"THE PATHOGENESIS OF HASHIMOTO'S DISEASE AND ITS INVESTIGATION BY THYROID TISSUE CULTURE."

by

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**PREFACE**

It is widely believed that the aetiology of Hashimoto's disease (lymphadenoid goitre, struma lymphomatosa) is that of an auto-immunising thyroiditis. This work was undertaken in the attempt to decide whether or not circulating auto-antibodies such as are found in Hashimoto serum have any direct toxic effect on thyroid cells.

The effect of Hashimoto serum on human thyroid cells in tissue culture was studied. In addition rabbit sera were prepared containing antibodies in high titre to pure thyroglobulin and to crude thyroid extract. The effect of these rabbit sera on human thyroid cells in tissue culture was also investigated.
PART I.

HASHIMOTO'S DISEASE.
1.

HASHIMOTO'S DISEASE

The Clinical Features.

According to Joll (1939), a firm goitre which appears in a middle aged patient, usually a woman, reaches its maximum in a few months, and involves every part of the gland, causes only moderate degree of dyspnoea, but produces neither serious pressure effects, thyrotoxic symptoms, pain, tenderness, pyrexia nor other inflammatory phenomena, is almost certainly a lymphadenoid goitre (Hashimoto's disease). If to these features is added the absence of any evidence of involvement of extra thyroid tissues and the presence of some degree of hypothyroidism, then Joll would have placed the diagnosis beyond reasonable doubt. A lymphadenoid goitre associated with myxoedema is shown in Figs. 1 and 2. Fig. 3 demonstrates a characteristic retro-tracheal extension of the goitre.

Nevertheless the purely clinical diagnosis of Hashimoto's disease is a matter of no small difficulty. Thus Marshall et al. (1948) had only a correct pre-operative diagnosis of 17 per cent and the figure of 21 per cent given by Lindsay et al. (1952) is not much better.
Fig. 1.
Lateral X-ray of neck showing marked increase in the pre-vertebral soft tissue shadow and striking forward displacement of the trachea.

Fig. 2.
Mrs. I.P. Age 59 years. Hashimoto's disease. Goitre of 2 years duration Note hypothyroid facies.
Lateral X-ray of neck showing marked increase in the pre-vertebral soft tissue shadow and striking forward displacement of the trachea. Same case as Figs. 1 and 2.
The full clinical picture as described above is not invariably present and the various signs and symptoms are in no way pathognomonic for they may be individually or collectively present in other forms of thyroid disease. The main differential diagnosis is between a simple diffuse nodular goitre and malignant disease.

Until very recently there were no laboratory aids to the diagnosis of Hashimoto's disease. Since 1953, however, an ever increasing amount of diagnostic assistance has proceeded from the laboratory so that at present there are a number of such tests which help to confirm or refute a clinical suspicion of the disease.

It was in 1953 that Fromm et al. reported raised serum gamma globulins in Hashimoto's disease. Cooke & Wilder (1954), Cooke & Luxton (1955) and Luxton & Cooke (1956) found similar serum protein abnormalities and in addition reported the occurrence of abnormal flocculation tests of liver function in a significant proportion of patients with this disease. The colloidal gold and thymol turbidity reactions appear to be the most significant.
Doniach & Hudson (1957) demonstrated normal or high thyroid uptakes of radioactive iodine (I\(^{131}\)) in Hashimoto's disease even in the presence of myxoedema, associated with a uniform distribution of isotope corresponding with the outline of the goitre. A short biological half-life and high plasma activity led these workers to suggest that in Hashimoto's disease there is a small thyroidal iodine pool with a high activity and rapid turnover.

The third laboratory aid to diagnosis developed from the observation of Roitt, Doniach, Campbell & Hudson (1956) that precipitation occurred when a saline extract of a normal thyroid gland was layered onto the serum of a patient with Hashimoto's disease. The precipitin, tanned cell and complement fixation tests (see later) are now in common diagnostic use and of these the precipitin test has the greatest practical value.

**Histo-Pathology.**

There has been little agreement on the histological features of lymphadenoid goitre since Hashimoto first described his four cases of unusual goitre in elderly females in 1912:
According to Hashimoto's classical description diffuse parenchymal changes, lymphoid infiltration and fibrosis are characteristic (Fig. 4). The parenchymal changes consisted of diminution in the size of the acini which were lined by a single layer of epithelium, sometimes infiltrated by mono-nuclear leucocytes. Further characteristic epithelial cell changes were subsequently described by Parmley & Hellwig (1946) and are illustrated in Fig. 5. The lymphoid infiltration in Hashimoto's series was widespread; there were lymphoid follicles with germinal centres and diffuse infiltration by lymphocytes and small numbers of plasma cells. The description of plasma cells by the original author is important because some workers (Paine et al., 1957) are reluctant to diagnose "Hashimoto's disease" where such cells are present. Fibrous tissue proliferation was present in all four of Hashimoto's cases. Hashimoto considered that his cases showed a progression of pathological changes, that fibrosis could be marked and that plasma cells were present.

Stuart & Allan (1958) studied the basement membrane changes in Hashimoto's disease and found them to be striking, severe and constant.
Fig. 4. (H.E. x 120).

Classical appearance of Hashimoto's disease showing prominent lymphoid follicles, small acini and moderate degree of interstitial round cell infiltration.
Fig. 5. (H.E. x 150).

Characteristic epithelial cell changes. The epithelium is plump and cuboidal with faintly granular oxyphilic cytoplasm. The nuclei vary in position and some acini contain fewer nuclei than usual. This appearance is sometimes called Askanazy, Hurtle or pink cell change. Small foci of Askanazy cell change are sometimes seen in thyrotoxicosis and in such instances the oxyphilia and nuclear pleomorphism is usually more marked than in Hashimoto's disease.
Fig. 6.

Normal thyroid: (Silver impregnation x 975)

The basement membrane in a normal gland stains intensely and evenly; it measures approximately 0.1 to 0.2 μ in width and is closely applied to the base of the epithelial cells. It forms a continuous unbroken lining and completely seals off the follicle from capillary vessels.
Hashimoto's Disease (Silver impregnation x 575)

The basement membrane is irregular, wavy, and fragmented.
They used the modified silver stain method of Slidders & Lendrum (1958), as illustrated in Figs. 6 and 7. Fragmentation of the basement membrane was characteristic and was usually but not always associated with the presence of chronic inflammatory cells in the affected part.

Pathogenesis.

Until recent years there was no clue to the pathogenesis of Hashimoto's disease, but, as already mentioned, in 1956 Roitt, Doniach, Campbell & Hudson showed that serum from patients with Hashimoto's disease contained an antibody which reacted with human thyroid extracts. They concluded that the raised serum gamma globulin observed in Hashimoto's disease and the presence of plasma cells in the thyroid gland was the result of an auto-immune process. Confirmation of this hypothesis was provided in the same year by Rose & Witebsky (1956) who produced thyroid lesions in rabbits somewhat similar to these of lymphadenoid goitre by immunising the rabbits against rabbit thyroid extract. Furthermore, it was easy to see that a reaction of this type, once initiated, might become self-perpetuated. Damage to the thyroid, caused by antibody
action, would release more antigen and hence enhance the level of circulating antibody. The cause of the initial release of antigen which was presumably responsible for setting this chain-reaction in motion, remained obscure; but it was not difficult to postulate that it might be some relatively trivial damage to the thyroid which perhaps was difficult or impossible to detect.

These initial observations stimulated much interest and the concept of Hashimoto's disease as the outcome of an auto-immune process is now widely accepted. Indeed the new name for Hashimoto's disease has become "auto-immunising thyroiditis."
PART II.

"THE CULTURE OF HUMAN THYROID CELLS IN MEDIA CONTAINING NORMAL HUMAN SERUM."
PREVIOUS WORK ON THYROID TISSUE CULTURE.

The cultivation of tissues and organs outside of the body is no recent achievement. Indeed a reference to thyroid culture in vitro was made as long ago as 1910 by Carrel & Burrows. Following the work of Harrison on the embryonic tissues of the frog, these workers cultured thyroid explants from adult dogs in plasma clots and noted that this was very easy. They observed that an abundance of long fusiform cells radiated from the tissue through the plasma.

Later, in 1925, Ebeling cultured the thyroid glands of 18-19 day chick embryos in plasma clots. A pure strain of thyroid epithelium was isolated and maintained in an active condition for 7 months. They noted that the thyroid cells grew at the surface of the coagulum as pavement epithelium and within the coagulum as a glandular structure. The cells did not de-differentiate and the lumen of the acini in cultures from a strain over 4 months old is reported to have contained colloid secretion morphologically similar to that from a freshly exterpolated thyroid gland. Furthermore, at the end of 7 months the rate of cell multiplication was as great as at the beginning.
of the experiment.

A technique for the "culture" of whole thyroid glands was described in 1937 by Carrel, whereby the intact thyroid gland was perfused in a Lindbergh apparatus. By this method the gland could be kept alive for at least some 3-21 days.

Cinemicroscopic studies of living thyroid cells of man and dog were recorded by Gey in 1937-8, and in the following year Gey & Bang (1939) reported the use of thyroid tissues for the culture of the lymphogranuloma inguinale virus. Oppenheimer, Tata & Rawson (1956) studied the morphology and function of young adult rabbit thyroids grown in chicken plasma clots and noted that the cells could be separated into fibroblast-like cells and epithelial cells. Moreover, these authors demonstrated that such cultures could convert radio-active iodine (I$^{131}$) which was added to their media to organic forms.

As distinct from previous studies, which have been concerned with the growth of thyroid cells from whole tissues, this work was done on human thyroid cells obtained from trypsin dispersed glands. This work proceeded
independently of the recent studies of Pulvertaft, Davies, Weiss & Wilkinson (1959) who very recently published an account of their work.
MATERIALS AND METHODS.

The technique employed in the present experiment was a modification of that described by Dulbecco & Vogt (1954).

The thyroid was transported from the Operating Theatre to the Laboratory within a matter of minutes after partial thyroidectomy had been performed. For this purpose a sterile large glass jar containing 300 mls. of unbalanced Hanks' solution was used. After the gland had been inspected and suitable portions taken for biopsy, between 5 and 10 G. of thyroid parenchyma were transferred to a petri dish containing unbalanced Hanks' solution and there cut up with fine scissors and forceps into pieces of about 1-2 mm. in diameter (Fig. 8). This chopped tissue was then washed in an effort to remove as much blood as possible and then transferred to 100 mls. of 0.25 per cent trypsin (Difco 1:250) in unbalanced Hanks' solution. After incubating in a water bath at 37° C. for 30 minutes the container was vigorously shaken by hand. The supernatant fluid was then decanted and replaced by a further 100 mls. of 0.25 per cent trypsin.

The thyroid tissue can be kept gently
Fig. 8.
Thyroid tissue cut up into small pieces and being washed in unbalanced Hanks' solution.
stirring by an automatic magnetic stirrer or more simply, but perhaps less satisfactorily, by vigorously shaking the container by hand every 15 minutes. Particularly when a magnetic stirrer was used the thyroid fragments adhered in a mucinous mass, and the fluid became cloudy with epithelial aggregates. A drop of fluid was examined at intervals until such time as a rich suspension of cells was obtained. In general, this took 2 to 3 hours. The suspension was then filtered through a single layer of sterile gauze and the cells centrifuged at 1500 r.p.m. for 3 minutes. The trypsin supernatant was then decanted and the cells re-suspended in just sufficient nutrient medium to ensure that when a drop was examined under a coverslip several cellular aggregates were seen in the field of 10x objective. The final volume of nutrient medium was generally 50 mls.

REAGENTS.

**RESIN PURIFIED WATER**

In order to obviate any adverse effects metallic ions might have had on cultured tissue all traces of tap water had to be removed from washed glass ware with water of very low ionic content. For this purpose, and also for the
preparation of salt solution, resin purified water was invariably used. This was obtained by passing metal distilled water through an "Elgostat De-Ioniser" (Elga Products, Railway Place, London, S.W.9.)

**HANKS' SALT SOLUTION**

All water used was resin purified. Salts used were "Analar" (BDH) grade. The composition of this saline was described by Hanks & Wallace (1949) and is as follows:-

Stock solution A -

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<tr>
<td>NaCl</td>
<td>160G.</td>
</tr>
<tr>
<td>KCl</td>
<td>8G.</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>4G.</td>
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These salts were dissolved in approximately 800 ml of water. 2.8 G. Calcium chloride were dissolved in approximately 100 ml of water. These two solutions were combined and made up with water to a litre. 2 ml of Chloroform were added as a preservative and the solution stored at 4°C.

Stock solution B -

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<th>Amount</th>
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<tr>
<td>Na₂HPO₄ 12 H₂O</td>
<td>3.04G.</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.2 G.</td>
</tr>
<tr>
<td>Dextröse</td>
<td>20G.</td>
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These were dissolved in approximately 800 ml water. 100 ml of 0.04 per cent phenol red solution were added and the volume made up to a
litre with water. 2 ml. of chloroform acted as a preservative and the solution was stored at 4°C. The stock solution of phenol red was prepared by placing 1G. of phenol red in the 250 ml. volumetric flask and adding N/20 NaOH from a burette with shaking until the powder was almost completely dissolved. NaOH was then added carefully until solution was complete and the colour was a deep ruby red. The volume was then made up to 250 ml. with water.

Hanks' unbalanced salt solution was prepared by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of resin purified water. The solution was sterilised by steaming for 90 minutes and kept at 4°C for not longer than 14 days. Balanced Hanks' solution was prepared by adding 0.5 ml. of sterile 1.4 per cent NaHCO₃ solution to each 20 ml. of unbalanced solution immediately before use. The bicarbonate solution was sterilised by steaming for 90 minutes and when cool was gassed for 10 minutes with a stream of CO₂. This was achieved by dipping a sterile, plugged Pasteur pipette into the flask containing the bicarbonate solution through the cotton wool plug and connecting the pipette to a tube containing a small piece of solid CO₂ (Drikold).
ANTIBIOTICS

Penicillin in unbalanced Hanks' solution (100 units per ml.), streptomycin in unbalanced Hanks' solution (100 micrograms per ml.) and mycostatin in distilled water (100 units per ml.) were used in all fluids routinely. The use of antibiotics, although of very great assistance, did not prevent infection of cultures by resistant gram-negative bacilli if the standard of asepsis was low. With careful handling, however, infection of cultures was rare.

TRYPsin

Crystalline trypsin (Difco 1:250) was used in a 0.25 per cent solution. The solution was made up in 200 ml. bottles at 2 per cent dilution in unbalanced Hanks' solution, Seitz filtered and stored at -20°C. After Seitz filtering the trypsin was tested for sterility in nutrient broth. Thyroid were lost whenever this regime was not strictly adhered to. For example, by keeping the trypsin at 4°C. for a few days the activity of the trypsin was readily lost and if it was not Seitz filtered it was liable to grow organisms.

CULTURE MEDIUM

The basic formula for all nutrient media
was:

\[
\begin{align*}
\text{Lactalbumin hydrolysate} & \quad 49 \\
\text{Serum} & \quad 20 \\
\text{Balanced Hanks' solution} & \quad 76 \\
\end{align*}
\]

**GLASSWARE.**

Rimless Pyrex tubes (125x 16 mm.) were principally used. A small rectangular strip cut from a coverslip was inserted into each tube to act as a flying coverslip. Small vials of ½ oz. capacity with screw cap tops (Kingsley Saunders bottles) were also found very useful being optically more satisfactory and carrying a larger flying coverslip. (Fig. 9). Large centrifuge bottles, baby's feeding bottles and medical flats proved suitable for maintaining a reservoir of thyroid cells in fair bulk. (Fig. 10). In addition, a small culture chamber of almost ideal optical quality was used (see later).

**STOPPERS**

Escho white phenol-free rubber bungs were used throughout, except for the large centrifuge bottles. New rubber stoppers were boiled in 0.5N NaOH for 10 minutes, rinsed thoroughly with tap water, boiled for 10 minutes in 4 per cent HCl, rinsed thoroughly with tap water and finally rinsed 5 times in resin purified water.
Fig. 9.

Kingsley Saunders bottle showing the flying coverslip submerged in nutrient medium.
Fig. 10.

A selection of the culture chambers used is illustrated. On the top shelf of the incubator are the slide culture chambers; on the middle shelf are two large centrifuge bottles on the left and two baby's feeding bottles on the right; on the floor of the incubator are racks of test-tubes.
STERILISATION OF APPARATUS

Flasks, tubes, bottles, coverslips and pipettes were sterilised in a hot air oven for 90 minutes at 160°C. Flasks were plugged with muslin covered cotton wool and the tubes were inverted in large tins (National Dried Milk). Pipettes were wrapped individually in Kraft paper or kept in metal canisters. Rubber bungs were autoclaved in small glass jars with aluminium screw caps or in jam jars plugged with muslin covered cotton wool and Kraft paper. They were then dried in an oven at 56°C for several hours (after loosening the screw cap).

WASHING OF GLASSWARE

New glassware was rinsed once in tap water to remove fragments of straw etc., and then left overnight in N/10 HCl. Following several rinses in warm tap water the glassware was then treated in the same way as used glassware.

Contaminated flasks and tubes were treated as follows:

The rubber bungs were removed and replaced in a beaker of boiling water. The glassware was immersed in a pail of dilute "Lissapol" (a neutral I.C.I. detergent), or .5 per cent white soap solution (Lux), and autoclaved. The tubes
etc. were brushed in this soap solution when the temperature had fallen to about 50°C. and rinsed once in tap water. The glassware was boiled in a soap solution (see above) for 1 hour and repeatedly rinsed with hot tap water. All glassware received a final rinse with two changes of resin purified distilled water. Calibrated pipettes used for measuring non-infectious material were rinsed once in cold tap water, and left over-night in a jar of cleaning fluid (98 per cent concentrated sulphuric acid + 2 per cent nitric acid). They were rinsed with warm tap water, boiled in soap solution and rinsed in a pipette washer. Before sterilisation, pipettes were soaked for 10 minutes in three changes of resin purified distilled water. Pasteur pipettes were made from soft glass and were discarded after use.

**DRUMS**

Stationary as opposed to roller drums were used throughout. They were constructed from perspex with holes of appropriate size to accommodate the culture tubes or Kingsley Saunders bottles. The holes were arranged in such a way that the axis of the culture vessel made a small angle with the horizontal. (Fig. 11).
Culture chambers of circa ideal optical properties were constructed according to the design of Constable & Huth (1958). The design is shown in the adjacent diagram (Fig. 11).

**Fig. 11.**
Stationary perspex drum mounted with test tubes, each tube containing 0.75 mls. cell suspension in which is submerged a flying coverslip.

At this stage all components of the chamber were cleaned to the requisite standard for tissue culture.

To assemble the chamber one cover glass was cemented over the hole in the slide on the surface on which there are no grooves. Two hypodermic needles were bent into the shape of a crank and were cemented into the grooves to
CULTURE CHAMBER

Culture chambers of almost ideal optical properties were constructed according to the design of Constable & Moffat (1958). The design is shown in the adjacent diagram (Fig. 12). The body of the chamber was made from a thin glass microscope slide, 3 in. x 1 in. x .8 mm., a hole approximately $\frac{1}{2}$ in. in diameter being cut in the centre and a number one cover glass (0.18 mm. in thickness) placed above and below to form the walls.

At each side of the perforation a short groove was cut in the glass, parallel to the long axis of the slide, and of sufficient depth to accommodate a stainless steel hypodermic needle no. 18. The needle should not project above the surface of the glass within the area of the cover glass seating.

At this stage all components of the chamber were cleaned to the requisite standard for tissue culture.

To assemble the chamber one cover glass was cemented over the hole in the slide on the surface on which there are no grooves; two hypodermic needles were bent into the shape of a crank and were cemented into the grooves so
Fig. 12.
Diagram of culture chamber as designed by Constable & Moffat (1958).
Fig. 13.

Diagram of the cutter used by Constable & Moffat (1958) to drill holes through the glass slides.
Fig. 14.
Changing the medium in a Constable & Moffat culture chamber.

That the points projected slightly into the chamber. A small piece of plasticine gave additional support to the needle butt. Finally, a cover glass was cemented in place to close the chamber; the only access to which was then cut holes in glass slides. The cutters consisted of a brass rod soldered to a length of copper tubing. The face of the cutting edge was slotted with a fine hack saw to increase its efficiency. In operating the tool was repeatedly fed with coarse carbonized. The drill speed was kept at moderate. It is important if cracking of the glass is to be avoided, that the slides should be placed on a perfectly flat support and that the drill pressure should be carefully regulated especially towards the end of the cutting. It
that the points projected slightly into the chamber. A small piece of plasticine gave additional support to the needle butt. Finally, a cover glass was cemented in place to close the chamber, the only access to which was then through the needle.

A satisfactory cement was found to be "Araldite" which is a two-component epoxy resin, obtainable from Aero Research Ltd., Duxford, Cambridge. At room temperature this adhesive takes at least 12 hours to set, but by raising the temperature to 160°C, this period was reduced to 90 minutes.

A power driven vertical bench drill was used to cut holes in glass slides. The cutter which proved to be effective is illustrated in Fig. 13. It consisted of a brass rod soldered to a length of copper tubing. The face of the cutting edge was slotted with a fine hacksaw to increase its efficiency. In operation the tool was repeatedly fed with coarse carborundum. The drill speed was kept at moderate. It is important if cracking of the glass is to be avoided, that the slides should be placed on a perfectly flat support and that the drill pressure should be carefully regulated especially towards the end of the cutting. It
is also important to detect the earliest moment when the drilling is complete or else further drilling will almost immediately result in breakage of the slide. Drilling a slide took about 5 minutes.

The needle grooves were cut with a carborundum wheel in a dental drill. No carborundum was necessary. This second operation took another two or three minutes. The complete chamber was sterilised in the hot air oven at 160°C. for 90 minutes when the cement set extremely hard and often became black.

**SETTING UP CULTURES AND CHANGING THE MEDIUM**

0.75 ml. of the final cell suspension in the appropriate medium was put in each test tube, 1.5 ml. in the half ounce vials and 10 ml. in the larger flasks. A cover slip of suitable size was placed in each tube and vial and care was taken to ensure that this cover slip was wholly immersed in the cell suspension. The tubes and vials were then labelled and set up in the stationary drums and placed in an incubator at 37°C. The larger flasks lay horizontally.

The proportion of tubes, vials, flasks and culture chambers used depended on the particular experiment, but generally some 30
tubes, 12 vials, 3 chambers and 1 or 2 flasks were employed on each occasion.

The medium could be changed either by pouring off the old medium and adding an equal volume of fresh medium or by extracting the cover slip and placing it in a fresh tube with fresh medium.

Slide tissue culture chamber is filled with a suspension of cells in a suitable medium at a density of such that several small cellular aggregates were seen under the 10x objective. This cell suspension was injected through a syringe directly attached to one of the needle fittings, and by tilting the slide upwards virtually all the air inside the chamber could be excluded. Thereafter the needle ends were sealed with sterile wool pledgets followed by paraffin wax to prevent evaporation of the medium on incubation. The chambers were then inverted on an egg-rack for some hours to allow the cells to settle on the lower cover glass. In this way a mono-layer of cells is obtained and when the chamber was turned the right way up this cellular mono-layer was adherent to the upper cover glass and thereby ideally situated
for photomicrography.

The medium in the slide culture chamber could be readily changed by removing the occluding wax and wool from the needle and by connecting the chamber to a couple of syringes. As the fresh media was injected by the one syringe the old media pushed out the plunger of the second syringe. (Fig. 14).

EXAMINATION OF CULTURES

As a rapid screening test for the presence or absence of growth the tubes and vials could readily be examined under the 4x or 10x objectives of the microscope. A perspex cradle to fit the moving stage of the microscope and which could accommodate either the test tubes or the vials was of much assistance when a large number of tubes and vials were to be examined.

PHOTOGRAPHY

The cells in suspension settled under the influence of gravity onto the flying cover slips in the test tubes and vials. The cells became adherent to the glass of the cover slip. By rotating the test tubes or vials through 180° the cover slip came uppermost and was separated from the convex surface of the container wall by only a thin layer of medium. Indeed, in the
case of the vials most of the fluid drained off between the cover slip and the wall of the container. (Fig. 15). For photography the cover slip could be placed in a fresh container so that cells adherent to the inside of the container wall did not interfere. The curvature of the container wall limited the optical resolution obtainable, but \( x 65 \) magnification was still capable of giving a sharp image. Better photographs were obtained with the vials than with the test tubes, the glass of the vials being thinner and the curvature less convex. Condensation of the wall of the container was difficult to avoid and showed up as black dots on the final print.

Phase contrast microscopy was used when photographing at higher magnifications. It was used exclusively with the slide culture chamber.

Colour microphotography was employed with stained preparations.

**STAINING**

The flying cover slips with their adherent cells could readily be removed, washed in unbalanced Hanks' solution and fixed in absolute methyl alcohol and stained Giesma or fixed in Zenker's fluid and stained by the Altman-Kull method as recommended by Baker.
Fig. 15.

Direct photography of the cellular monolayer adherent to the flying coverslip.
Washing in saline prior to fixation is to be avoided if good cytoplasmic staining is to be achieved. No benefit could be detected from fixation in osmium tetroxide rather than absolute methyl alcohol.
The appearance of human thyroid cells in culture is independent of the in vivo histology of the parent gland; that is to say, cells derived from toxic or from non-toxic goitres were indistinguishable in culture. Fig. 16 shows a low power view of a section from a hyperplastic thyroid gland used for tissue culture and Fig. 17 shows the appearance of the cells in situ at the same magnification as the photomicrographs of cells in tissue culture to be illustrated later.

After trypsinisation the thyroid cells were spherical and adhered to one another in aggregates (Fig. 18). This clumping of cells, which made cell counts quite impossible to do, was particularly marked with thyroid cells as opposed to guinea pig lung, monkey kidney or HeLa cells. Also peculiar to thyroid tissue was the rapidity with which the cells adhered to glass. The cells were adherent within half an hour or so. When examined in a slide culture chamber the originally spherical cells were seen to extrude pseudopodia within a few hours. These pseudopodia were constantly throwing off sequestrated fragments which retained the power of independent motility. The cells gradually became fusiform and by 24 hours had migrated.
away from their initial clusters to form a mono-layer. Fig. 19, which was stained after 18 hours in culture, demonstrates this well. If not overcrowded, these wandering cells characteristically retained contact with one another at each end so that they formed a network or reticular pattern, as shown in Fig. 20 and also in Fig. 31. If re-trypsinised the cells once more reverted to their spherical form and tendency to form aggregates.

The culture media required to be changed about twice per week, the colour of the medium being a rough guide as to when this was necessary. The acid products of cellular metabolism gradually changed the colour of the phenol red indicator contained in the medium from red towards yellow through amber. With experience one could virtually tell from the appearance of the medium alone whether or not the cells "growing" in it were viable. The mono-layer of fusiform cells remained virtually unchanged for 14 days or more, but after this time many gradually disintegrated and fell off the glass while the survivors became more and more elongated.

As shown in Fig. 30 there may be more than one nucleus per cell. The cytoplasm of the
in culture contains only a few very fine and long mitochondria. (Figs. 21 and 22). Mitoses was not observed in any of the many hundreds or even thousands of thyroid cells examined. "Transformation" to malignancy and the occurrence of rapid mitoses in thyroid cells in culture is stated by Pulvertaft et al. (1959) to occur invariably within 6 weeks. In the present study thyroid cells were cultured for 5 weeks and remained essentially constant in their appearance with no evidence of mitoses or other sign of undergoing "transformation."

The cytology of thyroid cells in tissue culture is broadly the same as that of cells from other organs. Somewhat limited experience with foetal guinea pig lung, monkey kidney and HeLa cells bears this out. (Figs. 23 and 24). However, the impression was gained that certain minor differences might exist. By comparing Fig. 20 and 24 it would seem that the nucleus of the thyroid cell as shown by phase contrast contains less dark material than that of the HeLa cell.

The pattern of cellular arrangement in the mono-layer also appeared to be different for each gland of origin. The reticular pattern
Figs. 16 & 17 illustrate the histology of a thyroid gland used for tissue culture. The patient was thyrotoxic and the gland had been prepared for surgery by carbimazole and potassium iodide. The follicles are generally of large size and have good colloid content, but the epithelium, which is columnar, shows marked hyperplastic papillary ingrowths.
After trypsinisation the thyroid cells have a marked tendency to adhere together in aggregates, making cell counts impossible.

By 18 hours the thyroid cells have begun to migrate away from the cellular aggregates and have begun to form a monolayer. The individual cells are now fusiform rather than spherical.
Fig. 20. Phase contrast x 450.

The typical reticular pattern of the fusiform thyroid cells after 48 hours in culture is illustrated.
Fig. 21. Altman-Kul x 1,800.

The long and thin mitochondria of the human thyroid cell in culture is illustrated.

Fig. 22. Altman-Kul x 1,300.

A different field of the same preparation as shown in Fig. 21.
Fig. 23. Giemsa x 525.
Monkey kidney cells from continuous culture. Three days after trypsin-dispersal.

Fig. 24. Phase contrast x 400.
HeLa cells from continuous culture. Three days after trypsin-dispersal.
of the thyroid cells was not so prominent with HeLa cells as may be seen by comparing again Figs. 20 and 24. It is doubtful however if one could confidently state the gland of cell origin from the appearance of the individual cells in culture and from their relationship to one another.

Pulvertaft et al. (1959) have shown that, although they appear morphologically de-differentiated, thyroid cells in tissue culture retain the ability to take up iodine and to synthesise thyroxin and its intermediaries. These workers have also shown that thyroid cells in tissue culture are a suitable vehicle for the culture of certain viruses. For instance they showed that fresh thyroid is susceptible to Coxsackie A.9 and to ECHO 6 and ECHO 9 viruses. In this respect fresh thyroid cells closely resemble monkey kidney and human amnion. Pulvertaft's transformed cells behaved like other transformed lines, in that they were susceptible to Coxsackie A.9 or ECHO 6 or 9.

In summary the thyroid is perhaps the most readily available source of human tissue for the preparation of trypsin-dispersed cells. The conditions of the thyroid for which the gland is removed are in general neither inflammatory nor
neoplastic so that theoretical objections for its use for experimental purposes do not arise. Furthermore, thyroidectomy is a common operation performed in certain selected cases of goitre and the performance of the operation is done entirely for the patient's benefit. The excised gland survives well in transit to the laboratory and its cells are sufficiently hardy to withstand the technique of trypsin-dispersal. Provided reasonable standards of sterility are maintained infection of cultures with cell death is rare. It is of the greatest importance, however, as in all tissue culture work that the glass ware be meticulously clean.
PART III.

"THE CULTURE OF HUMAN THYROID CELLS IN MEDIA CONTAINING HASHIMOTO AND OTHER ANTI-SERA."
MATERIALS AND METHODS

Apart from variations in the culture media the material and methods were identical with those described in Part 1.

Culture Media:

The basic formula for all nutrient media used was

Lactalbumin hydrolysate 4%
Serum 20%
Balanced Hanks' solution 76%

5 different variants of this basic formula were used according to the constitution of the serum component (see Fig. 25). All the sera were Seitz filtered. Fresh human serum was used to insure the presence of complement. The term human serum (inactivated) means that the serum was heated at 56°C in a hot water bath for 30 minutes.

Hashimoto sera: Hashimoto serum was obtained from patients who were diagnosed clinically as suffering from Hashimoto's disease and whose serology was strongly positive as regards the gel precipitation test, the tanned cell haemagglutination test and the complement fixation test. (See Table on page 61).
<table>
<thead>
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<th>Number of Nutrient Medium</th>
<th>Constitution of Serum component</th>
<th>% age of total medium</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Human serum (inactivated)</td>
<td>20</td>
</tr>
<tr>
<td>II</td>
<td>Human serum (inact)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Fresh human serum</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>Hashimoto serum</td>
<td>15</td>
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<tr>
<td></td>
<td>Fresh human serum</td>
<td>5</td>
</tr>
<tr>
<td>IV</td>
<td>Anti pure thyroglobulin rabbit serum</td>
<td>15</td>
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<td>Fresh human serum</td>
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<td>V</td>
<td>Anti crude thyroglobulin rabbit serum</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Fresh human serum</td>
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</table>

Fig. 25.

The constitution of the serum components of the five different media used.
Rabbit anti-sera: Crude thyroid extract (prepared by homogenising pooled post mortem thyroid or thyroidectomy specimens and extracting with saline) and pure thyroglobulin were injected intramuscularly in 1 ml. amounts at weekly intervals into two sets of 3 rabbits. After the fourth week the rabbits were bled and the serum from each was separated and stored in the deep freeze at -20°C.

Testing of Sera for Antibodies:

As already mentioned three methods were employed for the qualitative and quantitative estimation of antibodies in the different sera:-

1. Gel precipitation technique.
2. Tanned cell haemagglutination test.
3. Complement fixation test.

1) Gel precipitation technique: A modified Oudin technique was adopted (Oudin, 1948; Oakley & Fulthrope, 1953; Feinberg, 1956).

The material used included thyroid extract and clarified agar. The thyroid extract was prepared from human thyroid gland obtained at thyroidectomy or from fresh post mortem thyroid. The gland was frozen, cut into .5 - 1 mm. slices with a razor blade and put into 2-3 volumes of 0.9 per cent NaCl at 2°C, left overnight and centrifuged using the supernatant
for the test. The agar was clarified by suspending 1 per cent W/V New Zealand agar in 0.9 per cent NaCl, and the agar dissolved by heating in a boiling water bath. The solution was then rapidly filtered twice through well rinsed glass wool in a Buchner funnel to remove grossly insoluble particles. 1 per cent sodium azide was added as a preservative. One or two per cent W/V of a mixture of equal parts of powdered bentonite and "Hyflo Super-Cel" (Johns Manville, Co. Ltd.) was then added and the whole shaken vigorously to disperse the clarifying agents. The suspension was stored at 56° C for several days, the clarifying agents being re-suspended daily by gentle inversion of the bottles. When the cloudy flocculus was completely precipitated from the supernatant by the bentonite "Super-Cel" mixture the clarified agar was carefully decanted. The clarified agar was then filtered through a fluted Whatman No. 5 paper in a heated funnel into a bottle standing in hot water. The first 25 ml's. or so were returned to the funnel for filtering.

To carry out the test the clarified agar solution was dissolved by heating the container in a boiling water bath and thereafter the agar
was kept molten by placing the container in a 42°C bath. 0.2 ml. of serum were mixed with 0.2 ml. of agar solution in a 3 cm. x ½ cm. tube at 42°C and the mixture was placed at the bottom of a Widal tube with a long Pasteur pipette at room temperature, taking care to avoid bubbles. When this layer was set, equal amounts of agar solution and normal saline were mixed at 42-45°C and a 0.8 cm. column of this mixture was placed on the serum layer. When the second layer was set, the antigen layer consisting of equal amounts of agar solution and thyroid extract was added (Fig. 26).

The antigen of the thyroid extract and the antibodies in the patients serum diffused slowly towards the neutral saline zone. A line of precipitation became visible where they met in optimum concentrations (Figs. 26, 27 and 28). If the serum contains a large amount of antibody the precipitation becomes visible in the saline layer after 2-3 days and thickens as time goes on. When the antibody titre is low, the precipitation may take up to 14 days to become clearly visible and is more diffuse.
DOUBLE DIFFUSION GEL PRECIPITATION TEST
(modified from Oakley and Fulthorpe.)

Thyroglobulin or Thyroid extract in 1% agar.
1% Agar with zone of precipitation.
Serum (antibody) in 1% agar.

Fig. 26.
Illustrated photograph of the modified Oudin technique of gel precipitation using, in this instance, Hashimoto serum.
Fig. 27.

Modified Oudin technique using rabbit serum containing antibodies to pure thyroglobulin.
Fig. 28.
Modified Oudin technique using rabbit serum containing antibodies to crude thyroid extract.
2) Tanned cell haemagglutination test:
This test was elaborated by Boyden (1951) and Stavitsky (1954). The principal is that treatment of human group 0 erythrocytes with suitable concentration of tannic acid renders them capable of absorbing certain protein molecules from solution in saline. Red cells treated in this way and washed are agglutinated by the homologous anti-protein sera. Thus sera can be titrated for antibodies against antigens absorbed on the cells exposed to tannic acid; furthermore, small amounts of antigens can be detected by their power to inhibit haemagglutination of the treated cells.

A 4 per cent suspension of washed human group 0 cells were added to an equal volume of 1:20,000 dilution of tannic acid in saline at pH 7.2. After standing at room temperature for 30 minutes the mixture was centrifuged gently and washed 3 times in buffered saline and then re-suspended to give a 2 per cent suspension.

An equal volume of suitably diluted antigen (usually 1:20 to 1:50, but 1:1000 if pure thyroglobulin was used) was added to the 2 per cent solution of red cells and the mixture allowed to stand at room temperature for 30
minutes. The cells, which are now sensitised, were then washed 3 times with 1:200 rabbit serum to prevent lysis and to aid the re-suspension of cells after centrifugations.

The antigen sensitised red cells were exposed to the test serum by setting out two rows of 11 tubes for each test serum. 0.1 ml. serum diluted 1:10 was placed in the first tube of each row and thereafter in subsequent tubes in doubling dilutions to a titre of 1:10,000. To the front row was added 0.1 ml. of tanned antigen coated cells and to the back row 0.1 ml. of tanned cells. Saline controls were made by placing 0.1 ml. tanned antigen coated cells and 0.1 ml. saline in each of two tubes, and by placing 0.1 ml. saline and 0.1 ml. tanned cells in each of another two tubes. A negative serum and a positive serum were always put up with each batch of tests.

Finally, all racks were shaken vigorously and left at room temperature for 2-4 hours. The final reading was taken the following morning. A positive result together with a negative control is illustrated in Fig. 29. The titres obtained from the different sera are shown in the Table on page 61.
Fig. 29.

Positive haemagglutination test:

The tube on the left is a negative control and shows a compact button of cells. The tube on the right shows a carpet of agglutinated cells with a crenated well defined border.
3) **Complement fixation test**: An immune serum may contain antibodies which, along with antigen, fix or absorb complement; and a complement-fixing antibody is therefore spoken of.

To test for this effect, the haemolytic system was used as an indicator. If complement has been removed by fixation to the antigen-antibody complex, then when the haemolytic system is added, no haemolysis will occur. This haemolytic system consists of red blood corpuscles in isotonic saline plus a haemolytic anti-serum which has been heated at 56°C for half an hour to annul complement (i.e. red cells plus specific antibody only). The test is made quantitative by determining at what titre haemolysis first occurs.

**Antibody Titres in the Various Sera:**

The antibody titres in the various sera that were used in this experiment are shown in the following Table.
Table showing the antibody titres as determined by the three serological methods of the various sera used.
RESULTS.

The growth of human thyroid cells in tissue culture in medium containing normal human serum has already been described and illustrated in Part I of this Essay.

1. Human thyroid cells cultured in normal human serum and in Hashimoto sera.

Human thyroid cells were found to grow equally well in media containing normal human serum and Hashimoto serum. This observation is illustrated in Figs. 30 and 31. The presence or absence of added complement made no obvious difference. As already mentioned in Part I human thyroid cells are easy to culture and they were cultured in media containing normal human serum and Hashimoto sera on numerous occasions and for prolonged periods without evidence of cellular damage. Furthermore, the results were the same whether fresh thyroid cells were used or whether thyroid cells were used which had been maintained in culture for some days or even weeks.

2. Human thyroid cells cultured in rabbit serum containing antibodies to pure thyroglobulin.

Human thyroid cells cultured in rabbit serum containing antibodies to pure thyroglobulin
and thrived equally as well as those cultured in normal human serum (Fig. 32). Once again the presence or absence of added complement had no effect and the results were the same whether fresh thyroid cells or cells that had been maintained in culture for some days or even weeks were used.

3. **Human thyroid cells cultured in rabbit serum containing antibodies to crude thyroid extract.**

No successful growth was ever observed in cells incubated in rabbit serum containing antibodies to crude thyroid extract. Some difficulty was experienced in recording this cytotoxic effect for in the presence of this medium the cellular aggregates of the freshly trypsinised cells failed to adhere to the glass and consequently failed to form a mono-layer. Phase contrast microscopy was therefore impracticable and there was no point in attempting to stain the flying coverslips in the absence of adherent cells.

This difficulty was overcome by setting up a number of slide culture chambers, half of them containing normal human serum and the other half containing rabbit serum with antibodies to crude thyroid extract. After 24 hours incubation the
chambers were examined and photographed by
direct light. (Figs. 33 and 34). This
experiment was repeated on 3 occasions with
consistent results.

Summary of Results.

Neither Hashimoto serum nor rabbit serum
containing antibodies to pure thyroglobulin had
any deleterious effect on trypsin-dispersed
human thyroid cells in tissue culture, but the
rabbit serum containing antibodies to crude
thyroid extract proved to be markedly cytotoxic.
**Fig. 30.** Giemsa x 525.

Human thyroid cells growing in nutrient medium with normal human serum as the serum component.

**Fig. 31.** Giemsa x 525.

Human thyroid cells growing in nutrient medium with Hashimoto serum as the serum component. Note the healthy appearance of the cells.
Fig. 32. Giemsa x 525

Human thyroid cells growing in nutrient medium with rabbit serum containing antibodies to pure thyroglobulin as the serum component. Again note the healthy appearance of the cells.
Fig. 33. Direct light x 225.

Human thyroid cells growing in nutrient medium with rabbit serum containing antibodies to pure thyroglobulin as serum component. Photographed in a slide culture chamber. As shown in greater detail in Fig. 30 these cells are healthy.

Fig. 34. Direct light x 225.

Human thyroid cells incubated in medium with rabbit serum containing antibodies to crude thyroid extract as serum component. Photographed by same technique as Fig. 33. Note the distinct cytotoxic effect of this serum.
PART IV.

DISCUSSION.
Clearly, this work constitutes in vitro evidence which may not be strictly applicable to what takes place in vivo, but it is of some interest that human thyroid cells survive in Hashimoto serum and are yet destroyed by antibodies to crude thyroid extract.

Nature of the Thyroid Antibodies.

It was first shown by Witebsky & Rose (1956) that thyroid antibodies had an unusually high degree of organ specificity. Stuart (1959a) found that positive precipitin tests were obtained only with extracts of thyroid gland and not with other organs. Saline extracts of thyroid are unusual in that they contain a high proportion of one constituent, mainly thyroglobulin and, therefore, it seems likely that this is the antigenic material in auto-immune thyroiditis. There appears to be more than one thyroglobulin and Derrien, Michel & Roche (1948) demonstrated by salting out procedures the presence of several thyroglobulin fractions. The purified thyroglobulin used in this study was virtually homogeneous on electrophoresis although ultra-centrifugal analysis showed it was only 88 per cent homogeneous and that two other small components
were present. This chemical evidence for the existence of more than one thyroglobulin is supported by the existence of more than one antigen. The pleurality of antigens is seen in Fig. 27, where at least two bands of precipitation have formed when rabbit serum containing antibodies to pure thyroglobulin was tested against crude thyroid extract in an Oudin tube.

Roitt, Campbell & Doniach (1958) pointed out that as judged by the Ouchterlony test the antigens present in crude thyroid extract are also present in purified thyroglobulin. By immune electrophoresis in agar they showed the antigens in crude extracts had identical mobilities with that of thyroglobulin. They prepared a rabbit anti-serum to purified human thyroglobulin and placed this anti-serum with a Hashimoto serum known to give 3 lines in an Ouchterlony plate with thyroglobulin in the central well. The precipitation curves merged completely which suggests that the same antigens were responsible for stimulating production of antibody in both rabbit and man. Accordingly it seems likely that the antibody detected in man by precipitation and tanned cell haemagglutination tests is in fact directed against the
thyroglobulins.

Early experience with complement-fixation techniques led Trotter, Belgavin & Waddams (1957) to suggest that quite a different antigen may be involved in this reaction. This suggestion has now been confirmed and amplified by Roitt & Doniach (1958). Reactions with this unknown antigen can at present only be detected by complement fixation. Sera containing both types of antibody can be completely absorbed with thyroglobulin, leaving the complement fixation reaction with the other antigen unaffected. The richest source of the new antigen has been found to be thyroids from patients with thyrotoxicosis, though it has also been detected in normal thyroids. Differential centrifugation in sucrose shows that it is deposited in the microsomal fraction, and thus strongly suggests that it is an intra-cellular component.

It was therefore seen that antibodies can be formed both against thyroglobulin contained in the acini and against an unknown substance contained in the thyroid cells.

The Interpretation of the Experimental Results.

Care was taken in choosing the Hashimoto
sera employed in the present experiments to ensure that these sera contained antibodies in high titre both against thyroglobulin (as estimated by the precipitin and tanned cell tests) and also against the intra-cellular or microsomal antigen (as estimated by the complement fixation test). The fact that human thyroid cells in tissue culture thrive in media containing such Hashimoto sera may be interpreted in two ways: either, the circulating auto-antibodies which are demonstrable in Hashimoto thyroiditis have no direct toxic effect on the thyroid cells and are therefore of purely secondary aetiological importance; or, alternatively, the rhetorical question may be asked as to whether cells maintained in tissue culture may not perhaps lose their antigenic properties and so fail to demonstrate any cytotoxic effect. It is possible that cells in culture may perhaps undergo immunological as well as morphological de-differentiation, but if so then the process must be so rapid as to be virtually inconceivable for no toxic effect was observed when expressly fresh thyroid cells were used.

In view of the fact that the Hashimoto sera proved non-toxic, it was only to be expected that the rabbit serum containing antibodies
against pure thyroglobulin would likewise prove to be nutritious rather than the contrary.

The immunological pattern of rabbit serum prepared against crude thyroid extract was no doubt very complex and its markedly cytotoxic effect was probably due to a species antibody unrelated to organ specificity. At present work is in progress in an attempt to produce anti-thyroid-cell serum by injecting rabbits with a pure line of thyroid cells maintained in tissue culture.

It would therefore appear that the circulating auto-antibodies demonstrable in Hashimoto serum are not cytotoxic to human thyroid cells. This leaves the possibility that Hashimoto thyroiditis may yet be due to an auto-immunising process in which "cell-bound" rather than circulating antibodies are responsible.

Discussion.

A number of clear-cut examples of cell damage caused by the injection of serum antibodies are known; the passive transfer of heterologous antibodies against placenta (Seegal & Loeb, 1940), kidney (Smadel, 1936), erythrocytes (Damashek & Schwartz, 1940),
leucocytes (Chew, Stephens & Lawrence, 1936) and blood platelets (Ledingham, 1914) causes disruption of the elements against which they are directed, while the intra-peritoneal injection of rabbit anti-Ehrlich ascites-tumour serum plus complement increases the survival time of mice bearing the tumour and results in progressive cellular degeneration of the ascites cell (Flax, 1956). The lysis of red cells, Ehrlich ascites-tumour cells (Flax, 1956; Easty & Ambrose, 1957), Bragg rat lymphosarcoma cells (Schreck & Preston, 1956) in vitro and human skin and placenta in tissue culture (Bassett, Campbell, Evan & Earle, 1957) by their respective anti-sera have been demonstrated. In these examples of cell damage produced both in vivo and in vitro by circulating antibodies, it is probable that the antibodies were directed against "cell-surface" antigens.

This type of "delayed" hypersensitivity reaction may indeed have been operating in Rose & Witebsky's rabbit experiments, since Freund adjuvants (containing killed tubercle bacilli) had to be used in conjunction with thyroid extract in order to obtain any notable thyroid damage.

The observations of Buchanan, Anderson,
Goudie & Gray (1958) would be compatible with a mechanism of this type. They showed that intra-dermal injection of a thyroid extract provoked local skin reactions (similar to those seen after tuberculin injection in sensitised subjects) in patients with thyroid disease but not in the controls. These experiments would have been more significant if they had been made with preparations of the two antigens separately rather than an extract of thyrotoxic gland, which almost certainly contained both. Nevertheless they do seem to show that the tissues of patients with high levels of circulating antibody may become sensitised to some factor or factors in thyroid extracts. Some such process might conceivably account for other lesions (nephrosis, cirrhosis, Paget’s disease) observed by Luxton (1957) in patients with Hashimoto’s disease.

Finally, in considering the hypersensitivity hypothesis it should be remembered that the process need not be brought about by one of the antibodies which we have come to recognise. There may be others of the “incomplete” type which do not reveal their presence in the usual laboratory tests. Such undetected antibodies may cause damage to the thyroid, either directly or by means of a hypersensitivity reaction and
the ones that are measurable may merely be an indication that an immune response of some sort has occurred.

On the other hand, the suggestion afforded by the present experiment that the circulating antibodies demonstrable in Hashimoto's disease may be purely of secondary importance is supported by an increasing body of evidence that Hashimoto's disease is not simply an auto-immunising thyroiditis.

For example, the production of lymphadenoid lesions in the thyroid gland of rabbits by immunisation with rabbit thyroid extract as described by Rose & Witebsky (1956) has never been repeated in spite of numerous attempts by many workers (Stuart, 1959b). This casts serious doubt on one of the main arguments on which the auto-immunisation theory is based.

Again, experience with subacute thyroiditis (Roitt & Doniach, 1958) has shown that the postulated chain reaction following minor initial thyroid damage with subsequent leakage of antigen and further antibody formation does not invariably occur. In cases thought to be due to viral infection of the thyroid auto-antibodies do indeed appear in the blood
temporarily, but their subsequent disappearance shows that they have not caused any self-perpetuated damage to the gland.

Consequent upon the recognition of autoantibodies in Hashimoto serum much work has been done to determine the incidence of autoantibody reaction in other types of thyroid disorder besides Hashimoto's disease. The observations of three groups of workers (Roitt & Doniach, 1958; Owen & Smart, 1958; Anderson et al. 1959) on the incidence of positive precipitin, tanned cell haemagglutination and complement fixation tests in the various thyroid disorders have been summarised by Macgregor (1959) and are displayed in Figs. 35, 36 & 37. Allan, Clark, Fulton, Macgregor & Stuart (1959) have in addition determined the antibody titres as revealed by the three serological tests in the same range of thyroid disorders as well as in controls (Figs. 38, 39, 40 & 41). In myxoedema the occurrence of antibodies closely parallels that seen in Hashimoto's disease although there are more cases showing no antibody formation. Antibodies are also present in thyrotoxicosis using the tanned cell and complement fixation tests, but the tanned cell titres are usually lower than in Hashimoto's disease and
### Precipitin

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### Tanned Cell Agglutination

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Fig. 35.

Fig. 36.
### COMPLEMENT FIXATION

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**Fig. 37.**

Figs. 35, 36 & 37 illustrate the incidence of positive precipitin, tanned cell haemagglutination and complement fixation tests in the various thyroid disorders as summarised by Macgregor (1959).
**TANNED CELL HAEMAGGLUTINATION AND PRECIPITIN TESTS**

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</table>

![Graph](image)

*Fig. 38.*
TANNED CELL HAEMAGGLUTINATION AND PRECIPITIN TESTS

<table>
<thead>
<tr>
<th>TITRE 100,000</th>
<th>Simple goitre</th>
<th>Thyroid carcinoma</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>Red-Precipitin Positive</td>
<td>White-Precipitin Negative</td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 39.
COMPLEMENT FIXING ANTIBODIES

<table>
<thead>
<tr>
<th>TITRE</th>
<th>Lymphadenoid goitre</th>
<th>Thyrotoxicosis</th>
<th>Myxoedema</th>
</tr>
</thead>
<tbody>
<tr>
<td>512</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>256</td>
<td>●</td>
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<td></td>
</tr>
<tr>
<td>128</td>
<td>●</td>
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<td></td>
</tr>
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<td>64</td>
<td>●</td>
<td>●</td>
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<td>●</td>
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<td>●</td>
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</tr>
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</tr>
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<td>4</td>
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<td>●</td>
<td>●</td>
</tr>
<tr>
<td>2</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Zero</td>
<td></td>
<td></td>
<td>Secondary Myxoedema</td>
</tr>
</tbody>
</table>

Fig. 40.
COMPLEMENT FIXING ANTIBODIES

Controls  Simple goitre  Thyroid carcinoma

Fig. 41.
precipitins are rarely detectable by gel diffusion. Antibodies are present in a few cases of thyroid carcinoma usually at a low titre. They are rarely found in simple goitre. These serological findings support the inference from purely histological studies (Bastenie, 1944; Douglas & Jacobson, 1957) that primary myxoedema and Hashimoto thyroiditis share the same pathological process. It is not suggested that all primary myxoedemas result from Hashimoto's disease, rather that they go through a sub-clinical stage without the formation of an obvious goitre. The significance of auto-antibodies in thyrotoxicosis is, however, difficult to assess.

It would seem that the term auto-immunising thyroiditis does not refer to one particular disease of the thyroid gland but to an immunological disturbance associated with pathological changes of the gland. These changes may be prominent enough to be recognised as a distinct clinical entity, Hashimoto's disease, or be so insignificant that their presence is only detectable by serological tests or histological examination of the gland when focal or patchy plasma cell and lymphocytic
infiltration may be detected.

As already mentioned (Part I) Stuart & Allan (1958) observed definite changes in the basement membrane in Hashimoto thyroiditis. Fragmentation of the basement membrane was nearly always associated with the presence of chronic inflammatory cells in the affected part, but in some instances damage to the membrane was seen in the absence of leucocytic infiltration. It therefore seems possible that fragmentation of the basement membrane is an early change which permits the escape of colloid into the interstitial tissue. Colloid is a tissue irritant (Ferguson, 1937) and the consequent inflammatory reaction would then explain the frequent co-existence of a pathological basement membrane with lymphocytes and plasma cells. Moreover, the escape of colloid would explain the production of auto-antibodies as a secondary phenomenon. The cause of initial injury to the basement membrane remains unknown and it may yet prove to be due to an auto-immunity reaction involving cell-bound antibodies.

**Conclusion.**

The evidence afforded by the present work adds further weight to the hypothesis that
perhaps Hashimoto's disease is not due to a simple auto-immunising process consequent upon the inter-action of thyroid antigen and circulating auto-antibodies. Evidence in support of alternative hypotheses is discussed.

Clearly there remain many unsolved problems relating to auto-immunity in thyroid disease. Their solution would be of considerable import not only to thyroid disease but also to a much wider field of clinical medicine.
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