edn. p. 47.

Boyland, E. and McLean, D. 1935. A factor in malignant tissues which increases the permeability of the dermis. J. Path. Bact. 41, 553.

Brain, E.B. 1949. An investigation into the dimensional changes of oral tissues during sectioning in Paraffin wax. Brit. dent. J. 87, 282.

Bunge, G. 1887. "Lehrbuch der physiologischen und pathologischen Chemie". F.C.W. Vogel. Leipzig.

Bunting, H. and White, R.F. 1950. Histochemical studies of skin wounds in normal and in scorbutic guinea pigs. Arch. Path., 49, 590.

Bourne, G.H. 1953. "An introduction to functional histology". Churchill, London.

Bunting, H. 1950. The distribution of acid mucopolysaccharides in mammalian tissues as revealed by histochemical methods. Arch. Path. 49, 590.

Cain, A.J. 1949. A Critique of the plasmal reaction, with remarks on recently proposed techniques. Quart. J. micr. Sci. 90, 411.

Cameron, G.R. 1952. "Pathology of the cell". Oliver and Boyd, London.

Campani, M., and Reggiani, O., 1950. Observations in the experimental animal on the nature of the metachromatic ground substance in granulation tissue. J. Path.Bact.,62,563.

Carleton, H.M. 1922. Note on the comparitive effects on tissues of isolonic saline and distilled water when used as solvent for mercuric chloride and formal in histological fixation. Quart. Journ. micr.Sci. 66, 501.

Carleton, H.M. 1938. "Histological technique". 10th Ed.Oxford.

Carsten, M.E. 1947. A rapid procedure for the embedding of eyes. Arch. Path., 44, 96.

Casella, C. 1942. Il permanganato di potassio quale assidante in alcune reazzioni istochimiche dei polisaccaridi. Anat. Anz., 93, 289.

Caspersson, T.O. 1940. Methods for the determination of the absorption spectra of cell structures. J.Roy. micr.Soc.60,8.

Caspersson, T.O. 1950. "Cell growth and cell function". Chapman and Hall, London.

Castor, C.W. and Baker, B.L., 1950. The local effect of adrenocortical steroids on epidermis and connective tissue of the skin. Endocrinology. 47, 234.

Catchpole, H.R. 1949. Distribution of glycoprotein hormones in the anterior pituitary gland of the rat. J. Endocrinol.6,218.

Cavallero, C., and Braccini, C. 1951. Effect of cortisone on the mast cells of the rat. Proc. Soc. exp.Biol. N.Y.78,141.

Caventou, J.B. 1826. Recherches chimiques sur l'Amidon et les diverses substances feculantes du commence. Ann.Chim.Phys., 31, 337.

Chain, E., and Duthie, E.S. 1940. Identity of hyaluronidase and spreading factor. Brit. J. exp.Path.21, 324.

Chamont, E.M., and Mason, C.W., 1930. "Handbook of Chemical Microscopy". N.Y.

Chapman, I.M.H. 1952. Personal communication.

Clark, E.R., and Clark, E.L., 1918. Phagocytosis of carbon and carmine granules in the transparent tails of tadpoles. Anat. Rec., 15, 151.

Clark, E.R. and Clark, E.L. 1933. Further observations on living lymphatic vessels in the transparent chamber of the rabbits ear. Their relation to tissue spaces. Amer.J. Anat. 52, 273.

Claude, A. 1939. Spreading properties of leach extract and the formation of lymph. J. Exp. Med., 66, 353.

Claude, A., and Duran-Reynals, F. 1934. On the existence of a factor increasing tissue permeability in organs other than testicle. J. exp. Med., 60, 457.

Coleman, L.C., 1938. Preparation of leuco basic fuchsin for use in the Feulgen reaction. Stain. Tech., 13, 123.

Colin, J.J. and de Claubery, E.G. 1814. Sur les combinaisons de l'iode avec des substances vegetales et animales. Ann. Chim, 90, 87.

Conn, H.J. 1948. "The history of staining". Biotech Publications. Geneva N.Y.

Cornil, V. 1875. Sur la dissociation du violet de methylaniline et sa separation en deux couleurs sous l'influence de certains tissus normaux et pathologiques, en particulier par les tissus en degenerescance amyloide. C.R. Acad.Sci, 80, 1288.

Cotta, H. 1806. Naturbeobachtungen uber die bewegung und funktion des saftes in den gewachsen. Wiemar, 16, 96.

Crippa, A. 1951. Sulla utilizzazione del tetracetato di piombo come ossidante in istochimica. Boll.Soc.Ital.Biol. sper., 27, 599.

Crowley, N. 1944. Hyaluronidase production by haemolytic streptococci of human origin. J. Path.Bact., 56, 27.

Cutler, O.I. 1935. Embedding in glycol stearate. Arch. Path. 20, 445.

Danielli, J.F. 1949. A critical study of techniques for the cytochemical demonstration of aldehydes. Quart.J. micr. Sci. 90, 67.

Danielli, J.F. 1953. "Cytochemistry - A critical approach". John Wiley & Sons, New York.

Davies, D.U. 1943. The staining reactions of normal synovial membrane with special reference to the origin of the synovial mucins. J. Anat. 77, 160.

Davies, D.U. 1952. Specificty of staining methods for mucopolysaccharides of the hyaluronic acid type. Stain. Tech. 27, 65.

Davies, H.G., and Wilkins, N.H.F., 1951. "Physical aspects of cytochemical methods." Cytochemistry commission of the Society of Cell Ciology. Stockholm.

Day, T.D., 1947. The nature and significance of the cementing substance in interstitial connective tissue. J. Path. Bact. 59, 567.

Dempsey, E.W., Bunting, H., Singer, M., and Wislocki, G.B. 1947. The dye binding capacity and other chemo-histological properties of mammalian polysaccharides. Anat.Rec. 98, 417.

Dempsey, E.W., and Singer, M. 1946. Observations on the chemical cytology of the thyroid gland at different functional stages. Endocrinology. 38, 270.

Dempsey, E.W., Singer, M., and Wislocki, G.B., 1950. The increased bosophilia of tissue proteins after oxidation with periodic acid. Stain Tech. 25, 73.

Dempsey, E.W., and Wislock, G.W., 1946. Histochemical contributions to physiology. Physiol. Rev. 26, 1.

Dische, Z. and Borenfreund, E. 1950. A spectrophotometric method for the microdetermination of hexosamines. J. biol. Chem. 184, 517.

Dorfmann, A. 1950. The in vitro action of hyaluronidase. Ann. N.Y. Acad.Sci. 52, 1017.

Driessen, A., 1905. Zur Glykogenfarbung. Zbl. allg. Path path. Anat., 16, 129.

Duran-Reynals, F., 1928. Exaltation de l'activite du virus vaccinal par les extraits de centaines organes. C.R.Soc.Biol. Paris, 99, 6.

Duran-Reynals, F. 1933. Studies on a certain spreading factor existing in bacteria and its significance for bacterial invasiveness. J. exp. Med., 58, 161.

Duran-Reynals, F. 1939. Studies on the localization of dyes and foreign proteins in normal and malignant tissues. Amer.J. Cancer, 35, 98.

Duran-Reynals, F. 1943. General permeability-increasing effect of factor from mammalian testicle on blood capillaries Yale J. Biol. Med. 11, 601.

Duran-Reynals, F., Bunting, H., and Wangenen, G., 1950. Studies on the sex skin of macaca mulatta. Ann. N.Y. Acac. Sci., 52, 1006.

Duran-Reynals, F., and Stewart, F.W., 1931. The action of tumour extracts on the spread of experimental vaccinia of the rabbit. Amer. J. Cancer. 15, 2790.

Dziewiatkowski, D.D., 1951. Isolation of chondroitin sulphate S-35 from articular cartilage of rats. J. Biol. Chem. 189, 187.

Ehrlich, P., 1879. Uber die specifischen granulationen des blutes. Arch.Anat. Physiol. Phys. Abr., 1879, 571.

Ehrlich, P., 1901. "Uber d dimethylamidobenzaldehyde reaction. Die Medic. Woche, 15.

Engstrom, A., 1946. Acta.Rudiol., Suppl., 63. quoted from Engstrom and Glick.

Engstrom, A., and Glick, D. 1950. The mass of gastric mucosa cells measured by X-ray absorption. Science, 111, 379.

Favilli, G. 1940. Mucolytic effect of natural and artificial spreading factor. Nature, Lond. 145, 866.

Feyrter, F. 1936. Uber ein sehr einfaches verfahren der markscheidenfarbung zugleich eine neve art der farberei. Virchows Arch., 296, 645.

Firminger, H.I. 1950a. Carbowax embedding for obtaining thin tissue sections and study of intracellular lipids. Stain Tech., 25, 121.

Firminger, H.I. 1950b. Ultraviolet absorption spectromicroscopy of interstial cells of testis using carbowax embedding. J. nat. Cancer Inst., 10, 1350.

Fischer, A. 1899. "Fixierung, Farbung and Bau des Protoplasmas". G. Fischer, Jena.

Flemming, W., 1882. "Zellsubstanz, Kern und Zellthcilung" Vogel, Liepzig.

French, D., and Edsall, J.T. 1945. The reactions of formaldehyde with amino acids and proteins. Adv.Prot.Chem.2,277.

Frey, H. 1868. Die Hamatoxylin farbung. Arch.mikr.Anat., 4, 345.

Frey, H. 1874. "Histology and Histochemistry of Man". Trans. Baker, A.F.J. London.

Frey-Wyssling, A. 1948. "Submicroscopic morphology of protoplasm and its derivatives" Elsevier. London.

Friberg, V., Graft, W., and Aberg, B. 1951. On the histochemistry of partly hydrolysed bacterial dextran. Scandinav. J. clin.lab. Invest. 3, 221.

Friodenwald, J.S. 1947. Discussion on metachromasia. Cold Spng. Harb. Symp. quant. Biol., 12, 141.

Fuson, R.C. 1950. "Advanced organic chemistry". John Wiley & Son, N.Y. p. 240.

Gage, S.H., 1917. Glycogen in the nervous system of vertebrates. J.comp.Neurol., 27, 451.

Garven, S.D. and Gairns, F.W. 1952. The silver diamine ion staining of peripheral nerve elements and the interpretation of the results; with a modification of the bielschowsky-gros methods for frozen sections. Quart.J.exp.Physiol., 37, 131.

Gersh, I. 1932. The Altmann Technique for Fixation by drying while freezing. Anat.Rec., 53, 309.

Gersh, I. 1947. Polysaccharide complex in individual follicles of the thyroid gland of the rat. Federation Proc., 6, 392.

Gersh, I. 1948. Application in pathology of the method of fixation by freezing and drying of tissues. J. tech. Methods, 25, 179.

Gersh, I. 1949a. Glycoproteins in the thyroid gland of rats. J. Endocrinol., 6, 282.

Gersh, I. 1949b. A protein component of the Golgi apparatus. Arch. Path., 47, 99.

Gersh, I., 1952. Some recent experiments in fixation by freezing and drying. Symposium "Freezing and Drying". Institute of Biology. London. Session IV p.

Gersh, I., and Catchpole, H.R. 1949. The organisation of ground substance and basement membranes and its significance in tissue injury, disease and growth. Amer.J.Anat., 85, 457.

Glegg, R.E., Clermont, Y., and Leblond, C.P., 1952. The use of lead tetracetate, benzidrine, O-dianisidine and a "Film test" in investigating the periodic acid-Schiff technic. Stain Tech. 27, 277.

Gleichen, W.F. von., 1778. Abhandlung uber die Saamen und Infusionsthierchen Nurnbeng. xii. p. 171., 34 pls. 4 to

Glick, D., 1949. "Techniques of Histo-and Cytochemistry" Interscience, New York.

Gomori, G., 1946. The study of enzymes in tissue sections. Amer. J. clin.Path., 16, 347.

Gomori, G., 1952. "Microscopic Histochemistry". University of Chicago press.

Goodspeed, T.H., and Uber, F.M. 1934. Application of the Altmann freezing-drying technique to plant cytology. Proc.nat.Acad.Sci., Wash., 20, 495.

Grishman, E. 1948. Histochemical study of mucopolysaccharides in various normal tissues. Bull int. Ass.mod.Mus. 28, 104.

Hack, M.H., 1949. Quoted from Pearse, 1953.

Hack, M.H., 1951. A new histochemical technique for lipids applied to plasmal. J. nat. Cancer Inst., 12, 225.

Hahn, L., 1945. A method for the quantitative determination of glucosamine and N-acetylglucosamine in the presence of other sugars. Ark.Kemi.Min.Geol. 22A no.2.

Hale, A.J., 1952. The effect of temperature and of relative humidity on sectioning of tissues embedded in polyethylene-glycol wax. Stain Tech. 27, 189.

Hale, A.J., 1953a. The effect of sodium hydroxide on the periodic acid-Schiff reaction. Stain Tech. 28, 160.

Hale, A.J., 1953b. Observations on substances that react weakly to the periodic acid-Schiff test. Quart. J. micr. Sci. 94, 303.

Hale, A.J., 1954. The relationship between alkali, formalin and periodic acid-Schiff sensitivity. (In preparation).

Hale, C.W., 1946. Histochemical demonstration of acid mucopolysaccharides in animal tissues. Nature, Lond., 157, 802.

Hamperl, H. 1951. quoted by Pearse, 1953.

Hardy, W.B., 1899. On the structure of cell protoplasm. J. Physiol., 24, 158.

Herlant, M., 1943. Recherces sur la localisation histologique des hormones gonadotropes femelles au niveau de l'hypophyse anterieure. Arch. Biol. Paris, 54, 225.

Hertwig, G., 1929. "Histochemische Methoden". in "Handbuch der mikroskopische Anatomie des Menschen." Berlin.

Haworth, W.N. 1946. Structure function and synthesis of polysaccharides. Proc. Roy. Soc. A, 186, 1.

Hempelmann, L.H. 1940. Staining reaction of the mucoproteins. Anat. Rec., 78, 197.

Hess, M., and Hollander, F. 1947. Permanent metachromatic staining of mucus in tissue sections and smears. J.Lab.clin. Med., 32, 905.

Hobby, G.L., Dawson, M.H., Meyer, K., and Chaffee, E. 1941. The relationship between spreading factor and hyaluronidase. J. exp. Med., 73, 109.

Hoerr, N.L., 1936. Cytological studies by the Altmann-Gersh freezing-drying methods. I Recent advances in technique. Anat. Rec., 65, 293.

Holmes, W.C. 1926. The chemical nature of metachromasy. Stain. Tech. 1, 116.

Horowitz, H.N. Ikawa, M. and Fling, M. 1950. Interference by sugars and amino acids in the Elson-Morgan method for hexosamine. Arch. Biochem., 25, 226.

Hotchkiss, R.D., 1948. A microchemical reacting resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., 16, 131.

Hudack, S., Blunt, J.W., Higbee, P., and Kearin, J.M. 1949. A probable acid mucopolysaccharide present in granulation tissue. Proc. Soc.exper.Biol. N.Y. 72, 526.

Hutcheson, H.E., 1943. A method of producing experimental granulating wounds. Lancet. 2, 75.

Immens, J., and Vasseur, E. 1950. Influence of sugars and amines on the colorimetric hexosamine method of Elson and Morgan and its possible elimination. Nature, Lond., 165,898.

Jeanloz, R. 1950. Hotchkiss reaction and structure of polysaccharides. Science 111, 289.

Jeanloz, R., and Forchielli, E. 1951. Studies on hyaluronic acid and related substances. Periodate oxidation. J. biol. Chem., 190, 537.

Jennings, R.S. 1951. A simple disposable apparatus for dehydration of frozen tissues, Amer.J. Path. 52, 195.

Johnston, J.P., Ogston, A.G., and Stanier, J.E., 1951. A modification of the Elson and Morgan method for the estimation of glucosamine. Analyst, 76, 88.

Jordan, R.H., and McManus, J.F.A., 1952. Lead tetracetate oxidation of 1, 2-glycols in histochemistry. J. nat. Cancer Inst., 13 (Suppl) 228.

Jorpes, E., Werner, B., and Aberg, B. 1948. The fuchsin-sulfurous acid test after periodate oxidation of heparin and allied polysaccharides. J. biol. Chem., 176, 277.

Julen, C., Snellman, O., and Sylven, B. 1950. Cytological and Fractionation studies on the cytoplasmic constituents of tissue mast cells. Acta Physiol. Scand., 19, 289.

Kelly, E.G., and Miller, E.G., 1935. Reactions of dyes with cell substances. II. The differential staining on nucleoprotein and mucins by thionine and similar dyes. J. biol. Chem. 110, 119.

Klein, G. 1929. "Practicum der Histochemie." Berlin.

Knight, T.A. 1803. Experiments on the descent of the sap in trees. Phil. Trons., 93, 277.

Krakow, 1897. Beitrage zur Chemie der Amyloidentartung. Arch. exper. Path. Pharmak., 40, 195.

Laguesse, E. 1921. La structure lamelluse et le devellopement du tissu conjonctif lache diez les mammiferes en general et chez l'homme en particulier. Arch. Biol. Paris, 31, 173.

Landsmeer, J.M.F., 1951. Some colloid aspects of metachromasia. Acta physiol. pharm. neerl., 2, 112.

Langhans, C. 1890. Ueber glykogen in pathologischen neubildungen und den menschlicken eihauten. Virchows Arch., 120, 28.

Layton, L.L. 1951. Effect of cortisone upon chondroitin sulphate synthesis by animal tissues. Proc.Soc.exp.Biol. N.Y., 76, 596.

Leeuwenhoek, A van. 1674. Microscopical observations on blood, milk, bones, the brain, spitle, cuticula, sweat, fatt and teares. Philos. Trans., 9, 121.

Leeuwenhoek, A. van. 1714. Epistolae physiologicae, 3 of Opera. Dalphis, 102. quoted from Cameron.

Lehmann, C.G., 1851. "Physiological Chemistry". Translated by Day, G.E. London.

Levine, M.D., Garzoli, R.F., Kurtz, R.E., and Killough, J.A. 1948. On the demonstration of hyaluronidase in cercariae of Schistosama Mansoni. J. Parasitol., 34, 158.

Lewis, F.T., 1942. The introduction of biological stains; Employment of Saffron by Vieussens and Leeuwenoek. Anat. Rec. 83, 229.

Lhotka, J.F., 1952a. Histochemical use of lead tetracetate. Anat. Rec. 112, 153.

Lhotka, J.F., 1952b. Histochemical use of sodium bismuthate. Stain Tech. 27, 259.

Lhotka, J.F., 1952c. Histochemical differentiation of cis-trans 1-2 glycols. Nature N. London, 170, 751.

Lillie, R.D., 1947. Reticulum staining with Schiff reagent after oxidation by acidified sodium periodate. J. lab. clin. Med., 32, 910.

Lillie, R.D., 1948. "Histopathological Technic". Philadelphia.

Lillie, R.D., 1949. Studies on the histochemistry of normal and pathological mucins in man and in laboratory animals. Bull. int. Assoc. med. Mus., 29, 1.

Lillie, R.D., 1950. Significance of the periodic acid-Schiff reaction. Anat.Rec., 108, 239.

Lillie, R.D., 1951a Simplification of the manufacture of Schiff reagent for use in histochemical procedures. Stain Tech., 26, 123.

Lillie, R.D., 1951b Histochemical comparison of the Casella, Baver and periodic acid - Schiff, leucofuchsin Technics. Stain Tech., 26, 163.

Lillie, R.D., 1952. Ethylenic reaction of ceroid with performic acid and Schiff reagent. Stain Tech., 27, 37.

Lillie, R.D., and Greco, J., 1947. Malt diastase and ptyalin in place of saliva in the identification of glycogen. Stain Tech., 22, 67.

Lillie, R.D., and Mowry, R.W., 1949. Histochemical studies on absorption of iron by tissue sections. Bull. int. Ass. med. Mus., 30, 91.

Linderstrom-Lang, K., 1936. Problems in histochemistry. Arch. exp. Zellforsch., 19, 231.

Lison, L., 1932. Sur la specificite du reactif de Schiff evers les aldehydes. Bull. Histologie, 89, 177.

Lison, L., 1935. Etudes sur la metachromasie, colourants metachromatiques et substances chromotropes. Arch. Biol. Paris, 46, 595.

Lison, L., 1936. "Histochemie Animale" Gauthier-Villars,Paris.

Longley, J.B., 1952. Effectiveness of Schiff variants in the periodic acid and Feulgen nucleal Technics. Stain Tech., 27, 161.

Lutwak-Mann, C. 1941. Enzymic decomposition of amino-sugars. Biochem. J., 35, 610.

Magnol, P., 1709. Sur la circulation de la seve dans les plantes. Histoire de l'Acad. roy des Sciences, Paris, p.44.

Malaprade, M.L. 1934. Etude de l'action des polyalcools sur l'acide periodique et les periodates alcalins. Bull. Soc. chim. Paris, 1, 838.

Malmstrom, B.G. 1951. Theoretical considerations of the rate of dehydration by histological freezing-drying. Exp.Cell. Res., 2, 688.

Malmstrom, B.G., and Glick, D. 1952. Simple and efficient freezing drying apparatus for the preparation of embedded tissue. Exp. Cell Res., 4, 125.

Malpighi, P. 1686. Opera omnia (London). quoted from Cameron.

Mancini, R.E. 1944. Estudio comparative de las metodos histoquimicos para investigar el glucogeno. Arch. Soc. argent. Anat. norm. patol., 6, 628.

Mancini, R.E. 1950. Investigacion histoquimica de las mucopolisacaridos. Rev.Soc.argent.Biol., 26, 139.

Madinavietia, J., Todd, A.R., Bacharach, A.L., and Chance, A.R.A. 1940. Mucolytic activity of diffusing factor preparations. Nature, Lond., 146, 197.

Mann, G., 1902. "Methods and theory of physiological histology" Oxford (Clarendon Press).

Massart, L., Coussens, R., and Silver, M. 1951. Metachromasie et structure des acides nucleiques. Bull. Soc. Chim. biol. Paris, 33, 514.

Medawar, P.B., 1941. The rate of penetration of fixatives. J.R. micr. Soc., 161, 46.

Meischer, J.F. 1874. Verh. naturforsch. gesellsch. in Basel. 6, H.i. quoted from Cameron.

Mendelow, H. and Hamilton, J.B. 1950. A new technique for rapid freezing and dehydration of tissues for histology and histochemistry. Anat. Rec., 107, 443.

Menkin, V. 1940. Effect of adrenal cortex extract on capillary permeability. Am. J. Physiol., 129, 691.

Meyer, K., 1945. "Advances in protein chemistry". [illegible] Academic Press Inc., New York.

Meyer, K., 1946. Combined staff clinics on rheumatoid arthritis. Amer. J. Med., 1, 675.

Meyer, K., 1947. The biological significance of hyaluronic acid and hyaluronidase. Physiol. Rev., 27, 335.

Meyer, K., 1950. The mucopolysaccharides of the interfibrillar substance of the mesenchyme. Ann. N.Y. Acad.Sci. 52, 961.

Meyer, K., 1952. Hyaluronic acid, chondroitin sulphates and their protein complexes. Proc. Faraday Soc. (Proff), August, 1952

Meyer, K., and Chaffee, E. 1941. The mucopolysaccharides of skin. J. biol. Chem., 138, 491.

Meyer, K., Chaffee, E., 1941. Hobby, G.L., and Dawson, M.H., Hyaluronidases of bacterial and animal origin. J. exp. Med., 73, 309.

Meyer, K., Dubos, R., and Smyth, E.M., 1937. The hydrolysis of the polysaccharide acids of vitreous humour, of umbilical cord, and of streptococcus by the autolytic enzyme of pneumoccus. J. biol. Chem., 118, 71.

Meyer, K.H., and Fellig, J. 1950. La constitution de l'acide hyaluronique. Experientia, 6, 186.

Meyer, K., Hobby, G.L. Chaffee, E., and Dawson, M.N., 1940 The hydrolysis of hyaluronic acid by bacterial enzymes. J. exp. Med., 71, 137.

Meyer, K.H., and Linker, A. 1951. Hydrolysis of glucuronidic linkage of hyaluronic acid. Fed. Proc., 10, 223.

Meyer, K.H., Odier, M.E., and Siegrist, A.E., 1948. Constitution de l'acide chondroitine-sulfurique. Helv.chim. acta., 31, 1400.

Meyer, K. and Palmier, J.W., 1936. On glycoproteins. II. The polysaccharides of vitreoushumor and umbilical cord. J.biol. Chem. 114, 689.

Meyer, K., and Rapport, M.M. 1951. The mucopolysaccharides of the ground substance of connective tissue. Science,113,596.

Michaelis, L., 1902. "Einfuhrong in die Farbstoffchimie fur Histologen" Berlin.

Michaelis, L., 1947. The nature of the interaction of nucleic acids and nuclei with basic dyestuffs. Cold Sprg. Harb. Symp. quant. Biol, 12, 131.

Michaelis, L., and Granick, S., 1945. Metachromasy of basic dyestuffs. J. Amer. chem. Soc., 67, 1212.

Miles, A.E.W., and Linder, J.E., 1953. Polyethylene glycols as histological embedding media: With a note on the dimension change of tissue during embedding in various media. J.R.micr.Soc. 72, 199.

Mitchell, A.J. and Wislocki, G.B. 1944. Selective staining of glycogen by ammoniacal silver nitrate: A new method. Anat. Rec., 90, 261.

Morgan, W.T., and Elson, L.A., 1934. A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Biochem. J., 28, 988.

Moller, K.M., 1951. Personal communication.

Muller, F., 1901. Beitrage zur kenntniss des mucins und einigen damit verwandter eiweifstaffe. Z.f.Biol.42,468.

Mullen, J.P. 1944. A convenient and rapid method for staining glycogen in paraffin sections with Best's carmine stain. Amer.J.clin.Path., 14, 9.

Munro, H. 1951. Personal communication.

Macallum, A.B., 1908. Die methoden und ergebuisse der mikrochemie in der biologischen furschung. Ergebn. Physiol., 7, 552.

McCullagh, D.R., Cassidy, J.W., Valentine, F and Tolksdorf, S. 1949. The stability and stabilisation of testicular hyaluronidase. Proc. Soc. exp. Biol. N.Y., 71, 295.

McCutcheon, M and Coman, D.R. 1947. Spreading factor in human carcinemas. Cancer Research, 7, 379.

McLane, S.R., 1951. Higher polyethylene glycols as water soluble matrix for sectioning fresh and fixed plant tissues. Stain Tec., 26, 63.

McLean, D. 1930. The influence of testicular extract on dermal permeability and the response to vaccine virus. J. Path. Bact., 33, 1045.

McLean, D., 1936. A factor in culture filtrates of certain pathogenic bacteria which increases the permeability of the tissues. J. Path. Bact, 42, 477.

McLean, D., Rogers, H.J. and Williams, B.N. 1943. Early diagnosis of wound infection. Lancet, 1, 246 and 355.

McManus, J.F.A., 1946. The histological demonstration of mucus after periodic acid. Nature, London, 158, 202.

McManus, J.F.A., 1948a. The periodic acid routine applied to the kidney. Amer.J.Path., 24, 643.

McManus, J.F.A., 1948b. Histological and histochemical uses of periodic acid. Stain Tech., 23, 99.

McManus, J.F.A., and Cason, J.E. 1950. Carbohydrate chemistry studied by acetylation techniques. J. exp. Med., 91, 651.

McManus, J.F.A., and Hoch-Ligeti, C. 1952. Modified periodic acid spot test for 1,2. glycols. Lab.Invest, 1, 19.

McMaster, P.D., and Parsons, R.J. 1939. Physiological conditions existing in connective tissue. J.exp. Med., 69, 265.

Nadkarni, M.V., Meyers, D.B., and Zopf, L.C. 1949. Effects of surface-active agents on the crystallinity of carbowax compounds. J.Amer.pharm.Ass. 38, 216.

Nicolet, B.N., and Shinn, L.A., 1939. The action of periodic acid on 1-amino alcohols. J.Amer.chem.Soc., 61,1615.

Nicolet, B.N., and Shinn, L.A., 1941. The determination of serine by the use of periodate. J.biol. Chem., 139, 687.

Nitschmann, H. and Hadorn, H. 1943. Die umsetzung des Caseins mit Formaldehyd. III quantitative verfolgung der formaldehydindung bei der hartung des. [Helv. chim. Act.26,1075] Cuseins mit massrigem und mit gasfommigem formaldehyd. ↘

Ogston, A.G., and Stanier, J.E. 1950. On the state of hyaluronic acid in synovial fluid. Biochem. J., 46, 364.

Olander, A. 1927. Uber die kinetik der oximbildung. A.phys. Chem., 129, 11.

Orimus, E., 1865. De l'emploi de la fuschine dans l'etude des elements Anatomique. J. de l'Anat., 5, 569.

Opsahl, J.C. 1949. Dermal spreading of Indian ink with or without hyaluronidase as influenced by hormones from adrenal cortex. Yale. J.Biol. and Med., 21, 248.

Opsahl, J.C., White, A., and Duran-Reynals, F. 1950. The effect of adrenocortical hormone on the dermal spreading of Indian ink in normal and adrenalectomized mice. Ann.N.Y. Acad. Sc., 52, 1061.

Orton, S.T., and Post, J., 1932. Some experiments with a new embedding material. Bull. neur. Inst., N.Y. 2, 302.

Ostling, S., and Virtama, P. 1946. Modified preparation of the universal buffer described by Teorell and Stenhagen. Acta phys. Scandinav. 11, 289.

Packer, D.M., and Scott, G.H., 1942. A cryostat of new design for low temperative tissue dehydration. Bull. inst. Ass. med. Mus., 22, 85.

Parat, M., 1927. A review of recent developments in histochemistry. Biol. Rev., 2, 285.

Patten, B.M. and Philpott, R. 1921. The shrinkage of embryos in the processes preparatory to sectioning. Anat. Rec., 20, 393.

Patzelt, V., 1928. "Fertschritte der Mikochemie". Klein and Strebingen, Vienna.

Pearse, A.G.E., 1949. The nature of Russell bodies and Kurloff bodies. J. clin. Path., 2, 81.

Pearse, A.G.E., 1950. The histogenesis of granular cell myoblastoma. J. Path. Bact., 62, 331.

Pearse, A.G.E., 1951. A review of modern methods in histochemistry. J. clin. Path., 4, 11.

Pearse, A.G.E., 1953. "Histochemistry: Theoretical and applied." Churchill, London.

Pirani, C.L. D.O.C.A. and wound healing. Report 75, Medical Nutrition Lab. Chiago, Illinois.

Pischinger, A., 1926. Die lage des isoelektrischen punktes histologischen elemente als ursache ihrer verschiedenen fanbankeit. Z.Zellforsch., 3, 169.

Pischinger, A., 1927. Uber die isoeloktrischen punkte der muskelbestandteile. Plug. Arch.ges.Physiol., 217,205.

Pischinger, A., 1937. Untersuchungen uber die kernstruktur, besondens uber die beziehungen zwischen struktur in leben und nach fixierung. Z.Zellforsch. mikr. Anat., 26,249.

Policard, A., and Okkels, H. 1932. "Die Mikroveraschung als histochemische Hilfs methode." in "Handbuch der biologischen Arbeitsmethoden" Abderhalden. Berlin.

Prönscher, 1901. Zur keuntniss der Erlich'schen dimethylamide-benzaldehydreaction. Z.f.physiol.Chem.31,520.

Ragan, C., Howes, E.L., Plotz, C.M., Meyer, K., and Blunt, J.W., 1949. Effect of cortisone on production of granulation tissue in the rabbit. Proc. Soc. exp. Biol. N.Y.72,718.

Raspail, F.V. 1825a. Developpement de la Fecule dans les organes de la fructification des Céréales, et analyse microscopique de la Fecule, suivie d'experiences propres à en expliquer la conversions en gomme premiere partie. Ann.Sci. nat., 6, 224.

Raspail, F.V., 1825b. As above. Seconde partie. Ann. Sci.nat., 6, 384.

Raspail, F.V., 1829. quoted from Pearse, Ann.Sc.Observation, 1, 72.

Richards, A.G., Anderson, T.F., and Hance, R.T. 1942. A microtome sectioning technique for electron microscopy. Proc. Soc. exp. Biol. N.Y. 51, 148.

Riechel, G.C. 1758. "De vasis plantarum spiralibus" Lipsiae. quoted from Lewis.

Rigby, W. 1950. Oxidations with sodium bismuthate. Glycol fission and related reactions. J. Chom.Soc. 1950,1907.

Rinehart, J.F. and Abul-Haj, S. 1951a. Histological demonstration of lipids in tissue after dehydration and embedding in polyethylene glycol. Arch.Path., 51, 666.

Rinehart, J.F., and Abul-Haj, S.K. 1951b. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. Arch.Path., 52, 189.

Ritter, H.B. and Oleson, J.J. 1950. Combined histochemical staining of acid polysaccharides and 1,2 glycol groupings in paraffin sections of rat tissues. Amer.J.Path., 26,639.

Roberts, G.B.S., and Jarret, W.F.H., 1947. The histochemical identification of the higher polysaccharides. Proc.Anat. Soc.G.B. April, p.3.

Rogers, H.J. 1946. An analysis of the products formed during the hydrolysis of hyaluronate by enzymes and acids, with observations on the nature of the amino sugar released from the polysaccharide. Biochem. J., 40, 782.

Rogers, H.J. 1948. The complexity of the hyaluronidases produced by micro-organisms. Biochem. J., 42, 633.

Romeis, B. 1932. "Histochemische Methoden" in "Taschenbuch der mikroscopischen Technik". Boehm and Oppel. Munich.

Scanlin, J.T., and Melin, C.G., 1937. The production of basic fuchsin suitable for the Feulgen Technic. Stain Tech., 12, 1.

Schloss, B. 1951. Colorimetric determination of glucosamine. Anal. Chem., 23, 1321.

Schrader, F., and Leuchtenberger, C. 1951. The cytology and chemical nature of sperm constituents. Chromosoma, 4, 404.

Schwann, T. 1847. "Microscopical researches into the accordance in the structure and growth of animals and plants". Sydenham Soc. London.

Schwarz, F. 1867. Die morphologische und chemische zusammensetzung des protoplasmas. quoted from Cameron.

Scott, G.A., 1933. A critical study and review of the method of microincineration. Protoplasma, 20, 133.

Scott,GH, and Williams, P.S. 1936. A simplified cryostat for the dehydration of frozen tissues. Anat. Rec., 66,475.

Seibert, F.B., Seibert, M.V., Atno, A.J., and Campbell, H.W. Variation in protein and polysaccharide content of sera in the chronic diseases, tuberculosis, sarcoidosis and carcinoma. J. Clin.Invest. 26, 90.

Seifter, J., Baeder, D.H., and Dervinis, A. 1949. Alteration in permeability of some membranes by hyaluronidase and inhibition of this effect by steroids. Proc.Soc.exp.Biol. N.Y., 72, 136.

Selye, H. 1947. "Textbook of Endocrinology" Acta Endocrinologica. Montreal.

Shimizu, N., and Kumamoto, T. 1952. A lead tetracetate-Schiff method for polysaccharides in tissue sections. Stain Tech., 27, 97.

Shuman, C.R., and Finestone, A.J. 1950. Inhibition of hyaluronidase in vivo by Adreno-cortical activation. Proc. Soc. exp. Biol. N.Y., 73, 248.

Sideris, C.P., Young, H.Y. and Krauss, B.N. 1938. The distribution of uncombined hexosamine in pineapple plants supplied either with ammonia sulphate or calcium nitrate salts. J. biol. Chem., 126, 233.

Singer, M. and Wislocki, G.B., 1948. The affinity of syncytium, fibrin and fibrinoid of the human placenta for acid and basic dyes under controlled conditions of staining. Anat.Rec., 102, 175.

Sjobring, N. 1900. Ueber das formol als fixirungsflussigkeit. Anat. Anz., 17, 273.

Sjovall, E., 1906. Uber spinalganglienzellen und markscheiden. Anat. Hefte, 1 Abt., 30, 259.

Sprunt, D.H. 1950. The ground substance in infection. Ann. N.Y. Acad. Sci., 52, 1052.

Stafford, R.O., and Atkinson, W.B., 1948. Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatase in rat tissues. Science,107,279.

Staughton, R. and Wells, G., 1950. A histochemical study on polysaccharides in normal and diseased skin. J.Invest. Dermat., 14, 37.

Stedman, E., and Stedman, E. 1943a. Chromosomin, a protein constituent of chromosomes. Nature, London, 52, 267.

Stedman, E. 1943b. Distribution of nucleic acid in the cell. Nature, London, 152, 503.

Stedman, E. 1944. Chromosomin and nucleic acids. Nature, London, 153, 500.

Steedman, H.F. 1945. Ester wax: A new embedding medium. Nature, Lond, 156, 121.

Steedman, H.F., 1947. Ester wax: A new embedding medium. Quart.J.micr.Sci., 88, 123.

Steedman, H.F., 1949. An ester wax for use in the tropics. Nature, Lond, 164, 1084.

Stowell, R.E., 1942. Photometric histochemical determination of thymonucleic acid in experimental epidermal carcinogeresis. J. nat. Cancer Inst., 3, 111.

Stowell, R.E., 1951. A modified freeze-drying apparatus for tissues. Stain Tech., 26, 105.

Strangeways, T.S.P. and Cant, R.G. 1927. The living cell in vitro as shown by dark ground illumination and the changes induced in such cells by fixing reagents. Quart.J.micr. Sci., 71, 11

Swammerdam, J. 1758. "The book of nature". London.

Sylven, B. 1941. Ueber das Varkommen van hochmelekularen Esterschwefelsauren in Granulationsgewebe und bei der Epithal regeneration. Acta. chir. scand., 86, Suppl. 66.

Sylven, B., 1945. Ester sulphuric acids of high molecular weight and mast cells in mesenchymal tumours. Acta. radiol. Stockh., Suppl., 59.

Sylven, B., 1950. The qualitative distribution of metachromatic polysaccharide material during hair growth. Exper.Cell.Res. 1, 582.

Sylven, B., 1951. On the advantages of freeze-vacuum dehydration of tissues in morphological and cytochemical research. Acta unio internationalis contra cancrum., 7, 708.

Sylven, B., 1952. Effects of freezing and drying compared with common fixation procedures. "Freezing and Drying". Institute of Biology, London, p. 169.

Sylven, B., and Malingren, H. 1952. On the alleged metachromasia of hyaluronic acid. Lab. Invest. 1, 413.

Tarkhan, A.A. 1931. The effects of fixation and other reagents on cell-size and tissue bulk. J.R.micr.Soc., 51, 387.

Tellyesniczky, K. 1898. Ueber die fixirungs (Hartungs) Flussigkeiten. Arch.mikr.Anat., 52, 202.

Tellyesniczky, K. 1902. Zur kritik der kernstrukturen. Arch.mikr.Anat., 60, 681.

Tellyesniczky, K., 1905. Ruhekern und Mitose. Arch. mikr. Anat., 66, 367.

Tellyesniczky, K., 1936. quoted from Baker.

Tracey, M.V., 1951. The determination of glucosamine by alkaline decomposition. Biochem. J., 49, xx.

Tracey, M.V., 1952. Determination of glucosamine by alkaline decomposition. Biochem. J. 52, 265.

Underhill, B.M.L., 1932. The rate of penetration of fixatives. J.R.micr.Soc., 52, 113.

Van Gieson, J., 1889. Laboratory notes of Technical methods for the nervous system. N.Y. Med.J., 50, 57.

Van Horme, R.L., and Zopf., L.C. 1951. Water soluble embedding materials for botanical microtechnique. J.Amer. pharm. Ass., 40, 31.

Vieussens, R., 1684. quoted from Lewis, F.

Virchow, R., 1860. "Die Cellutarpathologie". Berlin.

Wade, H.W., 1952. Notes on the Carbowax method of making tissue sections, Stain Tech., 27, 71.

Waldeyer, 1863. Untersuchungen uber den Ursprung und den Verlauf des Axsencylinders dei Wirbollosen und Wirbelthieren sowie uber dessen Endverhalten in der quergestreiften Muskelfaser. Heule u p feifer's Zts. f. rationelle Med., 3, Reihe, 20, 193.

Wang, K.J. and Grossman, M.I. 1949. A simplified vacuum dehydration technique for the preparation of sections by freezing-drying. J. lab. clin.Med., 34, 292.

Wieland, H., and Scheying, G., 1921. Die fuchsin-schweiflige Saure and ihre Farbreaktion mit Aldehyden. Ber.deut.Chem. Gesell., 54. 2527.

Winzler, R.J., Devor, A.W., Mehl, J.W. and Smyth, I.M. Studies on the mucoproteins of human plasma. 1948. J. clin.Invest., 27, 609.

Wislocki, G.B., Bunting, H., and Dempsey, E.W., 1947. Metachromasia in mammalian tissues and its relationship to mucopolysaccharides. Amer.J.Anat., 81, 1.

Wislocki, G.B., and Soggnaes, R.F., 1950. Histochemical reactions of normal teeth. Amer.J.Anat., 87, 239.

Wolfrom, M.L. Madison, R.K., and Cron, M.J. 1952. The structure of chondrosino and of chondroitinsulfuric acid. J. amer. Chem. Soc., 74, 1491.

Wolman, M. 1950. Staining of lipids by the periodic acid-Schiff reaction. Proc. Soc. exp. Biol. N.Y., 75, 583.

Wolman, M., and Greco, J., 1952. The effect of formaldehyde on tissue lipids and on histochemical reactions for carbonyl groups. Stain Tech., 27, 317.

Young, J.Z. 1935. Osmotic pressure of fixing solutions. Nature, London, 135, 823.

Zernicke, F. 1942. Phase contrast, a new method for the microscopic observation of transparent objects. Physica. 9, 656, 914.

Zupinger, A. 1874. Eine methode axencylinderfortsatze der Ganglienzellen des Ruckenmarkes zu demonstriren. Arch. Mikr. Anat., 10, 255.