AUTORADIOGRAPHIC STUDIES WITH RADIOACTIVE ISOTOPES

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I N T R O D U C T I O N

AUTORADIOGRAPHIC STUDIES WITH RADIOACTIVE ISOTOPES

In the technique of autoradiography radioactive material is localised accurately in a tissue section by the image produced in a photographic emulsion closely apposed to the section. The study of the distribution of labelled chemical compounds in normal and diseased tissues can often throw light on the mechanism of disease. This thesis is an account of the application of autoradiography, as a biophysical method, to a variety of problems in pathology, and is arranged in 6 parts in volume 1. Volume 2 contains tables and illustrations.

In Part I the technique is described. It is then established that inorganic radio-sulphate is utilised specifically in the synthesis of sulphated mucopolysaccharides, in the living animal, and the cellular origin of some of those compounds is demonstrated. From this base line the investigation is extended to the problem of fibrogenesis, and evidence is presented to support the view that fibroblasts form and secrete acid mucopolysaccharides into the surrounding tissues during collagen formation. As complexes with protein the acid mucopolysaccharides very probably form further complexes with the fibrous proteins as fibrogenesis proceeds. The mast cell is known to produce the sulphated mucopolysaccharide heparin, and it is postulated that in certain fields of fibrogenesis heparin may have an inhibitory role. The pattern of mast-cell recovery following

administration of the histamine-liberator compound 48/80 is next studied and no evidence is found that new mast cells originate in perivascular areas.

In Part II radio-sulphate is used to label specifically sulphated mucopolysaccharides of the calcified tissues, both in vitro and in vivo. A test for the viability of cartilage grafts is described, and illustrated by a clinical example. The experimental disease lathyrism is next investigated and a comparison is made between the distribution of sulphated mucopolysaccharides and labelled proteins in the skeletal lesions. Radioactive amino acids are used as protein precursors in this case. The results support the hypothesis that the lathyrus factors act by blocking complex formation between protein and chondroitin sulphates A and C. The distribution of sulphated mucopolysaccharides is established in normal teeth, and evidence is presented for the synthesis of these compounds in odontoblasts, ameloblasts, and cells of the dental pulp and stellate reticulum. The distribution-pattern suggests that these compounds play a part in the mechanism of calcification of dentine and enamel. Fluoride administration is shown to alter the pattern of dentinal calcification, and radio-sulphate is found in the proximal zone of the calciotraumatic response. This again indicates the participation of sulphated mucopolysaccharides in calcification.

Part III records an experimental approach to the problem of amyloidosis. The model used is amyloid occurring in the golden hamster

which is infected with Leishmania donovani. Since there is chemical evidence that amyloid consists of protein with a small mucopolysaccharide component, labelled amino acids are used as protein precursors, and radio-sulphate as a specific label for sulphated mucopolysaccharides. In this way the distribution-patterns of protein and polysaccharide produced as amyloid develops are studied. The results support a new hypothesis for the pathogenesis of amyloid. A glycoprotein, the carbohydrate part of which is sulphated, is formed by proliferating cells of the plasma series mainly in splenic red pulp. This glycoprotein circulates and is a soluble precursor of amyloid. Endothelial cells form another sulphated mucopolysaccharide which gives an insoluble complex with the glycoprotein. This complex is amyloid. Amyloid has a characteristic subendothelial distribution, and therefore there is increased capillary endothelial permeability for which the glycoprotein may be responsible.

In Part IV the isotope used is iodine-131 given as iodide preoperatively to patients proceeding to thyroidectomy. The autoradiographs prepared from sections of the resected glands show the distribution of organically bound radio-iodine. Inorganic iodinated compounds are washed out in histological processing of the tissue. The series consists of 159 pathological thyroid glands representing thyroid adenoma and carcinoma, non-toxic and toxic nodular goitre, diffuse colloid goitre, thyroid cyst, dyshormonogenetic goitre, Graves' disease, and Hashimoto's thyroiditis. From the various

distribution-patterns of organic iodine-131 several conclusions are made. The differentiation between neoplasia and hyperplasia when most difficult histologically is not made easier by the picture of radio-iodine utilisation. The uptake of iodine-131 by malignant tumours is, from the therapeutic standpoint, disappointingly meagre. Nodular goitre and dyshormonogenetic goitre differ distinctively in distribution-patterns and in histological structure. The differences are sufficiently marked to be diagnostic. In Graves' disease, treated with large doses of iodine-131, and in dyshormonogenetic goitre, there is evidence of epithelial utilisation of radio-iodine. The Askanazy-cell of Hashimoto's thyroiditis represents hyperactivity rather than degeneration or involution, but the hyperactivity may be a final stage before involution or degeneration occurs.

In Part V radio-chromate and radio-iodate are used in an examination of the histochemistry of chromaffin tissue in vitro. It is demonstrated that the main cause of failure in the chromaffin reaction for catechol amines is failure to control the pH of the dichromate solution used. The pigment formed in this reaction is probably a mixture of adrenaline black and a chrome-adrenochrome complex. The pigment formed in the iodate reaction for nor-adrenaline is considered to be a non-iodinated oxidation product of noradrenaline.

Part VI describes an autoradiographic investigation of the merits

of colloidal radio-gold in the treatment of human carcinoma. The radioactive material is injected interstitially near accessible tumours, and an examination of its distribution in the regional lymph nodes is made. The series comprises 23 tumours and 247 lymph nodes are sectioned for autoradiography. It is shown that no radio-gold is present in lymph nodes wholly replaced by tumour, and that in tumour-free or partially replaced nodes the uptake is variable.

P A R T I

1. Autoradiography
2. Radio-sulphate Distribution in the Normal Animal
3. Sulphur-35 in Connective Tissue Formation
4. Regeneration of the Tissue Mast Cell

1. AUTORADIOGRAPHY

Autoradiography demonstrates the position of radioactive isotopes in a tissue section or other specimen by the production of an image in a photographic emulsion applied to the section. The radiation from the isotope is responsible for the image which therefore overlies the site of radioactivity.

RESOLUTION

The degree of resolution obtained varies with several factors including radiation energy, section thickness, emulsion thickness, space between emulsion and section, and emulsion grain size. At one end of the scale a coarse grain emulsion 12 μ thick, such as Kodak AR50, used with high energy β particles, say from iodine-131, will give a resolution down to 15 μ. On the other hand, with a fine grain emulsion such as Kodak V-1042 2 μ thick, and low energy β particles from tritium, a resolution of 2 μ or better is obtainable in practice. Emulsions in gel form are also available for high resolution work and examples are Ilford G5 with a grain size of about 0.27 μ and Ilford L4 with a grain size of 0.15 μ. Smaller grain size emulsions are being developed and it is probable that this will be the ultimate limitation of resolution. Useful electron microscope autoradiographs have been described recently (Pelc et al, 1961; Przybylski, 1961).

TECHNIQUE

The basic method used is the stripping film technique (Doniach

and Pelc, 1950) with Kodak AR10 and AR50 plates. The sections are mounted on slides coated with a thin film of the following solution:-

Gelatine	5 g.
Chrome alum	0.5 g.
Water to	1 litre.

The sections are brought down to water and the emulsion is applied in a dark-room with Wratten series 1 (red) safelight. Rectangles of film about 5 x 3.5 cm. in size are cut and stripped gently from the plate. They are floated emulsion side down on clean distilled water at 24°C. The emulsion swells and the rectangles of film after 2-3 mins. are quite flat on the surface of the water. The portions of film are now picked up on the slides bearing the sections so that the emulsion is smoothly apposed to the section and overlaps the slide edges. The preparations are dried in a current of clean air at room temperature and placed in a light-tight box for exposure. The box is stored at 4°C to reduce background fogging, and it is an advantage to include a drying agent.

After an exposure time which is empirically determined and varies with concentration of isotope in the tissue, half-life of isotope, radiation energy, emulsion sensitivity, and the factor of latent image fading, the autoradiographs are developed and fixed. Developing is carried out with Kodak D 19b or DX80 developer for 5 minutes at 20°C. The autoradiographs are then rinsed in water at

20°C and fixed with Amfix or Kodak acid fixer at the same temperature for twice the time taken to clear. Further washing follows for about 30 minutes in running water. The autoradiographs are dried and are now ready for examination by phase contrast microscopy, or for staining.

STAINING

I have developed a staining method which gives excellent contrast between image and stained section. The autoradiographs are overstained for 4 hours or more in this solution:-

Light green 0.5 per cent aqueous	20 ml.
Absolute alcohol	50 ml.
Distilled water to	100 ml.

They are then destained in Sörensen's buffer at pH8 until the emulsion is a lighter shade of green than the section. They are next overstained in 0.1 per cent aqueous Safranin O for 4 hours or more, and again destained in 0.001 N HCl until all the red dye is removed from the emulsion. The preparations are dehydrated in alcohol, cleared, and mounted in D.P.X. from a mixture of equal parts of xylol and D.P.X. Alternatively they may be dehydrated at 56°C and mounted in D.P.X. after soaking in the xylol-D.P.X. mixture. A less elegant but quicker staining method is 0.1 per cent aqueous neutral red for a few minutes followed by dehydration and mounting as before. The preparations are now ready for examination by ordinary light microscopy.

TISSUE PROCESSING

The method chosen of preparing the radioactive tissue for sectioning depends on the experimental design. With the usual fixation, dehydration, and clearing methods of histology radioactive compounds which are soluble in water or in the organic solvents used are leached out from the tissue. This is desirable when the experiment is concerned with protein-bound radioactivity, or with compounds insoluble in water and the ordinary histological reagents. This is particularly relevant to the work with radio-sulphate and radio-iodide where free sulphate and iodide are leached out and therefore do not complicate the distribution-pattern of the organically bound isotopes. Alcoholic formalin is a satisfactory fixative for this type of experiment, and tissues are processed to paraffin and cut in the usual way.

INTERPRETATION

This is discussed fully in the context of the experiments but some general points are made here. The essential data derived from study of autoradiographs is topographical. Quantitiative estimation of image intensities is fraught with many difficulties. Some of the variables are section thickness, emulsion thickness, exposure time, developing time, temperature of developer and degree of background fogging. However, it is a valuable and valid procedure to compare image intensities in different parts of a single preparation. When image intensities are compared in two or more preparations then every effort is made to ensure that the factors

affecting image intensity, which are common to the preparations compared, are standardised. Such comparison is considered to be a valid procedure particularly when it shows a gross difference in image intensity as, for example, the presence of a discrete image in one preparation and the absence of a discrete image in another. Data based on smaller differences are only valid if this difference is a constant feature of many preparations and is reproducible. These general provisions have been applied throughout the work.

2. RADIO-SULPHATE DISTRIBUTION IN THE NORMAL ANIMAL

I undertook this investigation to establish and interpret the normal pattern of sulphate-distribution as a necessary preliminary step to subsequent experiments. Some aspects of the work were reported by Curran and Kennedy (1955a). I was responsible for the experimental design related to the distribution study, I carried out all the autoradiographic work, and I made my own assessment of the data provided by the autoradiographs. The present account includes a considerable amount of new material which I have prepared and assessed quite independently.

MATERIALS AND METHODS

Sulphur-35 as sulphate at pH7 was given by intraperitoneal or subcutaneous injection to a total of 53 albino mice made up of 23 adults, 20 suckling animals, and 10 foetal animals. The last group received the isotope by subcutaneous injection of pregnant adults. The dose of radio-sulphate was 1 μc. per g. body-weight for suckling animals and 5 μc. per g. body-weight for adult animals. For the foetal animals the fraction of the maternal dose that crossed the placenta was not calculated.

The animals were killed 4-48 hours after isotope injection. Tissues were routinely fixed in 4 per cent neutral or alcoholic formaldehyde, in alcohol, or in a mixture of alcohol and acetone. Fixatives containing mercury were unsatisfactory because image artefacts were produced in the autoradiographs. Paraffin embedding

and celloidin-paraffin double embedding were employed. Sections were cut at 5-6 μ and autoradiographs prepared by the stripping film method with Kodak AR10 and AR50 plates. Sections adjacent to those used for autoradiography were variously stained with haematoxylin and eosin, alcian blue (Pearse, 1953), toluidine blue, by the periodic acid-Schiff method, and by Gordon and Sweets' method for reticulin. Occasionally sulphation metachromasia was demonstrated (Kramer and Windrum, 1953), and a few sections of cartilage were autoradiographed after treatment with testicular hyaluronidase. The autoradiographs were stained by the method already described (p. 10).

RESULTS

There is a good correlation between the distribution-pattern of radio-sulphate and the distribution-pattern of acid mucopolysaccharides stained by such a conventional method as alcian blue (table 1,1). This is most apparent in the foetal and suckling animals. The sulphate-uptake in older animals is less brisk.

In skin and other tissues mast cells invariably take up radio-sulphate avidly (fig. 1,1). This characteristic feature of mast cells is demonstrated below in human skin in vitro (fig. 1,41). Sulphate-distribution in tactile hair is illustrated in fig. 1,2. This is an example of comparison of image intensities in different parts of a single preparation at a given time to show that it is unlikely that sulphur-containing compounds in keratin are derived

from sulphated mucopolysaccharides (Sylvén, 1950).

In cartilage the effect of testicular hyaluronidase is mainly to remove extracellular sulphated compounds (figs. 1,3 and 1,4). The cellular origin of the sulphated mucopolysaccharides of the matrix is clearly revealed. Cellular utilisation of radiosulphate in other tissues is listed in table 1,2. Autoradiographs of growing bone (fig. 1,5) show a marked image over new lamellae. By taking autoradiographs of such a tissue at various intervals after isotope injection it is possible to build up a dynamic picture of the role of sulphated mucopolysaccharides in endochondral ossification. Some aspects of this problem are presented in the section on lathyrism (Part II, 2).

The corneal content of keratosulphate is known (Meyer and Chaffee, 1940; Asboe-Hansen, 1953). Fig. 1,6 confirms clearly the presence of a bound sulphated compound in cornea, and this is a good example of the assessment of an autoradiograph in the light of knowledge from chemical extraction studies. The fibrous tissue of the lens capsule also contains a sulphated mucopolysaccharide but the image intensity is less than that over cornea.

The presence of sulphated compounds in the cardiovascular system is of considerable pathological interest. The image intensities over heart valves (fig. 1,7) and vessel walls (fig. 1,8) are again greatest in the youngest animals. In adults these structures are often inert with respect to sulphate metabolism or show only minimal images. An endothelial contribution to the images

obtained cannot be excluded, but the major intensity in valves and cusps is over the connective tissue, and in large vessels over the media. The point of endothelial utilisation of sulphate is referred to in more detail in Part III.

Glands secreting mucin, and intestinal goblet cells are amongst the most active structures showing incorporation of radio-sulphate (figs. 1,9 and 1,10; table 1,2). It is also of interest that the pancreatic acinar cells take up the isotope, but the islet cells are inert in this respect (fig. 1,10). Giant cells, possibly megakaryocytes, are present in large numbers in the spleens of young animals and invariably show an intense sulphate-image (fig. 1, 11). These cells are inconspicuous in the normal adult.

In the nervous system a diffuse rather low level sulphate-image is obtained over brain, cerebellum, and peripheral nerves. At the time intervals after isotope injection studied it is not possible to ascertain a cellular origin of this sulphated material. The image intensity is considerably less than that over the dura mater, perineural connective tissue, and vascular media (figs. 1,12 and 1,13).

The distribution of radio-sulphate in dental tissues shown in table 1,1 is illustrated in detail in Part II, 3 where a dynamic picture is built up of the movement of sulphated mucopolysaccharides in dentine corresponding to the steps of appositional growth and incremental maturation.

DISCUSSION

The correspondence between sulphate-distribution studies and

conventional staining methods for acid mucopolysaccharides is good but presumptive evidence that the autoradiographic method is specific for sulphated mucopolysaccharides. Exceptions to the correspondence are obviously possible when the staining methods themselves are not specific. For example it has been reported that alcian blue stains polysaccharides other than acid mucopolysaccharides (Zachariae and Dyrbye, 1959). And radio-sulphate will not be incorporated into acid mucopolysaccharides other than the sulphated ones.

The case for specificity is made much stronger by further considerations. Firstly, sulphated mucopolysaccharides can be extracted chemically from many tissues which show a sulphate-image by autoradiography. For example, labelled chondroitin sulphate has been isolated from cartilage (Dziewiatkowski, 1951; Boström, 1952) and from skin (Boström and Gardell, 1953). There are numerous other instances in the literature of this type of correspondence in tendon, cornea, and bone. Occasionally there are discrepancies. Singher and Marinelli (1945) and Dziewiatkowski (1949) reported a high marrow content of radio-sulphate. This has not been confirmed in the present work by autoradiography, but there is abundant radio-sulphate in bone lamellae (fig. 1,5), supporting the view of Layton (1950b) that the high marrow counts were due to the presence of such lamellae in the samples estimated.

Secondly, it has been shown by Tarver and Schmidt (1939 and 1942) and Dziewiatkowski (1954) that radio-sulphate is not incorporated to any appreciable extent into sulphur-containing amino

acids. Boström and Åqvist (1953) support this view in a report that cystine, taurine and methionine show only a low or inappreciable uptake of sulphur-35. Their results are based on the chemical extraction of sulphur-containing compounds other than sulphated mucopolysaccharides. Further, the histological tissue processing leaches out any unbound inorganic radio-sulphate. Excretion studies have shown that injected sulphate is rapidly excreted as inorganic and ethereal sulphates (Laidlaw and Young, 1948; Dziewiatkowski, 1949; Everett and Simmons, 1952).

Thirdly, Schiller et al (1958) studied chondroitin sulphate metabolism in skin with labelled sulphate and acetate, and sulphate and glucose simultaneously. They showed that neither the sulphate nor acetyl groups underwent exchange, but were metabolised at the same rate as the polymers. Muir (1961) considers that this finding validates other work, such as the present, with labelled sulphate alone.

The autoradiographic method of demonstrating sulphated mucopolysaccharides has advantages other than specificity over the conventional staining techniques. One of the most important is the possibility of following the distribution path of a sulphated mucopolysaccharide at various times after injection of radio-sulphate. Engfeldt and Westerborn (1960) have studied normal epiphyseal cartilage in this way. At 2 hours after injection of sulphur-35 they found activity confined to the cells; at 24 hours the image was over cell periphery and matrix; at 7 days all the activity was

in the matrix. Their conclusion that the chondroitin sulphate of cartilage matrix is synthesised in the chondrocytes supports the view already given in the present work.

Another advantage is that autoradiography clearly shows differences in metabolic activity which are not apparent by the usual staining methods. This is very obvious in the intense images obtained in many young tissues compared with adult. Layton (1950b) and Layton and Denko (1952) have also reported this feature. The giant cell activity in the spleens of young animals is striking (fig. 1,11). The cells are thought to be megakaryocytes containing a sulphated mucopolysaccharide (Halmi and Davies, 1953), but their appearance in large numbers in the spleen of adult animals after caseinate injections (p. 105) is worthy of note. In such a reactive hyperplasia the giant cells again utilise radio-sulphate avidly. Their relation to experimental amyloidosis is discussed in Part III.

SUMMARY

The autoradiographic distribution-pattern of radio-sulphate in normal animals indicates the distribution of sulphated muco-polysaccharides.

These compounds are identified in intracellular and extracellular sites. It is very probable that the cells synthesise the compounds and secrete them into the extracellular tissues.

3. SULPHUR-35 IN CONNECTIVE TISSUE FORMATION

Curran (1952 and 1953) reported an examination of fibrous foci which developed after injection of quartz. He used staining methods to show that mucopolysaccharides were present in the cytoplasm of fibroblasts and extracellularly in the quartz foci. I suggested to him that an examination of sulphate-distribution in the quartz lesions might be rewarding. I injected radio-sulphate into a series of his experimental animals and obtained the auto-radiographic data which is the basis of the brief report published (Curran and Kennedy, 1955b). Figs. 1,14 and 1,15 show quite clearly that fibroblastic proliferation is associated with uptake of radio-sulphate, and that sulphated mucopolysaccharides are involved in the early stages of fibrogenesis. A more detailed study of the role of sulphated mucopolysaccharides in connective tissue formation was necessary. I carried this out and the major part of the following section has been published independently (Kennedy, 1960).

Robb-Smith (1954), in a review of the morphogenesis of connective tissue, stated that there are 2 schools of thought on the source of the mucopolysaccharides of the ground-substance, referred to the work of Gersh and Catchpole (1949) as favouring the fibroblast, and cited that of Asboe-Hansen (1951) in support of the view that the mast cell is the source.

More recently Asboe-Hansen (1957) has claimed that the mast cell is the only connective-tissue cell yet shown to contain mucopolysaccharide, but Taylor and Saunders (1957) were unable to

demonstrate an association between the intercellular acid mucopolysaccharides and tissue mast cells. Curran (1953) observed that in an organising quartz focus the fibroblasts produce a mucopolysaccharide, and that mast cells do not congregate around the focus. Curran and Kennedy (1955b) showed that radio-sulphate was utilised by fibroblasts in silicotic foci and suggested that fibroblasts form and secrete sulphated mucopolysaccharides into the surrounding tissues during collagen formation. The same authors (1955a) showed a high level of sulphate-utilisation by mast cells.

The present work is an autoradiographic examination of sulphur-35 utilisation and distribution, as an index of sulphated mucopolysaccharide formation and distribution, in conditions of rapid growth of new connective tissue, as seen in foetal, embryonic, and neonatal animals, in tumours, in granulation tissue, and in experimental cirrhosis. The animals in the last group were made available for autoradiographic studies by R.S. Patrick. A comparison is also made between the parts played by fibroblasts and mast cells in connective-tissue formation.

MATERIAL AND METHODS

Sulphur-35 as sulphate at pH 7 was administered to a total of 158 small rodents and 4 chick embryos grouped as under (groups 1-4). The human tissues in group 5 were from surgical biopsies and sulphur-35 utilisation was determined *in vitro* in a nutrient medium containing 1 μc. per ml. radio-sulphate as described by Gibson *et al*,

(1955). In the case of foetal mice, the pregnant adults were given the isotope subcutaneously, and in some suckling animals the isotope was again given to the parent and reached the young via the milk. In the chicks the radio-sulphate was injected into the yolk-sac or on to the chorio-allantoic membrane. Direct injection in the other animals was intraperitoneal or subcutaneous. The dose of sulphur-35 ranged from 1 to 5 μc. per g. body-weight for direct injection. For the indirect routes the fraction of the maternal dose that reached the foetus across the placenta, or the suckling animal in the milk, was not calculated. The injection into the chick embryos was 100 μc. in each case.

The animals were killed with ether 12-48 hours after isotope injection, and the in-vitro tissues were fixed after incubation for 48 hours in the labelled medium. As a routine tissues were fixed in 4 per cent neutral or alcoholic formaldehyde, embedded in paraffin and cut at 5-6 μ. The method of decalcification used was that given by Collins (1949), and celloidin-paraffin double embedding was used for decalcified material. Autoradiographs were prepared by the stripping film method with Kodak AR 10 and AR 50 plates. Sections adjacent to those used for autoradiography were stained with haematoxylin and eosin, or Van Gieson, or by Gordon and Sweets' method for reticulin. Toluidine blue, azure A, and alcian blue (Pearse, 1953; Lison, 1954) were also used.

Group 1. Foetal and neonatal animals. (a) Eleven normal foetal albino mice and 2 normal embryo chicks; (b) Eighteen normal suckling

albino mice and 10 normal suckling Wistar rats.

Group 2. Experimental tumours. (a) Connective-tissue tumours were induced in 7 Wistar rats by a single subcutaneous injection of 0.2 ml. of a 0.5 per cent solution of 20-methylcholanthrene in olive oil. The tumours appeared 5-9 months later. (b) Epithelial tumours were induced in 13 albino mice by repeated cutaneous application of 0.5 per cent 20-methylcholanthrene in olive oil or 0.2 per cent 9,10-dimethyl-1,2-benzanthracene in acetone. In a few animals croton oil was used as a co-carcinogen. Tumours appeared after about 3 months.

Group 3. Granulation tissue. (a) Granulomata were induced in 14 guinea-pigs by the subcutaneous injection of 5 ml. of a normal saline suspension of crude carrageenin, and in 10 albino mice by 1 ml. of the carrageenin suspension (Robertson and Schwartz, 1953; D.S. Jackson, 1957). (b) Cutaneous ulceration and subcutaneous abscess formation were observed in 12 albino mice after repeated injection of sodium caseinate (in an investigation of amyloidosis) and in 10 Wistar rats after repeated injection of 2.5 per cent aqueous sodium fluoride solution (in an investigation on fluorosis). In one albino mouse a resolving peritonitis caused by intraperitoneal injection of Freund-type adjuvant was studied. Normal human skin grafts on the chorio-allantoic membranes of 2 chick embryos were also examined for sulphate-utilisation 4 days after grafting and 18 hours after injection of 100 μc. sulphur-35 (Pullar and Cochrane, 1957).

Group 4. Experimental cirrhosis. R.S. Patrick induced this lesion in 34 albino mice by repeated administration of carbon tetrachloride. In 25 of the animals and in a further 18 normal albino mice he implanted a portion of fine catgut in the liver. I injected 100 μc. radio-sulphate into the animals which were killed 24 hours later. I prepared and assessed autoradiographs of the cirrhotic livers, of the cirrhotic livers with catgut implants, of the normal livers with catgut implants, and of healing wounds. The effect of cortisone was examined in 9 of the animals with catgut implants only.

Group 5. Human biopsy material. (a) Portions of 10 mixed parotid tumours; (b) biopsy material from a case of urticaria pigmentosa.

The in-vitro technique was used for the material in this group.

RESULTS

Group 1(a). Foetal and embryonic animals (Table 1,3).

The transplacental carriage of sulphate in the mouse (Kennedy, J.S. and Kennedy, G.D.C., 1957) was confirmed; Layton et al, (1950) showed sulphate-transfer across the placental barrier in the rat. Transferred sulphate is utilised for tissue synthesis in the foetus; Boström and Odeblad (1953) observed a similar transfer and utilisation in the rabbit.

Mast-cell utilisation of radio-sulphate was an invariable finding in foetal tissues. The autoradiographic image produced was sharp and punctate and usually clearly related to the cell cytoplasm.

This characteristic image was not seen in any other cell type and gave a practically specific "stain" for mast cells (fig. 1,16).

Fibroblasts in developing fibrous tissue showed an image less intense generally than that of the mast cells, less sharply delineated, and often associated with a diffuse activity over early argyrophilic fibres lying between the cells. This type of uptake was seen, e.g., in chick gizzard (figs. 1,17 and 1,18), developing fascia (fig. 1,19) perichondrium, tail tendon, corium (fig. 1,20) and dura mater.

Mast cells were often associated topographically with fibroblasts and this was particularly evident in the corium. But there were many clear examples of fibrogenesis without any tissue mast cells being present, as, e.g., in tail tendon, and in chick gizzard where the autoradiographic image corresponded in fine detail with the distribution of fibroblasts and argyrophilic fibres shown in silver preparations (figs. 1,17 and 1,18).

Group 1(b). Neonatal animals (Table 1,3).

The carriage of maternal sulphate by the milk and its utilisation in tissue synthesis in suckling rats (Denko and Priest, 1959) were confirmed. The distribution of bound sulphate in connective tissue was similar to that in the foetus, and the mast cells and fibroblasts both showed evidence of sulphate-utilisation.

In muscle tendons (fig. 1,21) no mast cells were demonstrable, but there was evidence of bound sulphate in cells and developing

fibres; activity was greatest at the areas of origin or insertion. Fascial planes were frequently clearly outlined by radio-sulphate. Mast cells were observed in thyroid and tongue and, particularly in the latter site, were not associated with fibrogenesis.

Fibroblasts, young fibres, and mast cells occurred together in some regions and were well shown in developing fibrous tissue between muscles in the rat snout (fig. 1,22). The fibroblasts and associated fibres showed a lower, more diffuse uptake than the mast cells, which were not confined to the fibrous bands, but were also distributed irregularly in muscle, Again the mast-cell image was sharply cellular, whereas activity in fibroblasts and fibres could not be clearly separated.

Group 2(a). Experimental connective-tissue tumours. (Table 1,4).

The tumours were 2-6 cm. in diameter and up to 55 g. in weight. Local invasion was a constant feature and muscle, kidney, spleen and liver were variously involved. No distant metastases were seen. Areas of haemorrhage and necrosis occurred in all the neoplasms. Microscopically they were predominantly spindle-cell sarcomata showing areas of pleomorphism. The fibre content was variable from tumour to tumour and from one part of a given tumour to another, but collagen and fine argyrophilic fibres were demonstrable in every case. The number of active mast cells varied from field to field. It was highest in areas showing well differentiated spindle-cells separated by fine argyrophilic fibres, lowest in pleomorphic areas, often on an invading margin, showing little or no fibrogenesis (fig. 1,23).

It tended to be low in regions where more mature collagen was stainable. There was no general massing of mast cells on the periphery of the tumours, although small numbers of mast cells were distributed irregularly on invasion edges. The mast-cell images were again sharply cellular.

The tumour cells generally showed a lower, more diffuse, utilisation of sulphate than the mast cells. Quite sharply localised images were produced over regularly orientated bands and whorls of tumour cells and fibres, and the uptake in such cells and fibres was distinctly higher than that in the more irregularly distributed and more pleomorphic tumour cells (fig. 1,24). In some cases isolated bands of collagen were demonstrated to contain radio-sulphate (figs. 1,25 and 1,26), but in most autoradiographs it was not possible to separate the cell image from the fibre image. Where silver stains showed fine irregular argyrophilic fibres the spindle-cell utilisation of sulphur-35, although present, was diffuse and of a lower order: such regions often showed very numerous mast cells. In the capsule or pseudo-capsule of spindle-cells and collagen fibres the image again was darker than in the undifferentiated parts of the tumour. This feature is illustrated in a fibrous band, with stainable collagen, dividing 2 tumour nodules (figs. 1,27 and 1,28). Metachromasia was sometimes demonstrable in capsular cells and fibres, but the autoradiographs gave a more constant positive result. A regular feature of many sections showing areas of infarction was an increased sulphate-utilisation by surviving tumour

cells immediately adjacent to the infarct.

Group 2(b). Experimental epithelial tumours. (Table 1,4).

The tumours were squamous-cell papillomata, or carcinomata with local invasion but without metastases.

Sulphate-distribution contrasted sharply with that in the connective-tissue tumours. The epithelial tumour cells did not utilise the isotope, but stromal uptake was a constant feature (fig. 1,29). The image was diffuse over fibroblasts and fibres, which in most Van Gieson preparations stained as collagen. Stromal mast cells were rather sparse and irregularly distributed, but large numbers of mast cells were frequently present in groups at the periphery of the tumours, and, in some cases, in the dermis underlying areas of normal or hyperplastic squamous epithelium at their margins. Their punctate images were again quite characteristic compared with the more diffuse picture over fibroblasts and related fibres. There was no constant relation between stromal fibrogenesis and mast-cell population or distribution.

Group 3(a). Carrageenin granulomata (Table 1,5).

The carrageenin injections caused lesions similar histologically to those described by Williams (1957). An initial polymorphonuclear leucocyte and macrophage response was followed by a granulomatous reaction and the formation of new connective tissue on the periphery of the focus. Sulphate-utilisation by the new connective tissue was not clearly demonstrable until the 5th day after carrageenin

injection, and corresponded then to a zone with numerous fibroblasts and fine argyrophilic fibres, and few, if any, mast cells. The intensity of the autoradiographic images increased up to the 9th day after carrageenin injection, the zones of activity became broader, and where fibroblasts were infiltrating muscle, activity could also be shown. From 10 to 15 days the zonal peripheral activity decreased (figs. 1,30-1,32). Fine fibres were stainable with silver at 4 days and young collagen fibres by the Van Gieson method at 6-7 days after injection. The most intense images were in zones rich in fibroblasts and early collagen fibres; they were diffuse over cells and fibres.

Mast-cell distribution remained normal as the lesions developed, i.e., most mast cells were present in the usual position in the dermis and showed no tendency to aggregation on the periphery of the focus. Occasional mast cells were seen in the new fibrous tissue, but most fields of fibrogenesis were devoid of mast cells. Metachromatic methods for staining mast cells proved difficult to interpret because of the presence of numerous macrophages containing carrageenin, which is metachromatic. The autoradiographs were unequivocal, because only the mast cells showed the characteristic focal punctate image. For the first 4 days after carrageenin injection mast-cell utilisation of sulphate was less than normal: from the 5th day it was of the usual intensity and did not decrease at 10-15 days. This pattern differed from that in the zone of fibrogenesis, where there was a clear fall in radioactivity at 10-15 days after carrageenin

injection.

Group 3(b). Other granulation tissue (Table 1,5).

The common feature in this group was the utilisation of sulphur-35 by granulation tissue (figs. 1,33-1,35). In every case the autoradiographic images were most intense over zones containing fibroblasts associated with young collagen. The images were diffuse, not sharply localised to cells or fibres, but present over both. Where fibrin was demonstrable, no sulphate-utilisation was seen, and in abscesses the central area of polymorphonuclear leucocytes was negative in the autoradiographs. In the peritonitis case, and in the skin graft on chick chorio-allantoic membrane, no mast cells were seen in the granulation tissue. With abscesses and ulcers, occasional mast cells in adjacent tissues showed sulphate-utilisation, but there was no constant topographical association of mast cells with the new connective-tissue cells or fibres. Capillaries in the granulation tissue generally did not show endothelial utilisation of sulphur-35.

Group 4. Experimental cirrhosis. (Table 1,6)

The radio-sulphate distribution-pattern corresponded exactly to the pattern of cirrhosis (fig. 1,36). The image intensity varied somewhat and there was no clear correlation with extent or duration of cirrhosis. No mast cells were seen.

The new connective tissue developing around implanted catgut also showed utilisation of radio-sulphate. The images were most

intense 7-10 days after implantation and on the whole were more intense than those over the cirrhotic lesions (figs. 1,36 and 1,37). With absorption of the catgut and production of more mature fibrous tissue the image intensity decreased (fig. 1,38). Cortisone reduced the image size but not its intensity (fig. 1,39). This effect was also observed in the healing wounds. Occasional mast cells were present in both the catgut lesions and the healing wounds but there was no constant topographical association with the new connective tissue cells or fibres. Radio-sulphate was not present in stitch abscesses.

Group 5(a). Mixed parotid tumours (Table 1,7).

Evidence of in-vitro sulphate-utilisation by the epithelial cells in this group of pleomorphic adenomata was equivocal, but in occasional autoradiographs, where stromal cells were clearly distinguishable from neoplastic cells, activity was demonstrable in fibroblasts lying in abundant hyaline material (fig. 1,40). The uptake was clearly cellular and the dense fibrous tissue adjacent to the cells showed no radioactivity. There was no evidence in such fields of new fibre formation. Mast cells were not present.

Group 5(b). Urticaria pigmentosa (Table 1,7).

The skin biopsy showed clumps of mast cells which were sometimes distributed around vessels in the dermis. Silver stains showed fine argyrophilic fibres between the mast cells. No

collagen was present in the lesions, and the collagen bundles of the surrounding connective tissue stopped short fairly sharply on the periphery of the mast-cell groups. A few inactive spindle-cells were seen in the lesions. In-vitro sulphate-utilisation by the mast cells was demonstrable (fig. 1,41). The image was somewhat more diffuse than that obtained in mast cells in all the other material examined, but on the edges of the lesions, where the distance between mast cells was greater, the cellular nature of the uptake was clear and the image could not be related to the fine argyrophilic fibres. No activity was seen in the mature dermal collagen.

DISCUSSION

Group 1. Foetal, embryonic and neonatal animals.

It has been demonstrated that both the mast cell and the fibroblast utilise inorganic sulphate, but that there is an easily recognisable difference in the type of image they give on autoradiography. This difference between the sharp intense cytoplasmic image of the mast cell and the diffuse less intense image of the fibroblast and its related fibres may be explained tentatively on the hypothesis that the mast cell stores much more sulphated mucopolysaccharide than the fibroblast.

Jorpes et al, (1953) showed that sulphate groups in heparin or in heparin precursors within mast cells exchanged more slowly than those of other sulphated polysaccharides, and suggested that more than 18 days were required for the exchange of half of the sulphate groups.

Bélanger (1954) ascribed a synthetic and storage function to the mast cell because radio-sulphate was retained for 2 days, in which period it had disappeared from other connective tissues. In the present work persistence of undiminished mast-cell images for periods up to 7 days after injection of radio-sulphate has been demonstrated. In this period, in young animals, other intracellular activity had greatly decreased, or, in most cases, had disappeared. In the synthesis and secretion of the sulphated components of the ground-substance of new connective tissue, where the growth rate is rapid, as in this group, the type of image given by fibroblasts is much more appropriate than that given by mast cells, and this is particularly so where fibroblasts and fibres are closely related with a diffuse image over both.

The chemistry of the sulphated compound demonstrable in mast cells is not known with certainty, but there is evidence that it is heparin (Jorpes, 1935; Holmgren and Wilander, 1937; Wilander, 1939). The intensity of the mast-cell image with radio-sulphate would be consistent with the presence of a compound with a high sulphate content. According to Bettelheim-Jevons (1958), Wolfrom et al, (1950) and Foster and Huggard (1955) have postulated a structure for heparin with 5 sulphate groups per tetrasaccharide unit. Asboe-Hansen (1950) has suggested that mast cells are the main source of hyaluronic acid, and that the sulphated compound they contain may be a heparin-like precursor of it. Meyer et al, (1957) have stated that there is no evidence for the occurrence in tissue of a sulphated

hyaluronic acid. There is also no chemical evidence to support the view that hyaluronic acid is synthesised by way of a heparin-like precursor.

The identity of the sulphated compound shown in fibroblasts and apparently secreted by them is not known. It is postulated that it is a sulphated mucopolysaccharide of the ground-substance and that it may be one or other or a mixture of the chondroitin sulphates found in collagenous tissue. In electron-microscope studies on the formation of connective-tissue fibres, Porter and Vanamee (1949) described a component of the ground-substance scattered rather sparsely among the fibres, but were unable to decide whether it was related in any way to collagen formation. Grossfield et al, (1955) showed that cultures of fibroblasts produced a substance forming a characteristic mucin clot. That the substance was a mucopolysaccharide was shown by treatment with testicular and bacterial hyaluronidase which inhibited mucin-clot production. Lastly, S.F. Jackson (1957) has described cytoplasmic granules in collagen-forming cells when intercellular material is about to be or is being deposited. The granules have been shown to contain both protein and mucopolysaccharide.

The mast cell is not then the only connective-tissue cell that has been shown to contain mucopolysaccharides. It is generally accepted that such compounds play a part, probably an early one, in fibrogenesis. The sulphate-distribution supports this view and constantly indicates the fibroblast as the dominant cell in the synthesis

and secretion of the sulphated mucopolysaccharides of the ground-substance. On topographical grounds it is also probable that the fibroblast rather than the mast cell is responsible for hyaluronic acid production. Production of ground-substance and fibre occurs for example in tendon where mast cells are not demonstrable, and mast cells are present in considerable numbers in such tissues as tongue where fibrogenesis is minimal. In the umbilical cords of the foetal animals of this group mast cells were scanty and fibroblasts numerous.

Only in the subcutaneous tissues was there a fairly constant relation between fields of fibrogenesis and mast-cell population. Riley et al, (1955) have suggested that the function of heparin from tissue mast cells may be concerned with events in the tissues rather than with the coagulability of the circulating blood. It may be that this is a controlling or inhibiting function in certain types of fibrogenesis.

Group 2. Experimental tumours.

In the sarcomata, mast cells were most numerous in regions where the tumour cells were well differentiated fibroblast-like spindle-cells separated by a fine network of argyrophilic fibres. The mast-cell count decreased as pleomorphism increased.

Holmgren and Wohlfart (1947) found a similar pattern and made the point that there was no obvious connexion between diffuse tissue metachromasia and mast-cell content; they suggested that

the mast cells played a part in the reaction of the system against tumour cells. Earlier, Sylvén (1945) had surveyed a series of human fibrosarcomata and found that the number of mast cells was greatest in and around tumour vegetations in active growth. He suggested that the acid mucopolysaccharide produced by the mast cells played a part in tumour invasion of normal tissues. Such a distribution was not seen in the present group. The available evidence favours the view that the mast cell is involved in a defence mechanism in which heparin influences fibroblastic proliferation. Oliver et al, (1947) demonstrated a very high heparin content in canine mast-cell tumours. Paff et al, (1947) showed that such mast cells prevented growth of other connective-tissue cells in vitro, and suggested heparin as the active agent. In a later paper Paff et al, (1952) showed that heparin suppressed fibroblastic proliferation in vitro. Heilbrunn and Wilson (1949) investigated the effect of heparin on cell division and showed that it could block mitosis in Choetopterus eggs.

The mast-cell distribution in the present group of tumours, showing a very high production of sulphated mucopolysaccharide by mast cells in predominantly fibroblastic regions, again favours a defence mechanism with release of a heparin-like compound as an inhibitor of fibroblastic proliferation. When such a defence mechanism is overwhelmed, as in pleomorphic areas and in frank invasion, the mast cells are few in number or absent.

Sulphate-utilisation by the tumour cells in the sarcomata tended

to vary directly with the degree of differentiation, and in any given autoradiograph was maximal where the spindle-cells were regularly orientated in bands and associated with young collagen fibres. The images were diffuse over cells and fibres. When the fibres present were of the fine argyrophilic type the cellular images were less intense although still diffuse. The tentative conclusion is that in fibrogenesis the part played by the sulphated mucopolysaccharides is quantitatively at a maximum at the stage when stainable collagen appears. At the presumably earlier stage of differentiation of fine argyrophilic fibres, sulphate is still bound, but to a less degree. Where there was no fibrogenesis, as in pleomorphic areas, there was no appreciable sulphate-uptake by the tumour cells. This conclusion will be discussed more fully in the carrageenin granuloma group. The intense images at the edges of infarcts could usually be related to increased fibrogenesis.

A defence reaction has also been postulated to explain the mast-cell distribution in experimental epithelial tumours by Cramer and Simpson (1944) who described dermal mast-cell aggregates associated with pre-invasive epithelial changes.

Bunting and White (1950) suggested that mast cells modified ground-substance to form a heparin-like compound. Campani (1951) observed that, in embryonic tissues and in healing wounds, ground-substance appeared before mast cells and again postulated utilisation of ground-substance by mast cells in

the production of a sulphated compound. A remarkable increase in dermal ground-substance in rabbit skin treated with 3,4-benzpyrene was described by Prodi (1955). This preceded epithelial neoplasia, but accompanied hyperplasia in some regions.

The distribution of mast cells in the epithelial tumour group and their utilisation of sulphate may be explicable on the hypothesis outlined above, but there is no direct chemical evidence for the synthesis of heparin by way of a sulphated or non-sulphated ground-substance precursor. It is perhaps more probable that the common factor in the tumour group is the inhibitory function of heparin in cellular proliferation.

Sulphate-utilisation by the stromal fibroblasts in the epithelial tumours was a constant feature. The pattern of distribution in cells and fibres was in accord with the hypothesis that the sulphated mucopolysaccharides are secreted by the fibroblasts, and take part in the process of fibrogenesis.

Group 3. Granulation tissue.

In the carrageenin granulomata, sulphate-distribution was roughly quantitated by image intensity as fibrogenesis proceeded.

Williams (1957) described the appearance of fine reticulin fibres on the 5th day and their maturation to adult collagen by the 14th day after carrageenin injection. The appearances in the present work were similar, but the terminology used is that advocated by Robb-Smith (1958) who pointed out that the immature

argyrophilic fibres found in embryonic and regenerating connective tissue merge very closely with, and indeed appear to be incorporated and replaced by, developing collagen fibres. They differ from basement-membrane reticulin, which is not a precursor of mature collagen.

Sulphate-utilisation in the present series became obvious on the 5th day with the appearance of such fine argyrophilic fibres. It reached a maximum about the 9th day and decreased from 10 days onwards. D.S. Jackson (1957) found the absolute maximum amount of collagen present about the 9th day. The type of image obtained in autoradiographs was consistently diffuse over fibroblasts and fibres. Slack (1956), in a chemical examination of the metabolism of sulphated polysaccharides in carrageenin granulomata, found that the polysaccharide fraction released by papain digestion reached its maximum concentration about the 7th day and fell steadily thereafter, and suggested that this fraction was closely associated with the fibrous collagens. The present sulphate-distribution pattern supports this view, and it is probable that the sulphated polysaccharide giving the autoradiographs is this papain fraction. I have demonstrated in the present work that papain releases sulphate-labelled polysaccharide from rabbit ear cartilage in vivo. The mechanism is unknown, but Bryant et al, (1958) have suggested a proteolytic action at or near points of attachment of chondroitin sulphate to cartilage protein.

The role of the sulphated mucopolysaccharides in fibrogenesis

is obscure.

Partridge (1948) suggested that chondroitin sulphate might act as a multivalent ion cementing together the protein molecules to form fibrous macromolecules and eventually fibre bundles. Meyer (1951) put forward the idea of a mucopolysaccharide template in fibrogenesis. The fibroblasts produced acid mucopolysaccharides as well as a globular native protein which was precipitated on the polysaccharide, whose regularly spaced acidic groups formed the template on which the fibrous proteins were built up. Kramer (1952, quoted by Robb-Smith, 1954) postulated an anionic detergent role for the mucopolysaccharides by which linkages in the globular form of collagen were broken down, and some degree of orientation of the collagen molecules promoted. The collagen protofibrils then crystallised as the carbohydrate moiety was lost by dissociation due to change in local electrolyte concentration or enzyme activity. Chondroitin sulphate was found to play a part in the stabilisation of tendon (D.S. Jackson, 1954). He suggested that a quarter of the stabilising linkages are between chondroitin sulphate and collagen, and that they consist of 40 per cent salt-like links and 60 per cent hydrogen bonds, and that some other mucopolysaccharide or mucoprotein might also be concerned in this stabilisation. Possible colloid complexes between collagen and mucopolysaccharide were discussed by Loeven (1955), who objected to the term salt-linkage in this connexion. In an examination of the composition of some protein

fractions from calf skin Bowes et al, (1956) described two non-collagenous fractions, both associated with relatively large amounts of hexosamine and with collagen. In a report published after the present work Partridge et al, (1961) described a chondroitin sulphate-protein complex in cartilage in which the protein was not derived from collagen. They suggested that it was this complex which combined loosely with collagen fibres and soluble collagen to give the tissue its characteristic physical properties and insolubility.

The sulphate-utilisation pattern in the autoradiographs supports the view that sulphated mucopolysaccharides play a part in the growth of new collagen fibres. The sulphated compounds appeared with the first histologically demonstrable fibres and reached a maximum concentration at about the same time as total collagen was maximal.

The mast cell played little or no part in fibrogenesis in the carrageenin granuloma. The apparent inhibition of sulphate-utilisation by mast cells for 4 days after injection of carrageenin might well have been a direct local result of the injection. Similarly, in the granulation tissue of resolving inflammation, and in the granulation tissue underlying the skin grafts in chick embryos, mast-cell utilisation of sulphate was topographically remote from, and apparently unrelated to, active fibrogenesis. But labelled sulphated compounds were consistently related to fibroblasts and fibres as before. Layton (1950a) examined

sulphate-fixation by granulation tissue in vitro and suggested that fibroblasts might be the active cells. No evidence was found of endothelial utilisation of radio-sulphate in any of the examples of granulation tissue studied. This is at variance with the view of Curran (1957) but supports the contention of Stehbens (1962) that it is not yet certain that vascular endothelium is one of the tissues with a marked capacity to concentrate radio-sulphate.

Group 4. Experimental cirrhosis.

The distribution-pattern of radio-sulphate supports the view that in hepatic cirrhosis there is active formation of new fibrous tissue (fig. 1,36), rather than passive condensation of existing fibrous tissue (Popper, 1954; Popper and Elias, 1955). In an independent experiment on amyloidosis I found that cirrhosis occurred in some of the hamsters used (Part III). It is worthy of note that the autoradiographs with sulphur-35 again showed the same picture of active utilisation of the isotope in the cirrhotic lesions (fig. 3,11). However the images over new fibrous tissue around the catgut implants were always more intense than those over the cirrhotic lesions (fig. 1,37), and therefore it is possible that some condensation of pre-formed fibrous tissue also occurs in cirrhosis. There is no evidence of any part played by the mast cell in this type of lesion.

In the animals given cortisone there was quite definite evidence

of utilisation of radio-sulphate in fibrogenesis around the catgut implants although the volume of new fibrous tissue was reduced (fig. 1,39). This is at variance with reports that the synthesis of sulphated mucopolysaccharides is inhibited by cortisone (Layton, 1951; Nordlie and Fromm, 1958; Kowalewski, 1959). Again there was no clear evidence that the mast cell was involved in this sort of fibrogenesis.

Group 5. Human biopsy material.

Willis (1953) has pointed out that in the pleomorphic adenomata of salivary glands the stromal connective tissue and the epithelial mucinous material might mingle intimately, but in Van Gieson preparations they could be rendered distinct. Sulphate-utilisation was clearly demonstrable only in spindle-cells in stromal areas. Thus, in sparsely cellular mature fibrous tissue in vitro there was evidence that the fibroblast could bind inorganic sulphate. In the other groups discussed above, where there was active fibrogenesis, it was not possible to separate the images due to activity in fibroblasts from those due to activity in fibres.

In the urticaria pigmentosa biopsy an interesting point was the presence of fine argyrophilic fibres in a lesion predominantly composed of mast cells which utilised sulphate in vitro.

Drennan and Beare (1954) described a mast-cell naevus of embryonic type in which the primitive mast cells formed reticulin. In another solitary mast-cell naevus, in which the cells were of

more mature type, reticulin and collagen fibrils were present but showed no distinct stromal arrangement. This lesion was similar to that of urticaria pigmentosa. The fine argyrophilic fibres in the present case resemble those of Drennan and Beare's case 2. It is suggested that they represent a degenerative process in pre-existing collagen, rather than new fibre formation by mast cells. Vanamee and Porter (1951) investigated the solvation and reconstitution of collagen and found that a decrease in pH caused collagen to dissociate into filaments. Williams (1957) found that intradermal injection of carrageenin caused collagen breakdown with the appearance of narrow argyrophilic fibres.

There is no direct evidence to suggest that mast cells play a part in collagen degradation, but heparin and carrageenin are similar in that they are both highly sulphated acidic polysaccharides, and there is strong evidence for the synthesis of heparin by mast cells.

In all groups the trend of the argument is towards ascribing a positive role to the fibroblast in the production of ground-substance and the formation of new fibres, and a negative or inhibitory role to the mast cell. Where fibre formation is not topographically related to mast cells, e.g., in tendon or cartilage, the tissue is compact, circumscribed, clearly bounded by sheath or perichondrium. Where fibre formation is topographically related to mast cells, e.g., in subcutaneous tissue or sarcoma, the tissue

is not compact, not circumscribed, not clearly bounded, and the mast cells present may exert an inhibitory or moulding influence on fibrous development. Where granulation tissue is formed in reactive or repair processes the mast cell generally has no clear topographic association with fibrogenesis, but it has been noted by Sylvén (1945) and Asboe-Hansen (1954) that in keloid there is a large increase in the number of mast cells. This may again be indicative of a defence mechanism.

SUMMARY

The distribution of organically bound radioactive sulphate has been examined in new connective-tissue formation in foetal, embryonic and neonatal animals, in tumours, in granulation tissue, in experimental cirrhosis, and in the lesions in a case of urticaria pigmentosa. The presence of bound sulphate represents the presence of sulphated mucopolysaccharides.

Mast cells produce and tend to store such compounds, and it is probable that heparin is the principal example. Fibroblasts produce, and in active fibrogenesis secrete, such compounds, and it is probable that the chondroitin sulphates are the principal examples.

The role in fibrogenesis of the sulphated mucopolysaccharides of the fibroblast has been discussed. The distribution-pattern supports the view that as protein complexes they may form further complexes with the fibrous proteins as fibrogenesis proceeds,

particularly from the stage of fine argyrophilic fibres to young collagen.

It has been postulated that in certain fields of fibrogenesis the mast-cell sulphated mucopolysaccharide may have a defensive or inhibitory role, and properties of heparin have been discussed in this respect.

4. REGENERATION OF THE TISSUE MAST CELL

My interest in the utilisation of radio-sulphate by mast cells led to discussion with W.C. Watson who had studied by tinctorial methods some aspects of mast cell topography in heart (Watson, 1958). A collaborative experiment was arranged in which I applied the autoradiographic technique to the study of regeneration of mast cells after administration of the histamine-liberator compound 48/80 which is a condensation product of p-methoxyphenethyl-methylamine and formaldehyde (Riley and West, 1955). In the report which was subsequently published (Watson and Kennedy, 1960) I was responsible for the autoradiography, and made a personal assessment of the data derived from study of the preparations. This aspect of the work is now presented.

Fawcett (1954) had reported that the peritoneal cavity of the rat was an ideal site for exposing *in vivo* a large population of mast cells to the action of a chemical histamine-liberator. Riley and West (1955) described a recovery phase after 48/80 in which new cells are formed from connective-tissue precursors in the adventitia of small blood vessels, the septa of fat cells, and the milk spots and connective tissue framework of the omentum. In the present experiment radio-sulphate was used as an index of mast cell functional activity in autoradiographs of mesenteric spreads from animals which had been given 48/80 followed by the isotope at various time intervals.

MATERIALS AND METHODS

Compound 48/80 was given daily for 14 days by intraperitoneal injection to 11 adult albino rats. The dose was increased from 100 μg. to 1 mg. and a total dose of 10.85 mg. was given to each animal. Radio-sulphate was injected 24 hours before sacrifice and the animals were killed at intervals of 1-23 days after the course of 48/80. The serial numbers used (table 1,8) refer to the day of injection of the sulphur-35. The dose of the isotope was 500 μc. in each case. Autoradiographs were prepared of mesenteric spreads using Kodak AR10 and AR50 plates. The exposure time and photographic processing were kept as uniformly constant as possible throughout the experiment. The autoradiographs were stained with toluidine blue and assessed firstly by counting all labelled mast cells in the coarse grain preparations, and secondly by counting the number of labelled mast cells per 50 mast cells in 2 fine grain preparations for each animal. In the coarse assessment the results are presented on the scale 0 to ++++, and in the fine assessment the numbers of labelled cells are given (table 1,8).

RESULTS

Functional activity is present in mesenteric mast cells within 24 hours of the cessation of a prolonged course of 48/80, but there is no evidence of an orderly pattern of increasing numbers of mast cells utilising radio-sulphate at greater time

intervals thereafter (table 1,8). The attempt at quantitation in this respect shows merely a random number of cells which utilise the isotope unrelated to the interval between cessation of the course of 48/80 and the time of injection of sulphur-35.

There is no evidence of any particular anatomical distribution of radioactive cells in the mesentery at any time studied after 48/80 administration. Perivascular labelled mast cells were never more numerous than labelled mast cells in other positions in the mesentery. Again the distribution appeared random.

Examination of numerous radioactive mast cells showed that the radioactivity was cytoplasmic (fig. 1,42). It was also apparent that swollen ghost cells with no granular affinity for toluidine blue could utilise radio-sulphate.

DISCUSSION

Riley and West (1955) found that after 5 days on 48/80 no mast cells were visible in the mesentery, and throughout a longer period of injection there was little evidence of recovery of mast cells. They described a substantial restoration of mast-cell pattern in skin at 4 days, and in mesentery at about 13 days after 48/80. The mesenteric mast cells appeared to have been derived in part from numerous very small mast cells in the adventitia of vessels. Riley and West used toluidine blue staining to recognise mast cells. It is possible that the

tinctorial method is a less reliable one than the autoradiographic as an index of mast cell viability, and that the obvious difference in results is due to this factor. Certainly the distribution-pattern of radio-sulphate 24 hours after 48/80 indicates the presence of viable mast cells, and would suggest that the method is more sensitive than the tinctorial one.

However, it is not necessarily true that heparin and histamine metabolism run in parallel. Compound 48/80 releases histamine but Riley et al, (1955) have shown that with a 94 per cent loss of histamine by 48/80 there is only a 53 per cent loss of heparin, and no change in the clotting time in rat blood. It can thus be argued that the utilisation of radio-sulphate by mast cells after 48/80 is a doubtful measure of the effect of 48/80. But the same argument applies to tinctorial methods which stain acid mucopolysaccharides such as heparin. Ideally this experiment should be repeated using instead of radio-sulphate a labelled precursor of histamine.

SUMMARY

Mast cells can metabolise radio-sulphate within 24 hours of a 14 day course of the histamine-liberator 48/80. There is no evidence of a regeneration pattern developing from perivascular mast cells. Synthesis of heparin and histamine may be dissociated, and sulphate-utilisation by mast cells is a function of heparin synthesis only.

P A R T I I

1. In-vitro Uptake of Radio-sulphate by Cartilage Cells
2. Autoradiographic Studies in Experimental Lathyrism
3. Radio-sulphate Distribution in Normal Teeth
4. The Effect of Sodium Fluoride on the Utilisation of Radio-sulphate in Teeth

1. IN-VITRO UPTAKE OF RADIO-SULPHATE BY CARTILAGE CELLS

From the finding already described in Part I, 2 of the avidity of cartilage cells for radio-sulphate *in vivo* it was an obvious step to suppose that only living cartilage cells should show this functional activity. A surgical colleague, T. Gibson, was interested in the problem of viability of cartilage grafts, and in discussion it seemed reasonable to put to test the assumption that uptake of radio-sulphate *in vitro* might be an index of viability of such grafts. Layton (1950) had described a suitable nutrient medium. I made up batches of this medium containing radio-sulphate, and incubated in it specimens of cartilage supplied by Gibson. After incubation for 24-48 hours the cartilage was fixed and autoradiographs prepared with Kodak AR10 and AR50 plates. This preliminary work, establishing the technique, was published (Gibson *et al*, 1955) and my part in it is given here.

Figure 2,1 shows radio-sulphate utilisation by chondrocytes in a specimen of human cartilage incubated as described above. The image is punctate and sharply confined to the cells. There is no evidence of a diffuse image which might be ascribed to absorption of inorganic labelled sulphate as such. The cellular activity was demonstrable in all specimens of normal human cartilage removed at operation and incubated with as little delay as possible. Cartilage killed by boiling did not show any cellular utilisation of radio-sulphate.

Subsequently Gibson *et al*, (1958) used the sulphate-uptake

test to study survival of cartilage homografts in man. They found that not only do the chondrocytes survive transplantation but they do not suffer from the homograft reaction which affects almost all other tissue cells. Their cartilage implants were excised at intervals which varied from 2 weeks to 2 years. More recently Gibson brought me a portion of maternal ear cartilage graft which had been transplanted into a child 19 years before. I incubated the cartilage in the medium containing radio-sulphate and prepared autoradiographs. The chondrocytes were found to be still alive and actively metabolising the isotope (fig. 2,2).

2. AUTORADIOGRAPHIC STUDIES IN EXPERIMENTAL LATHYRISM

I became interested in experimental lathyrism as a disease of connective tissue probably involving the mechanism of fibrogenesis, and through the courtesy of Hans Selye obtained a supply of the toxic nitriles which cause the lesions. With my brother G.D.C. Kennedy of Glasgow Dental Hospital I undertook an examination of the disease using the autoradiographic technique. His part was an assessment of the dental changes. I designed and carried out the whole experimental programme, and composed the paper (Kennedy, J.S., and Kennedy, G.D.C., 1962) on osteolathyrism which is the basis of this section. Another paper, composed by G.D.C. Kennedy, on dental aspects of lathyrism has been submitted for joint publication and is not used here.

The lesions of experimental lathyrism are striking but it is not known how they are produced. There is evidence that a ground-substance change may be responsible (Menzies and Mills, 1957; Ham, 1960). Other observations have suggested an alteration in collagen (Hurley and Ham, 1959; Levene and Gross, 1959). In the present work the specificity of labelling of sulphated mucopolysaccharides *in vivo* with sulphur-35 given as sulphate (Part I, 2) has made it possible to determine the distribution of these sulphated compounds by autoradiography, and their relation to lathyritic lesions. In a similar way protein metabolism in the lesions has been investigated with sulphur-35 methionine and carbon-14 glycine as precursors. The

effect of lathyrogenic agents on the distribution of sulphur-35 sulphate labelled compounds in healing fractures has also been investigated. The results sustain the hypothesis that the essential change in lathyrism is a failure of formation of chondroitin sulphates A and C complexes with protein, and a subsequent defect in fibrogenesis.

MATERIALS AND METHODS

A sweet pea diet, β-amino-propionitrile (BAPN) and amino-acetonitrile (AAN) were used to produce experimental lathyrism in 52 Wistar rats. The animals were 4-7 weeks old at the beginning of the experiment. Both sexes were used. Cystamine, reported as lathyrogenic (Dasler and Milliser, 1958), was given to 12 weanling rats. Sweet pea (Geiger et al, 1933; Ponseti and Baird, 1952). The diet consisted of Lathyrus odoratus peas and pellets of standard rat diet crushed in equal parts by weight. It was given to 11 rats for 3 months to 1 year. Two of the animals received in addition 30 mg. and 80 mg. of BAPN (Wawzonek et al, 1955) in 15 and 20 divided doses daily by subcutaneous injection before isotope injection.

AAN (Wawzonek et al, 1955). This compound was given daily in 10-20 mg. doses in water, orally or subcutaneously, to 41 rats. The total dose per animal ranged from 60 mg. to 550 mg. Five of the animals received in addition deoxycorticosterone acetate (DOCA) in 1 mg. daily subcutaneous doses (Selye and Bois, 1957).

The total dose of DOCA per animal ranged from 15 mg. to 55 mg. and the corresponding AAN range was 250-550 mg.

Cystamine (Dasler and Milliser, 1958). As the dihydrochloride cystamine was given daily in 10-40 mg. doses in water orally to 12 rats. The total dose per animal ranged from 240 mg. to 1,050 mg.

Fracture group. In 15 rats tibial fractures were produced (Selye, 1957a). One third of the animals in this group were given thereafter 10 mg. daily doses of BAPN orally with a total dosage range of 80-360 mg. One third were given 10 mg. daily doses of AAN orally with the same total dosage range. The remaining third were not given any lathyrogenic factors. An animal from each sub-group was killed 9, 14, 24, 32 and 42 days after the fracture.

Sulphur-35 sulphate was given by intraperitoneal injection to the 11 rats on the sweet pea diet and to 20 rats on AAN. The dose ranged from 1-5 μc./g. body-weight and in some cases was repeated at intervals before sacrifice. Most animals were killed 24 hours after radio-sulphate injection. The range was 16-96 hours.

All the animals in the fracture group were given sulphur-35 sulphate 24 hours before sacrifice. The dose was 5 μc./g. body-weight.

Sulphur-35 DL-methionine was given by intraperitoneal injection to 11 rats on AAN. The dose was 1-2 μc./g. body-weight. Most animals were killed 24 hours after radio-methionine injection.

The range was 16-96 hours.

Carbon-14 glycine. The uniformly labelled compound was given by intraperitoneal injection to 5 rats on AAN and glycine-1-^{14}C was given in the same way to a further 5 rats on AAN. The dose for both compounds was 1 μc./g. body-weight. The animals were killed 24 hours after radio-glycine injection.

Comparison of the distribution of the radioactive compounds used was made mainly in animals which had received the same amount of AAN.

The distribution of the radioactive compounds was also established in normal control animals.

The animals were x-rayed after sacrifice and a selection of skeletal and other tissues taken for examination. The tissues were fixed in 4 per cent neutral or alcoholic formaldehyde. Decalcification, where necessary, was achieved with Susa solution, or a formic acid-picric acid-formalin mixture (Menzies and Mills, 1957), or with a formic acid-citric acid mixture (Collins, 1949), or by agitation in a 10 per cent formic acid-cation exchange resin mixture. Celloidin-paraffin double embedding was used for decalcified material. Autoradiographs were prepared of 5-6 μ sections by the stripping film method with Kodak AR10, AR50 and V1042 plates. Sections adjacent to those used for autoradiography were stained variously with haematoxylin and eosin, toluidine blue, azure A, periodic acid-Schiff, Verhoeff-Van Gieson, orcein-haemalum-tartrazine (Menzies and Mills, 1957) and gentian violet (Selye, 1957b).

RESULTS

Methods of inducing experimental lathyrism

AAN was the most potent agent used (Gross et al, 1960). The report (Dasler and Milliser, 1958) that cystamine administration produced lathyritic lesions could not be verified. No gross or microscopic changes were seen in the animals given this compound.

The lesions produced in experimental lathyrism

Skeleton. The gross (fig. 2,3) and microscopic skeletal lesions obtained have been described previously in numerous publications and reviewed by Selye (1957b) and Dasler (1957). The early changes seen in epiphyseal cartilage corresponded closely to those recently described by Ham (1960) as sharply localised to the zone of maturing chondrocytes with fibrillation in the matrix (fig. 2,4).

Fracture healing. AAN administration caused excessive formation of fibrous tissue, cartilage and bone as described by Selye (1957a)and Storey and Varasdi (1958). The cartilage and bone appeared about the same time as in fractures without AAN administration. BAPN caused a similar but less marked increase in size of callus. Particularly in the AAN treated animals there was some retardation of union but this was not a striking feature (fig. 2,6). The persistence of PAS-positive globules in new woven bone in lathyritic animals was confirmed (Storey and Varasdi, 1958).

Vessels. No gross or microscopic vascular changes were seen in any of the animals. In particular no dissecting aneurysms of aorta were

seen even in animals with very advanced skeletal lesions. The small group of animals which received both AAN and DOCA also showed no evidence of aneurysm formation although the skeletal changes of lathyrism were observed.

AUTORADIOGRAPHIC RESULTS

Sulphur-35 sulphate. There was no evidence of inhibition of cellular utilisation of this compound nor of any significant abnormal accumulation of labelled sulphated mucopolysaccharide in the lathyritic lesions. The pattern of distribution however was abnormal.

In epiphyseal cartilage in lathyritic animals the most interesting observation was that radio-sulphate consistently failed to give an image over the abnormal fibrillar matrix. This was most obvious in the early linear lesion described by Ham (1960) and appeared as a clear band in the autoradiographs (fig. 2,5). In controls there was an uninterrupted image over the whole epiphyseal plate and, depending on the time between isotope injection and sacrifice, this activity could be related mainly to cells or to matrix. In the lathyritic animals there was no evidence that utilisation of sulphate by cartilage cells was affected, and in histologically normal parts of the matrix radio-activity was demonstrable (fig. 2,5). It was only in the abnormal fibrillated areas that incorporation of sulphate was deficient. In later lesions where the epiphyseal plate was disorganised by

irregular proliferation of chondrocytes the image was again dominantly cellular and the abnormal matrix between clumps of cartilage cells showed no uptake of radio-sulphate (figs. 2,7 and 2,8).

In normal control animals the process of endochondral ossification was associated with the appearance of sulphate-labelled trabeculae of cartilage matrix in the metaphysis (fig. 2,9). And more distally appositional deposits of bone matrix on the trabeculae also showed the incorporation of radio-sulphate. An image was apparent occasionally over the peripheral osteoblasts, but in most cases the interval between sulphate-injection and sacrifice was such that the sulphated mucopolysaccharides had been secreted into the matrix. In lathyritic animals the metaphyseal trabeculae of cartilage matrix generally showed a less intense sulphate-image. The appositional deposition of sulphate-labelled bone matrix was unaffected.

In periosteal proliferation in the lathyritic animals all stages of ossification in membrane were seen, and the small basophilic globules described by Selye (1957b) were demonstrable. Initially the image in the osteogenic field was light and uniformly diffuse over cells and fibres (figs. 2,10 and 2,11). At the next stage of fibrocellular condensation there was an obvious increase in image intensity and the image was again diffuse over cells and matrix (fig. 2,12). Later, presumptive trabeculae were outlined by osteoblasts, and at this stage of woven bone development the

trabecular content of sulphated mucopolysaccharide was greatest as estimated by image intensity (figs. 2,13-2,15). The diffuse nature of the image persisted, and fields containing numerous basophilic globules showed the same pattern of radioactivity as fields devoid of these structures. Appositional deposition of lamellar bone on the woven bone followed, and here, as in endochondral ossification, radio-sulphate was clearly utilised in the formation of the new bone matrix by the osteoblasts. The image was confined to a narrow zone over the peripheral osteoblasts and immediately subjacent matrix (fig. 2,16). In normal appositional growth from periosteum the zone of activity at longer time intervals was displaced from the formative cells by a further layer of unlabelled bone (fig. 2,17).

In healing fractures sulphate-utilisation was demonstrable in the osteogenic tissues in both control animals and those receiving AAN or BAPN. The images were related to endochondral ossification, ossification in membrane, and appositional bone growth and were very similar to those already described in the intact animal. In occasional lathyritic animals fibrillar changes were apparent in cartilage matrix in the callus. In such cases there was again, as in the intact animal, obvious utilisation of sulphate by related groups of chondrocytes but no image over the altered matrix. There was also a trend in the lathyritic group to a lesser uptake of radio-sulphate by appositional bone matrix, but this was an inconstant feature. The presence of the bone globules

(Storey and Varasdi, 1958) did not affect the pattern nor the intensity of the autoradiographic images. In vascular areas of the osteogenic field it was clear that capillary endothelium did not give a ring-type image of greater intensity than the image given by the immediately related pre-osteoblasts and osteoblasts. However, due to limitations of resolution, it was not possible to exclude endothelial activity, although the major utilisation of sulphate was clearly by the osteoblast series of cells. The epiphyseal plates of the fractured bones failed to show the lathyritic changes seen in intact animals. In fact, they were narrower than normal in control and lathyritic animals, but the pattern of sulphate-utilisation was unchanged.

In aorta and great vessels in both experimental and control animals there was evidence of incorporation of radio-sulphate in medial sulphated mucopolysaccharides, but there was no evidence of any significant difference in pattern or intensity of distribution. In occasional autoradiographs capillary endothelial utilisation of sulphate was observed. The capillaries were in joint peri-capsular connective tissue related to lathyritic lesions. Capillaries related to ossification in cartilage and in membrane did not show any convincing evidence of endothelial utilisation of sulphate in control or experimental animals.

Labelled amino acids. The findings with sulphur-35 methionine and carbon-14 glycine were similar and are described together. As for

inorganic sulphate there was no evidence of inhibition of cellular utilisation, but there was a significant difference in the pattern of distribution in the lathyritic lesions.

In epiphyseal cartilage in lathyritic animals there was consistently a definite image over the abnormal fibrillar areas in the matrix (figs. 2,18-2,22). This was most obvious in fairly advanced lesions and presented a quite striking positive picture compared with the negative obtained in the sulphate group. Cartilage cells were also active in utilisation of the labelled amino acids but generally the image intensity was lower than that of the matrix.

In normal control animals the amino acids were taken up by cartilage cells of the epiphyseal plate and appeared in the matrix. The distribution of activity was diffuse throughout the cartilage. The image was most intense in the zone of proliferating cells and least intense in the zone of resting cells. In the time intervals studied very little radioactivity was carried down into the calcified cartilaginous trabeculae in the metaphysis. However, there was consistently a heavy image related to appositional bone deposits on the trabeculae. This image had the same distribution but was more intense than the corresponding sulphate one. The intensity was not affected by the lathyrogenic factors.

In periosteal proliferation in the lathyritic animals the autoradiographic-pattern was very similar to that given with radiosulphate. In fields of imminent osteogenesis the image was most

intense over the most cellular areas and was diffuse. Later there was an intensification of activity over discrete islands of matrix with persistence of a lower diffuse activity over related cells. And in appositional growth on new woven bone the image was maximal and located in the bony trabeculae just below the row of peripheral osteoblasts which showed less intense radioactivity (fig. 2,23). This appositional type of image was also evident related to the normal periosteum and endosteum. In some cases the zone of activity was displaced from the formative cells by a further layer of unlabelled appositional bone.

In vessels generally a light rather diffuse mural image was seen in control and experimental animals with no significant difference in intensity or location. There was no evidence of selective endothelial utilisation of the labelled amino acids.

DISCUSSION

The results of the present work suggest that the essential change in osteolathyrism is neither a defect in acid mucopolysaccharide production (Ponseti and Shepard, 1954; Ponseti et al, 1956) nor a defect in preformed collagen (Levene and Gross, 1959), but a defect, partial or complete, in fibrogenesis due to failure of complex formation between non-collagenous protein and chondroitin sulphates A and C. There is considerable evidence, some of which has been already reviewed (Kennedy, 1960), that acid mucopolysaccharides as protein complexes, form further loose complexes with the fibrous

proteins as fibrogenesis proceeds. In lathyrism the evidence presented here points to a failure in the formation of the initial non-collagenous protein complex with the sulphated mucopolysaccharides found particularly in epiphyseal cartilage, for this structure bears the brunt of the damage. The result is the production of an abnormal fibre, weaker than usual, (Enzinger and Warner, 1957). Hence there is a stimulus to the observed hyperplasia of the formative cells. Chondroitin sulphates A and C are the main acid muco-polysaccharides of epiphyseal cartilage, and it is postulated that in lathyrism these compounds do not form complexes with protein. Other tissues which form chondroitin sulphates A and C will also be subject to lathyritic lesions but not necessarily so severe, because other acid mucopolysaccharides may be available, e.g., chondroitin sulphate B, keratosulphate, and hyaluronic acid. In periosteum the result of formation of abnormally weak collagen is again hyperplasia of formative cells and a tendency to periosteal elevation, particularly at insertions of muscles. In this way a stimulus is provided for the observed subperiosteal osteogenesis which is such a marked feature of lathyrism.

Epiphyseal cartilage. Ham (1960) demonstrated that an early effect of AAN in cartilage is a fibrillar change in the matrix occurring in the zone of maturation of chondrocytes. It is now shown that the matrix in such a zone does not contain sulphated mucopolysaccharides, although elsewhere in the epiphyseal plate at this stage there is clear evidence of utilisation of radio-sulphate by chondrocytes and

of the presence of labelled sulphated compounds in the matrix. It is also shown that in areas of fibrillar change in cartilage matrix there is an active utilisation of labelled amino acids. New labelled protein is present in the affected areas, but chondroitin sulphates are not. It is therefore very probable that there is a failure in the extra-cellular binding of chondroitin sulphates to protein, and that the fibrillar change observed histologically is evidence of consequent abnormal new fibre formation. Menzies and Mills (1957) suggested that the basic lesion in lathyrism is a local accumulation of chondroitin sulphate accompanied by a disintegration of argyrophil fibrils. Their findings are based on histochemical methods and the present tracer studies fail to show an abnormal accumulation of labelled chondroitin sulphate. Unbound chondroitin sulphate is water soluble and on the present hypothesis that a complex with protein is not formed its loss from lathyritic cartilage matrix would be expected.

The role of sulphated mucopolysaccharides in calcification is not precisely known but such compounds appear wherever calcification takes place (Sobel, 1955; Kennedy, J.S., and Kennedy, G.D.C. 1957). They also appear in many tissues not normally calcified, and Meyer (1955) suggested that there must be a difference in the nature or quantity of the substances necessary for calcification. Ham (1960) pointed out that AAN impaired calcification of cartilage <u>in vitro</u>. It would appear reasonable to suggest that,

because the cartilage matrix is altered in lathyrism, and the fibres formed are deficient in sulphated mucopolysaccharide, the process of calcification is therefore impaired, since the sulphated compounds are very probably involved in initiating mineralisation (Bachra and Sobel, 1959). Electron-microscopic examination of bone provided data which suggested that mineral crystals were formed on mucopolysaccharide-protein bridges connecting the doublet bands of collagen fibres (Robinson and Watson, 1953). Freeman (1956) extended this hypothesis to epiphyseal cartilage and suggested that the change in matrix which confers on it the property of calcifiability may be an increased formation of chondroitin sulphate, and a coupling of chondroitin sulphate molecules to themselves and to matrix collagen protein, that is bridge formation, as in bone. In lathyrism the protein-polysaccharide bridges may not form and thus the fibre structure is weakened and the calcifiability of the matrix is also affected.

Periosteum. The essential lathyritic lesions are hyperplasia of periosteal cells, elevation of periosteum, and osteogenesis, mainly in membrane, but occasionally in cartilage. Angevine (1959) reported absence of ossification but it has not been possible to confirm this observation. The above sequence of events is again explicable on the hypothesis that fibrogenesis is impaired with production of weakened collagenous fibres. There is in this case no such clear cut difference in the autoradiographs between the utilisation of labelled sulphate and labelled amino acids

as was observed in the lesions in epiphyseal plate. This supports the view that the lathyrogenic compounds have a specific blocking action on protein binding of chondroitin sulphates A and C, and not of other sulphated mucopolysaccharides, which of course are labelled indiscriminately by inorganic sulphate in vivo. Rogers (1951) noted that the amount of chondroitin sulphate recovered from bone matrix as part of a protein-polysaccharide complex accounted for only a small part of the total hexosamine. Meyer (1956) found hyaluronate, chondroitin sulphate A, keratosulphate, and other unidentified sulphated fractions in bone, but only chondroitin sulphates A and C in hyaline cartilage. It would appear reasonable to suggest that the effect of lathyrogenic agents in tissues other than actively growing cartilage is less severe because of the smaller content of chondroitin sulphates A and C and the presence of other acid mucopolysaccharides.

Fractures. The major effect of the lathyrogenic factors on healing fractures is a proliferation of osteogenic tissue to give an abnormally large callus (Selye, 1957a; Storey and Varasdi, 1958). The rate of tissue differentiation in the osteogenic fields is not obviously altered, and although calcification and bone formation is abnormal, it is not possible to confirm the claim of Clemmons and Angevine (1957) that the callus matrix does not calcify. The sequence of changes is again explicable on the hypothesis that in lathyrism there is a failure of protein binding of chondroitin

sulphates A and C with resultant weakness in collagen fibres formed, and thus a stimulus to hyperplasia of the formative cells. As in the periosteal changes the utilisation of radiosulphate may appear normal in autoradiographs because of its incorporation in other sulphated mucopolysaccharides. However, in cartilaginous areas of callus the fibrillar changes in matrix were demonstrable in lathyritic animals, and the absence of sulphated mucopolysaccharides in such areas was again a feature of the lesion, just as in epiphyseal cartilage.

Impaired calcification may follow as discussed above. Storey and Varasdi (1958) pointed out that lathyrogenic factors caused the persistence of PAS-positive globules in new woven bone in callus and suggested that this indicated an abnormal production of acid mucopolysaccharide. In the present work it is obvious that new woven bone with such globules contains abundant sulphated mucopolysaccharide giving a diffuse autoradiographic image. Selye (1957b) has reported that the globules contain calcium. A possible explanation is that the globules represent sulphated mucopolysaccharide bound to calcium. More free chondroitin sulphates A and C are available in lathyrism and may bind calcium and thus remain in the tissues as the bone globules. Kowalewski and Emery (1960) found that the lathyrogenic factors reduced the amount of sulphur-35 in callus. Their findings are consistent with the present work which amplifies their general conclusion that BAPN inhibits a certain stage of the metabolism of sulphated mucopoly-

polysaccharides of the ground-substance.

Ossification. In normal endochondral bone formation it is confirmed that cartilage cells synthesise and secrete into the matrix its protein and sulphated mucopolysaccharide components (Leblond and Greulich, 1956). In lathyrism the calcification of the matrix may be impaired (Ham, 1960), but appositional bone growth on cartilaginous trabeculae occurs in the metaphysis, and it has been shown that the proteins and sulphated mucopolysaccharides of the bone matrix are the products of the related osteoblasts. At no stage in endochondral ossification is there any evidence that vascular endothelium may be a source of the sulphated muco-polysaccharides involved in the process.

Fields of intramembranous ossification are produced regularly in lathyrism, mainly as a result of periosteal elevation. Depending on the age of the lesion all stages are seen from imminent osteogenesis to mature lamellar bone. Initially there is a diffuse uniform image in the osteogenic field with both labelled amino acids and sulphate. Next, in more cellular areas which are quite circumscribed and are the presumptive trabeculae, there is an increased synthesis and secretion of protein and sulphated mucopolysaccharides. At the end of this stage peripheral osteoblasts appear quite sharply delimiting the trabeculae and appositional deposition of bone begins. The labelled amino acids are utilised by the osteoblasts and are quite clearly incorporated in the matrix of this new lamellar bone. Sulphated mucopolysaccharides

are also a product of the peripheral osteoblasts and appear in the new lamellar bone. Thus the results of the present work differ in detail from those reported by Curran and Collins (1957). They found little difference in the distribution of mucopolysaccharides in pathological and normal tissues showing intramembranous ossification. In lathyrism the distribution pattern is also unaltered. It is confirmed that intramembranous ossification takes place in a connective tissue rich in sulphated mucopolysaccharides, and that these compounds are present in larger quantities in new woven bone. Curran and Collins failed to demonstrate mucopolysaccharides in lamellar bone, but in the present study it is clear that sulphated mucopolysaccharides are synthesised by the osteoblasts and secreted into the matrix of lamellar bone laid down in apposition to woven bone. It is also clear that in normal appositional growth from periosteum and endosteum there is cellular synthesis of sulphated mucopolysaccharides which appear later as a linear zone of radioactivity deep to the formative cells. This zone is displaced into the shaft by further deposition of unlabelled lamellar bone (Kent et al, 1956; Leblond and Greulich, 1956). A similar type of appositional growth has been described in dentine (Kennedy, J.S. and Kennedy, G.D.C., 1957). In mature bone osteocytes are more active in protein than in sulphated mucopolysaccharide synthesis. This is in keeping with the low level of extractable mucopolysaccharide in

shaft bone (Rogers, 1951) and confirms the conclusion of Curran and Collins (1957) that osteocytes exhibit only slight sulphate-utilisation. However, their conclusion that sulphate-utilisation is a marked feature particularly of capillary endothelium in the osteogenic field could not be confirmed. In all areas of intramembranous ossification, whether complicated by lathyrism or not, pre-osteoblasts and osteoblasts were the cells dominating sulphate-utilisation. Occasionally in cellular areas the diffuse image encompassed capillary endothelium but it could not be ascribed with certainty to this structure. Recently Stehbens (1962) has also reported that he could not substantiate the claim (Curran, 1957; Curran and Collins, 1957) that endothelium takes up labelled sulphate avidly to synthesise acid mucopolysaccharide. No endothelial image of the ring-type described in experimental amyloidosis (Part III) could be found in fields of intramembranous osteogenesis.

Vessels. The failure to produce aortic aneurysm in the present series cannot be explained on dose level of lathyrogenic agents or on age of experimental animals, but animal strain may be a factor (Lalich, 1956). Levene (1961) found it impossible to differentiate histologically with any certainty between normal and lathyritic chick aortae, and Ponseti et al, (1956), who used sulphur-35 autoradiography, found no difference in aortic images, although they did describe aortic aneurysm. I have found it impossible to differentiate between aortae of control and experimental animals on histological or autoradiographic grounds, but it is possible that

a difference in fragility existed of degree insufficient to cause aneurysm (Levene, 1961). The apparently normal utilisation of radio-sulphate in the autoradiographs is again explicable because sulphated mucopolysaccharides other than chondroitin sulphates A and C are present in aorta (Meyer, 1956).

The nature of the change in lathyrism. Muir (1961) observed that the different sulphated mucopolysaccharides are metabolically quite distinct and must have separate functions. Since the physico-chemical properties of the three chondroitin sulphates appear so similar (Mathews, 1959) their separate functions may be due to the formation of different types of protein complex in each case. It is demonstrated here that an effect of the lathyrogenic factors is a failure of sulphated mucopolysaccharide-protein complex formation in epiphyseal cartilage which is known to be rich in chondroitin sulphates A and C. Partridge et al, (1961) have described a chondroitin sulphate-protein complex in cartilage in which the protein is not derived from collagen. It appears to be this entity which enters into loose combination with collagen fibres and soluble collagen to give the tissue its characteristic physical properties and insolubility. In lathyrism the physical properties of collagenous tissues are altered (Enzinger and Warner, 1957; Levene and Gross, 1959). There is a marked increase in the amount of salt and alkali-soluble collagen which can be extracted (Levene and Gross, 1959; Mikkonen et al, 1959-60). Collagen fibres fail

to form large bundles (Krikos and Orbison, 1960). Moreover, in lathyrism there is a significant increase in serum hexosamine and mucoprotein levels (Schwartz, 1959; Grant et al, 1960), and abnormal sulphate-labelled compounds are present in urine (Engfeldt et al, 1961). The identity of these sulphated compounds has not been established. It has been shown that there is a large decrease in hexosamine content of epiphyseal plates in experimental lathyrism with no significant difference in hydroxyproline content (Castellani and Castellani-Bisi, 1958). Follis and Tousimis (1958) also found that in lathyritic cartilage the collagen content based on hydroxyproline estimation was not abnormal but on electron-microscopic examination there was a lack of collagen fibrils. They suggested that the defect was a failure of the tropocollagen molecule to form collagen fibres. Electron-microscopically normal collagen fibres were reported in tail tendon of lathyritic animals by Ponseti and Shepard (1954) but, on the present hypothesis that the chondroitin sulphates A and C complexes with protein are the target compounds, this would be expected as tendon is rich in chondroitin sulphate B which does not occur in cartilage or bone (Meyer, 1956). It is also of interest in this connection that the ratio of chondroitin sulphate B to chondroitin sulphates A and C content of pig skin varies strikingly with age. In the adult animal the ratio is 640 whereas in the embryonic animal it is 0.48 (Meyer, 1959). A general dramatic increase in fragility has been described in the lathyritic chick embryo (Levene and Gross,

1959). It may well be that the accepted decrease in efficacy of the lathyrogenic factors with the age of the experimental animal is a result of altered tissue content of chondroitin sulphates A and C.

SUMMARY

Autoradiographic distribution studies with radio-sulphate and labelled amino acids have suggested a hypothesis for the mode of action of the lathyrogenic factors.

The matrix of epiphyseal cartilage is altered in experimental lathyrism. Labelled amino acids are incorporated into protein in the damaged areas but sulphated mucopolysaccharides are not demonstrable. It is concluded that the lathyrogenic factors act by blocking the combination of chondroitin sulphates A and C with non-collagenous protein. A defect in fibrogenesis follows.

No such clear cut difference in distribution-pattern is seen in other tissues, but sulphated mucopolysaccharides other than chondroitin sulphates A and C are normally present in these tissues. This is confirmatory evidence for the view that the lathyrogenic factors act specifically by blocking complex formation between protein and chondroitin sulphates A and C.

3. RADIO-SULPHATE DISTRIBUTION IN NORMAL TEETH

This work was undertaken to establish a base line for subsequent work on experimental dental caries, now in progress in collaboration with G.D.C. Kennedy, and not used here. I invited my brother to participate in the preliminary work as an expert in dental anatomy and physiology with whom I could discuss my autoradiographic findings. The entire experimental programme, assessment of results, and composition of the published report (Kennedy, J.S. and Kennedy, G.D.C., 1957) was carried out by me.

The localisation of mucopolysaccharides in teeth has been the subject of several recent investigations. Using chemical methods (Stack 1951, 1954) showed that dentine contained 0.2 per cent by weight of mucopolysaccharide, and that enamel contained a glycoprotein. Hess and Lee (1952) isolated chondroitin sulphuric acid, as its potassium salt, from dentine with a yield of 0.64 per cent. The same authors demonstrated the presence of galactosamine in dentine by paper chromatography.

Histochemical methods were used by Wislocki and Sognnaes (1950). Acid mucopolysaccharides were detected by metachromatic staining reactions by Sognnaes (1955), and Bevelander and Johnson (1955) distinguished between acid mucopolysaccharides and neutral mucopolysaccharides, again by histochemistry.

The technique of autoradiography was employed by Bélanger (1954, 1955) to show that, in the experimental animal, injected radiosulphate was incorporated in dentine and enamel. Leblond *et al*,

(1955) have studied the problem of calcification in bones and teeth, and used autoradiography to localise injected isotopes including sulphur-35.

The present work follows on the description given in Part I, 2 of a close correspondence between the localised uptake of inorganic sulphur-35 in vivo, and the presence, at the sites of uptake, of mucopolysaccharides as revealed by the usual staining methods. It was shown that the autoradiographic method is a specific means of identifying the presence in tissues of sulphated mucopolysaccharides.

MATERIALS AND METHODS

Sulphur-35 as sulphate at pH7 was administered intraperitoneally or subcutaneously to a total of 37 small rodents (table 2,1). In the case of the foetal mice the mothers were given the isotope subcutaneously. The dose of sulphur-35 ranged from 1-5 μc./g. body-weight except for the foetal mice where the fraction of the maternal dose which crossed the placenta was not determined.

The animals were killed with chloroform 5-169 hours after injection. The foetal mice were fixed intact in a mixture of equal parts of alcohol and acetone for 2 hours before slicing in the coronal plane. Fixation of the slices was continued for a further 6 hours. Thin blocks were taken from the jaws of the older animals, usually in the sagittal plane, and fixed for 24 hours in a mixture of equal parts of alcohol and acetone.

Alcoholic formalin, absolute alcohol, and 10 per cent neutral formalin were equally satisfactory. Five per cent formic acid for up to 72 hours was used for decalcification. Embedding in 2 per cent celloidin for 24 hours was used prior to vacuum embedding in paraffin for 1½ hours. Sections 5-6 μ thick were floated on water at 40°C, and those required for autoradiography were mounted on gelatine-coated slides. The stripping film method was used with Kodak AR10 and AR50 plates.

Sections adjacent to those used for autoradiography were stained with alcian blue (Pearse, 1953), by the alcian blue-chlorantine fast red method of Lison (1954), by the periodic acid-Schiff technique, and for metachromasia with toluidine blue at pH 2.6 and 4.9.

RESULTS

Adult animals. The distribution of sulphur-35 and the intensity of the autoradiographic images varied with the age of the animal and with the time interval between injection of the isotope and the death of the animal. In the adult animals the distribution-pattern and the intensity remained relatively constant with time (table 2,2).

The highest uptake was found uniformly in the predentine and no species difference was noted (fig. 2,24). The maximum uptake was less than the maximum found in young animals (table 2,3). The cells and matrix of the dental pulp showed a rather diffuse uptake

in most preparations, but were negative in the oldest teeth in which the pulp cavity was smallest. There was occasional focal activity over cells of the dental pulp. No activity was seen in the dentinal matrix, and the odontoblasts, enamel matrix, and ameloblasts showed only an occasional weak uptake.

Foetal and suckling animals. The uptake-pattern was more complex in this group than in the adult. The maximum intensity was found consistently in predentine, in dentinal matrix, and in the dental pulp. The variation in the uptake-pattern with time was studied in the following structures (table 2,3).

Dentinal matrix. The spatial distribution of radioactivity in this tissue varied most with the time interval between injection and sacrifice. Between 5 and 19 hours after injection the autoradiographs showed a dense band of activity in the predentine (fig. 2,25). As the time interval increased, the intensity of this image over the predentine decreased, and a further band of activity appeared in the dentinal matrix. This active zone in the dentinal matrix increased in intensity with time up to 135-139 hours after injection. The intensity decreased in the 164-169 hour period. The band also changed its position in the matrix. As the time interval increased the zone of activity was found progressively farther out towards the amelo-dentinal junction, and 164-169 hours after injection its edge coincided with the amelo-dentinal junction (figs. 2,26-2,27). The movement outwards,

however, was not uniform in all parts of the dentinal matrix, but was most rapid towards the apex. For example, 90-94 hours after injection, at the crown of a molar, the active band was found to be about equidistant from the amelo-dentinal junction and the predentine, while, at the apex, it had reached the amelo-dentinal junction. In many cases, in sections of incisors, the uptake in the dentinal matrix appeared on the convex sides of the teeth as two closely apposed bands, and on the concave sides as one band (fig. 2,28). The displacement outwards with time was again as described above. An appreciable uptake was still apparent, up to 169 hours after injection, in the predentine, with frequently a clear band of little or no activity between the predentine, and the densely active zone in the dentinal matrix. The activity in the dentinal matrix generally could not be related to the dentinal tubules, but appeared as quite sharply demarcated zones fairly constantly about the breadth of the layer of odontoblasts.

Enamel matrix. No such marked zoning of activity was seen. When a positive uptake occurred it appeared as a uniform stippling over the whole thickness of the matrix (fig. 2,29). This band was sometimes noted to be darkest at the apex of an incisor, and to shade off in intensity towards the incisal tip. No definite variation in intensity in the enamel matrix uptake was observed with time. At 164-169 hours there was a slight decrease, as in the case of the dentinal matrix uptake, but it cannot be claimed in either case that this marked a turning point.

Odontoblasts and ameloblasts. The cells showed activity up to 94 hours after injection (figs. 2,30-2,31). The activity was rather diffuse and, at times, weak, but in the ameloblasts at 10-14 hours it was possible in some cases to localise the maximum activity in the poles of the cells adjacent to the enamel matrix (fig. 2,32). When such a localisation was possible it was noted that it occurred in large, elongated ameloblasts. The stratum intermedium and the membrane between it and the ameloblasts both showed a fairly constant uptake for the period 5-94 hours after injection (figs. 2,30-2,32). As for the odontoblasts and ameloblasts this activity was not detected in the period 115-169 hours after injection.

Dental pulp. Radioactivity was present throughout the whole period of observation, and the intensity of the uptake showed a tendency to increase with time. Occasionally focal activity over cells of the dental pulp was noted (fig. 2,30), but generally the uptake was diffuse over cells and matrix. The maximum uptake was often seen at the apical part of the pulp, and this sometimes corresponded with an increased staining reaction for mucopolysaccharide. This localisation was not constant and, particularly for the period 115-119 hours after injection, the maximum activity was found in the central part of the pulp, and shaded off towards the odontoblasts in which no activity was seen at this time.

Other structures. In the earliest stages of the developing

tooth the internal and external enamel epithelia, the stellate reticulum, and the dental papilla showed a fairly constant image, up to 19 hours after injection. Activity in the dental papilla was usually greatest in the regions of future cusp development (fig. 2,33). In many cases focal cellular activity was noted in the stellate reticulum as well as more diffuse stippling over cells and matrix (fig. 2,34). At the bud stage the surviving connexion to the dental lamina showed weak activity with a heavier uptake in the mesodermal tissue on either side of the stalk (fig. 2,33). The squamous epithelium was inactive. At a later stage, the dental follicle showed sulphate-uptake which was also noted in the early periodontal membrane. The activity in these foetal and neo-natal structures was not observed, because of technical difficulties, for periods greater than 19 hours after injection.

No species variation in uptake was noted in this group of animals.

The staining reactions of the dental tissues showed quite marked variation by the methods used, and generally did not give as consistent a picture as the uptake pattern of sulphur-35 in corresponding sections (table 2,4).

Metachromasia was fairly consistently demonstrated in the matrices of the stellate reticulum, the dental papilla and the dental pulp. Predentine, dentinal matrix and enamel matrix, however, were more variable. The peripheral parts of the dentinal

tubules only stained metachromatically when the toluidine blue was buffered to pH 4.9 and were orthochromatic at pH 2.6. Weakly staining intracellular metachromatic material was seen occasionally in the supranuclear part of the ameloblasts. A similar weak reaction was demonstrable in a few sections in the odontoblasts. The cells of the dental papilla, dental pulp and stellate reticulum were more frequently metachromatic. Variable results were again obtained in the external and internal enamel epithelia, and the stratum intermedium was consistently orthochromatic.

Mucopolysaccharide methods were quite uniformly positive for the cells and matrix of the dental papilla and dental pulp, and in the large majority of cases for the cells and matrix of the stellate reticulum. The predentine, dentinal matrix and enamel matrix gave extremely variable results, particularly with the periodic acid-Schiff technique. The dentinal tubules however consistently stained for mucopolysaccharide by the alcian blue method. The positive result here was in the peripheral parts of the tubules, and frequently only for that portion of their length occupying the predentinal layer. The odontoblasts, ameloblasts, stratum intermedium and internal and external epithelia did not give consistent results, particularly with alcian blue.

In the staining reactions generally no species difference was observed, and the age of the animal did not cause as much variation in the results as it did in the case of the sulphur-35 uptake studies.

Only in the case of the dental pulp was it possible to grade the staining reaction and it corresponded closely to the sulphur-35 uptake grading. This was most evident with alcian blue and toluidine blue. Comparison of staining reactions of decalcified and undecalcified material showed no significant differences, although a tendency was noted for metachromasia to appear more frequently in the enamel and dentinal matrices of decalcified teeth. This, however, was not a constant finding.

DISCUSSION

Chemistry. Reference has already been made to the isolation of chondroitin sulphate from dentine by Hess and Lee (1952). They also showed that the chondroitin sulphate from dentine contained galactosamine, thus indicating its similarity to the chondroitin sulphate of cartilage which is a complex of acetylgalactosamine, glucuronic acid and sulphuric acid (Bray et al, 1944). Earlier work by Pincus (1949) led to the description of polysaccharide components of dentine and enamel, which, when exposed to sulphatase, may release sulphuric acid. Pincus stated that the polysaccharide component of dentine is chondroitin sulphate, and that of enamel may be mucoitin sulphate.

The present autoradiographic results were obtained mainly in decalcified preparations, and, therefore, it is very probable that the sulphur-35 localised was present in organic form. Any soluble

inorganic sulphate present, even in undecalcified tissues, is lost in histological processing and washing prior to autoradiography. Tarver and Schmidt (1939) showed that there was no significant incorporation of inorganic sulphur-35 in cystine, and in methionine Boström and Åqvist (1952) showed that again inorganic sulphur-35 was not significantly utilised. Taurine showed a very low uptake. Although Sognnaes (1955) suggested that there may be at least four different sources of organically bound sulphur in enamel (SH, SS, CH_3S, SO_4), it is not probable, on the evidence available, that inorganic sulphate is bound by the sulphur-containing amino acids in vivo. It has also been demonstrated in Part I,2 that inorganic sulphur-35 is not taken up by tissues containing sulphur compounds other than ester sulphates. It is thus very probable, when a positive autoradiographic uptake of sulphur-35 is demonstrated after the usual histological processing of the tissue that the sulphur-35 is incorporated in a sulphated mucopolysaccharide or in a sulphated precursor of a sulphated mucopolysaccharide. Inorganic radioactive sulphate labels sulphated mucopolysaccharides or their precursors in vivo. The present work supports this hypothesis.

Histochemistry. The extracellular dental tissues in which bound sulphur-35 has been demonstrated by autoradiography have been shown to stain more or less uniformly for acid mucopolysaccharides (Bevelander and Johnson, 1955). Wislocki and Sognnaes (1950) reported variability in the periodic acid-Schiff reaction in enamel and stellate reticulum. Sognnaes (1955) summed up histochemical

observations as favouring the interpretation that a Schiff-positive acid mucopolysaccharide component is present in the ground-substance of the stellate reticulum, of the dental papilla, and of the dental sac, in the interprismatic regions of the enamel, and in the peripheral regions of the dentinal tubules. Present observations confirm the correspondence between histochemical methods and sulphur-35 uptake in the ground-substance of the stellate reticulum, of the dental papilla, and of the dental pulp. In predentine, dentinal matrix, enamel matrix and dentinal tubules the staining methods used gave variable or negative results, and only occasionally was demineralisation associated with a definite metachromasia. In the case of the dentinal tubules it was not possible to localise accurately the sulphur-35 uptake-pattern.

Since chondroitin sulphate has been extracted from dentine, and since the sulphur-35 uptake-pattern in the predentine and dentinal matrix is so constant in numerous sections, it is suggested that the isotope distribution is a more sensitive and reliable index of ester sulphates than metachromasia or other histochemical methods.

Mode of uptake of sulphur-35. The mode of uptake of the inorganic sulphur-35 by the tissues cannot be determined with certainty in this investigation. Non-enzymic exchange of inorganic sulphate with ester sulphate is not significant, however, and enzyme-catalysed exchange of sulphate with preformed polysaccharide sulphate is now considered doubtful, because attempts to find such an enzyme

in mammalian tissue have consistently failed. Thus the uptake of sulphur-35 as sulphate is generally accepted as evidence for sulphated mucopolysaccharide synthesis. If it is postulated that hexosamines are esterified prior to the formation of polysaccharide then the lack of exact correspondence between the autoradiographic pattern and the pattern of metachromasia is explicable. The autoradiograph localises even the early stages of the synthesis, but metachromasia is not shown by the sulphated esters of low molecular weight.

More recently Schiller et al, (1958) showed that neither sulphate nor acetyl groups of acid mucopolysaccharides undergo exchange but are metabolised at the same rate as the polymers, thus validating work with radio-sulphate alone. Bélanger (1954, 1955) confirmed the appearance of radio-sulphate in the ameloblasts and suggested that only the ameloblasts of large type are capable of mucopolysaccharide synthesis and secretion. He gave no indication of whether radioactivity was observed in the odontoblasts or not. Leblond et al, (1955) favoured an extracellular sulphation hypothesis for dentinal mucopolysaccharide, and suggested that the bulk of the chondroitin sulphate of dentine is synthesised, or at least sulphated, at the predentino-dentinal junction. No evidence is given of the part played by the odontoblasts, if any. The present work does not confirm the idea of extracellular sulphation, and the distribution-pattern of sulphur-35 in predentine does not show any marked increase at the predentino-dentinal junction. The

present observations support the theory of cellular synthesis outlined above. In the case of the adult dental tissues the lack of definite activity in the odontoblasts and ameloblasts may be an index of a small turnover of sulphate as compared with the turnover in the dental tissues of the younger animals.

The focal activity over cells of the stellate reticulum and of the dental pulp, and the more diffuse activity over cells and matrices, may be interpreted as indicating the utilisation of radio-sulphate by fibroblasts in the production of the ground-substance (Part I,3).

Enamel. With regard to amelogenesis, the present work suggests that cellular activity in the stratum intermedium and in the ameloblasts is associated with the appearance of a labelled sulphated mucopolysaccharide in the enamel matrix, and supports the view of Marsland (1951) that the ameloblasts and stratum intermedium form a functional unit concerned with the formation of matrix. Marsland (1951, 1952) also claimed that there is a primary phase during which the enamel organ is concerned solely with the deposition of an enamel matrix, which, although it contains a certain proportion of calcium salts, is insoluble in acids. The matrix formation is incremental in character, but maturation begins only after the full width of the matrix has been laid down, and occurs rapidly throughout the full thickness of the tissue in a plane at right angles to the long axis of the tooth. There is no definite evidence of an incremental increase in the enamel

matrix mucopolysaccharide component, but it may be that the usual picture of a uniform band of radioactivity occupying the full width of the matrix is associated with maturation. Between 164 and 169 hours after injection a slight decrease in intensity was noted and, although no later observations were made, this decrease corresponds with the observations of Leblond et al, (1955) that mineralisation may be associated with metabolic transformation leading to a loss of sulphur-labelled compounds. They also suggested that the matrix is dispersed by the accumulation of mineral elements to explain a diffuse, broad uptake pattern obtained in enamel, 2 days after injection of sulphur-35.

Dentine. Cellular activity in the odontoblasts may also be related to the appearance of the bands of radioactivity in the predentine and in the dentinal matrix. And the movement with time of the dentinal bands of activity may be explained by appositional growth of dentine, with incremental maturation. The dentine in the rat is laid down in daily layers about 16 μ thick, and this rate of recession of the odontoblasts agrees quite well with the displacement of the bands of radioactivity. In the case of the double zoning in the dentinal matrix, on the convex side of an incisor, a possible explanation may be the relatively greater rate of growth on this side of the tooth. There may be a cyclical utilisation of sulphate corresponding to incremental maturation and, where the growth rate is a maximum, a second cycle is visualised before the blood level of radioactive sulphur-35 has fallen. Thus in

dentine, as in the enamel matrix, the sulphated mucopolysaccharides appear to play a part in matrix formation and in maturation. Leblond et al, (1955) suggested that in the region of the predentino-dentinal junction a sulphated mucopolysaccharide, and probably another carbohydrate, are added to the collagen base of predentine, and concomitantly calcium and phosphate ions are deposited as dentinal crystals. The results described here do not confirm the main initial activity at the predentino-dentinal junction, but show a more diffuse uptake throughout the predentine. There is also an increase in intensity of the dentinal radioactivity up to 135-139 hours after injection with a decrease in the last period observed.

Calcification. Sobel (1955), in an evaluation of the literature, pointed out that sulphate-containing mucopolysaccharides appear wherever calcification takes place, namely in dentine and enamel, in bone, and in abnormal calcification of the arteries. He suggested also that, for dentine and bone, chondroitin sulphate may be an integral part of the 'local factors(s)'. Lacroix (1954) postulated a role for sulphur-35 compounds in the formation of new osteons in bone, and Dziewiatkowski (1952) described a concentration of a sulphur-containing compound, or compounds, in centres of secondary ossification in the bones of young rats. In a later paper Dziewiatkowski (1954) found an increased accumulation of a sulphur-containing compound, in regions of active calcification, when vitamin A was administered to rats deficient of this vitamin.

It was shown by Engfeldt *et al*, (1954) that sulphur-35 was taken up in bone in the same sites as uptake of calcium-45. Rubin and Howard (1950) put forward some evidence that a change in the state, or concentration, of chondroitin sulphate in cartilage matrix leads directly to the interaction of this compound with calcium, and thereby confers the state of calcifiability on the matrix. On the other hand, Sognnaes (1955) has suggested that sulphated mucopolysaccharides, rather than being the 'local factor', may serve to maintain certain regions in the uncalcified state, and provide metabolic pathways through relatively avascular and acellular structures. Present results suggest that sulphur-35-labelled polysaccharides are distributed in dentine in a pattern corresponding with incremental maturation, and in enamel in a pattern corresponding with maturation *en bloc*.

SUMMARY

Sulphur-35 administered in inorganic form as sulphate is incorporated in organic form in the matrices of dentine and enamel, and in the ground-substances of the dental pulp and stellate reticulum.

A cellular origin of the organic sulphated compounds is postulated, and evidence is presented for their synthesis in the odontoblasts, ameloblasts and cells of the dental pulp and stellate reticulum. There is no evidence of utilisation of inorganic sulphate by the sulphur-containing amino acids. The sulphated compounds are considered to be sulphated mucopolysaccharides.

The distribution-pattern of the sulphated mucopolysaccharides suggests they may play a part in the mechanism of calcification of dentine and enamel.

4. THE EFFECT OF SODIUM FLUORIDE ON THE UTILISATION OF RADIO-SULPHATE IN TEETH

This experiment was also regarded as a necessary preliminary step towards an examination of the part played by fluoride in dental caries currently in progress in collaboration with G.D.C. Kennedy of Glasgow Dental Hospital. My brother co-operated in the present work with useful discussion but the entire experimental programme, assessment of results, and preparation of a published abstract (Kennedy, J.S., and Kennedy, G.D.C., 1959) was carried out by me, and the present composition is mine.

Sulphur-35 administered *in vivo* as sulphate selectively labels sulphated mucopolysaccharides (Part I, 2). The normal distribution of sulphated mucopolysaccharides in rodent teeth has been described (Part II, 3) and it is evident that there is a topographical association between such compounds and fields of calcification. Changes in calcification in dentine may be produced by a variety of agents including fluoride (Irving, 1957). In view of the interest in this compound as a prophylactic agent against dental caries the present experiment was designed to examine the effect of single and repeated doses of sodium fluoride on the dental distribution-pattern of radio-sulphate.

MATERIALS AND METHODS

Twenty-two Wistar rats of both sexes were used. Two of the animals were adult, the remainder aged 4-9 weeks. They received

1-9 subcutaneous injections of 0.4 ml. of a 2.5 per cent aqueous solution of sodium fluoride at intervals of 1-20 days. In each case the fluoride injection was followed by an injection of radio-sulphate at a constant interval of 1 day. Nine animals also received radio-sulphate with, and 1 day after the fluoride. Each dose of sulphur-35 was 4 μc/g. body-weight. Animals were killed with chloroform 1 day after the last dose of radio-sulphate or fluoride. The jaws were fixed in 10 per cent neutral formalin, decalcified in 5 per cent formic acid or a picric acid-formic acid-formalin mixture, and double embedded in celloidin and paraffin. Autoradiographs were prepared of 5-6 μ sections with Kodak AR10 and AR50 emulsions. Neighbouring sections were stained with haematoxylin and eosin, toluidine blue, by the periodic acid-Schiff method, and by the alcian blue-chlorantine fast red method of Lison (1954).

RESULTS

In every case the dentinal lesions observed corresponded well to the calciotraumatic response (fig. 2,35) described by Irving et al, (1949). For a single dose of fluoride a single response was obtained, and for multiple doses, multiple lesions were seen in the dentine. The distribution of radio-sulphate was consistently in the proximal or hypercalcified zone (figs. 2,36-2,39). Metachromasia, however, was not always obvious in this site. Occasionally a very light image was obtained over the calciotraumatic

line, but in no case was there any evidence of radio-sulphate in the distal or hypocalcified zone.

DISCUSSION

Irving et al, (1949) suggested that in the calciotraumatic response the hypercalcified zone is produced after the administration of the toxic agent, and that the hypocalcified zone represents the zone of predentine formed immediately prior to the experiment. Weatherell and Weidmann (1959) described wide seams of osteoid in skeletal lesions of fluorosis. They found that the calcification front was sharply defined by a metachromatic zone suggesting the presence of a polysaccharide ester sulphate. They found no evidence of hypermineralisation in the fluorotic lesions of bone, although they considered the calcification mechanism was defective.

The present work can give no data on degree of mineralisation of matrix, but confirms that there is histological evidence of a calcification defect in dentine, appearing as the calciotraumatic response. The localisation of radio-sulphate in the proximal zone of this lesion indicates the presence there of a sulphated mucopolysaccharide. If indeed this zone is hypercalcified (Irving and Weinmann, 1948) then clearly ester sulphate is involved in the process. And these data are consistent with the findings described in normal calcification of dentine (Part II, 3). On the other hand, irrespective of degree of mineralisation, the zone of radioactivity may correspond to the calcification front

following the toxic dose of fluoride. Again it is very probable that the sulphated mucopolysaccharides play an important, although as yet not clearly understood, part in the mechanism of calcification.

SUMMARY

Fluoride administration alters the normal distribution of sulphated mucopolysaccharides in rodent teeth.

Radio-sulphate is found in the proximal zone of the calcio-traumatic response, and whether this represents a hypercalcified zone or the calcification front, it indicates the participation of sulphated mucopolysaccharides in the process of calcification.

P A R T III

1. Sulphur-35 in Experimental Amyloidosis

1. SULPHUR-35 IN EXPERIMENTAL AMYLOIDOSIS

The material in this section has been published independently (Kennedy, 1962).

There is chemical evidence that amyloid consists of protein with a small mucopolysaccharide component (Hass and Schultz, 1940; Hass, 1942). Analysis of the polysaccharide fraction showed it to be a sulphated compound containing glucosamine, uronic acid and acetic acid (Meyer, 1947). The specificity of labelling sulphated mucopolysaccharides in vivo with sulphur-35 given as sulphate (Part I, 2) suggested an investigation of the pathogenesis of amyloid in laboratory animals using autoradiography to determine the distribution of the sulphated compounds and their relation to amyloid deposits. This is an account of such an experiment. In addition a small group of animals was studied with trace doses of sulphur-35 methionine and carbon-14 glycine to follow protein synthesis in experimental amyloidosis.

It will be shown that both radio-sulphate and labelled amino acids are incorporated in amyloid deposits. Cells of the plasma series, particularly in splenic red pulp, show evidence of utilisation of both radio-sulphate and labelled amino acids and therefore the synthesis of a glycoprotein is postulated. Endothelial cells related to amyloid show striking radio-sulphate utilisation but are not active with respect to labelled amino acids. It is therefore concluded that they synthesise a sulphated mucopolysaccharide. A new hypothesis is formulated in which amyloid is regarded as an insoluble complex formed by the

circulating glycoprotein of plasma cell origin and the sulphated mucopolysaccharide of local endothelial cell origin.

MATERIALS AND METHODS

Injections of sodium caseinate, Freund-type adjuvant, and Leishmania donovani were used to promote amyloidosis in mice and golden hamsters.

Caseinate (Teilum, 1952). Daily subcutaneous injections of 0.5 ml. of a 5 per cent casein solution in 0.25 per cent NaOH were given to 66 albino mice, 6 AKR strain mice and 20 C_3H strain mice. The animals were initially 15-25 g. in weight and were restricted to an oatmeal and water diet during the experimental period which ranged from 20 to 100 days. Both sexes were used. The effect of cortisone, corticotrophin and nitrogen mustard was examined in controlled groups (Teilum, 1954, 1956).

Freund-type adjuvant (Rothbard and Watson, 1954). The water in oil emulsion contained 8 mg. killed dried Mycobacterium butyricum. The oil phase consisted of 8.5 ml. Risella Oil 17 or Ondina Oil 17 with 1.5 ml. Crill 16 as emulsifying agent. Weekly subcutaneous injections of 0.2 ml. of the emulsion were given to a group of 19 albino mice for a period up to 14 weeks. A few intraperitoneal injections were made. Both sexes were used.

L. donovani (Fulton and Niven, 1951). Two infected golden hamsters were obtained from the National Institute for Medical

Research and the strain of L. donovani has been maintained in hamsters by intraperitoneal inoculations of 0.5 ml. splenic emulsions in Ringer-glucose, Earle's solution or normal saline. A group of 68 hamsters was used in the present work.

Some of the animals in each of the above groups were used for radioactive isotope studies. The remainder were examined by conventional histological methods.

Sulphur-35 sulphate was given by intraperitoneal injection to 62 albino, 5 AKR and 18 C_3H mice in the caseinate group, to 18 albino mice in the Freund-type adjuvant group, and to 21 hamsters inoculated with L. donovani. The dose ranged from 1 to 5 μc./g. body-weight and in some cases was repeated at intervals before sacrifice. Most animals were killed 24 hours after isotope injection: the range was 3 to 120 hours.

Sulphur-35 DL-methionine was injected intraperitoneally into 3 hamsters with leishmaniasis. The dose was 3 μc./g. body-weight and the animals were killed 48 hours later.

Carbon-14 glycine (uniformly labelled) was given by intraperitoneal injection to 2 hamsters with leishmaniasis. The dose was 1 μc./g. body-weight and the animals were killed 24 hours later.

The distribution of the radioactive compounds used was also established in normal control animals.

The tissues were fixed in 4 per cent neutral or alcoholic formaldehyde, embedded in paraffin and cut at 5-6 μ. Auto-

radiographs were prepared by the stripping film method with Kodak AR10 and AR50 plates. Ilford G5 nuclear emulsion as gel was also used occasionally for the preparation of coated autoradiographs (Messier and Leblond, 1957). Sections adjacent to those used for autoradiography were stained variously with haematoxylin and eosin, congo red, phosphotungstic acid haematoxylin, dahlia, periodic acid-Schiff, azure A, toluidine blue before and after peptic digestion (Braunstein and Buerger, 1959), methyl green pyronin, and with Giemsa.

RESULTS

Methods of inducing amyloidosis

Experimental kala-azar in the golden hamster was consistently the best method. All animals successfully infected with L. donovani developed amyloid, as described by Fulton and Niven (1951). Caseinate and Freund-type adjuvant injections were less successful in the animals used in this series. Amyloid did occur in 20 per cent of the caseinate group and in 5 per cent of the adjuvant group but its occurrence was fickle, unpredictable, and unrelated to strain, sex, age or material injected. Contrary to the findings of Teilum (1952, 1954) cortisone, corticotrophin and nitrogen mustard had no potentiating effect. Consequently the caseinate and Freund-type adjuvant groups were used mainly to study the cellular changes preceding and accompanying the early stages of amyloid formation.

The lesions produced in experimental kala-azar

The development of amyloid followed closely the descriptions given by Gellhorn et al, (1946) and Fulton and Niven (1951). The amyloid stained with congo red but gave equivocal results with metachromatic stains. After peptic digestion metachromasia with toluidine blue was demonstrable (Braunstein and Buerger, 1959).

Kidney. This was the first organ involved. Amyloid appeared in the glomerular tufts about 8 weeks after infection and was progressive. The material deposited appeared first in a pericapillary distribution and suggested by ordinary light microscopy basement membrane thickening (fig. 3,1). Later lesions were more diffuse in the glomerular tufts, and occasionally showed a nodular pattern not unlike Kimmelstiel-Wilson lesions (fig. 3,3). Periglomerular deposits were not seen, but in advanced cases interstitial amyloid occurred. In many animals colloid casts were present in dilated renal tubules and similar material was occasionally seen in glomerular spaces. Renal vein thrombosis was evident in 4 animals with advanced renal amyloidosis (fig. 3,5). There was no evidence of plasma cell proliferation in glomeruli, but occasional groups of plasma cells were seen related to arteries. They were not associated with amyloid deposits. Parasitised cells were not seen in kidney.

Liver. Amyloid appeared in liver from about 9 weeks after infection onwards. The amount was usually fairly small and was not as

rapidly progressive as in kidney. The deposits usually had a subendothelial distribution around branches of the portal and hepatic veins. In a few animals they later extended into the sinuses (fig. 3,6). The perivascular amyloid was frequently but not invariably associated with cellular foci composed mainly of the plasma cell series but also containing reticulum cells, lymphocytes, and occasionally eosinophil leucocytes (fig. 3,8). Such cellular foci were also seen in the absence of amyloid deposits. A recurrent feature was the presence of small groups of parasitised phagocytes in the sinuses, often in a mid-zonal position. Such clumps were also occasionally seen in small vessels and appeared to be embolic from spleen. Sometimes the cells were multinucleate and quite often they contained laminated basophil inclusion bodies (fig. 3,9). These basophil bodies also occurred extracellularly in the sinuses. There was no apparent topographical relationship between the clumps of parasitised cells and the deposits of amyloid. Portal vein thrombosis occurred in one animal with only minimal hepatic amyloid deposits.

Early cirrhotic changes were observed in 13 hamsters (fig. 3,10). There was no correlation with degree of hepatic amyloidosis.

Spleen. Amyloid appeared from about 9 weeks after infection onwards, had a characteristic perifollicular distribution (fig. 3,12) and was progressive. Occasionally in a congested spleen vascular spaces were obvious in the perifollicular zone and in such cases the amyloid had a perivascular distribution (fig. 3,14).

Perifollicular haemorrhage was a feature of many spleens showing early amyloid deposits. In all cases numerous groups of cells of the plasma cell series could be demonstrated in the red pulp (fig. 3,16) and a topographical relation to amyloid deposits was frequently but not invariably obvious. Splenic giant cells were seen but they were usually not numerous. A striking recurrent feature was the appearance of discrete clumps of parasitised phagocytes in the red pulp (fig. 3,18). At times such clumps occupied vascular spaces. These cells frequently also contained haemosiderin. They did not have any constant relationship topographically to amyloid deposits. Basophil bodies, as described in liver, were not seen. Splenic vein thrombosis occurred in one animal with only minimal splenic amyloid but with advanced renal lesions and renal vein thrombosis.

Other tissues. Amyloid was seen occasionally in the adrenal cortex involving the zona reticularis and the zona fasciculata (fig. 3,20). There was no evidence of any cellular proliferation preceding or accompanying adrenal cortical amyloidosis. Lymph nodes in some animals late in the disease showed discrete clumps of parasitised phagocytes as described in spleen. There was also an occasional proliferation of the plasma cell series in lymph nodes, but no amyloid was seen. The subcutaneous tissues were very oedematous terminally, but again no amyloid deposits were found.

The lesions produced by repeated injections of caseinate or Freund-type adjuvant

When amyloid occurred it was perifollicular in spleen and, less frequently, perivascular in liver. Renal amyloid was seen in only 4 animals and was never more than a minimal basement membrane thickening with light microscopy. The amyloid stained in the same way as in the hamster.

Spleen. There was invariably a proliferation of mononuclear pyroninophilic cells in the red pulp (fig. 3,16). Such cells were also seen occasionally in the Malpighian corpuscles. Some of these cells had the characteristic morphology of plasma cells, others of reticulum cells, and intermediate forms were numerous. These cells had an inconstant topographical relation to amyloid deposits. They were not invariably PAS or alcian blue positive, and there was no clear trend to an increase in the number of PAS positive cells and corresponding decrease in pyroninophilic cells with repeated injections. A large increase in the number of splenic giant cells was noted. They had no constant topographical relation to amyloid deposits and did not stain consistently with PAS, alcian blue or methyl green pyronin.

Liver. Groups of mononuclear cells with an occasional giant cell appeared on the periphery of the vessels and in the hepatic sinuses. The cells were mostly pyroninophilic and some were clearly plasma cells. They had the same staining properties with PAS and meta-

chromatic stains as had similar cells in the spleen. They were not invariably associated with amyloid deposits.

Kidney. Again occasional groups of pyroninophilic cells were seen, usually in relation to large vessels. No constant glomerular changes could be detected and, in particular, it was not clear that there was any proliferation of pyroninophilic cells. Amyloid occurrence was minimal and confined to glomerular tufts.

Other tissues. No amyloid was seen in random selections of tissues. Cutaneous ulceration and abscess formation occurred in some animals, and peritonitis in one of the Freund-type adjuvant group. These changes have been described in Part I, 3.

Autoradiographic results

The data presented here are consolidated from the complete series. Species or sub-group differences and exceptions are indicated where appropriate.

Sulphur-35 sulphate. This compound, incorporated in sulphated mucopolysaccharide, consistently gave definite images over amyloid whenever it occurred. The amyloid in some cases was labelled 4 hours after injection of the isotope. The image was usually diffuse and fairly uniform. There was evidence of utilisation of radio-sulphate by the plasma cell series and particularly by immature plasma cells. Endothelial cells related to the amyloid deposits were also active in sulphated mucopolysaccharide synthesis. Phagocytic cells were not active.

In liver a particularly striking composite image was obtained for perivascular amyloid deposits. The endothelial cells invariably gave an intense ring-image: outside this the image was less intense and diffuse over amyloid and related cells (fig. 3,7): by conventional staining methods most of these cells were pyroninophilic, and some were plasma cells. Occasional cells repeatedly showed rather heavier sulphate-utilisation, but their precise identification then was difficult because the image obscured cellular detail. In some cases it was possible to identify such active cells with reasonable certainty as immature plasma cells. An endothelial image was only given when the first traces of amyloid appeared.

In cases where there was no evidence of perivascular amyloid there was no endothelial radioactivity, but in such cases some of the vascular cuffing cells showed evidence of sulphate-uptake. A diffuse image was usually seen over plasma cells and cells in hepatic vessels. This was not an invariable finding. There was no clear evidence of sulphate-utilisation by Kupffer cells: the groups of parasitised cells in the sinuses were not radioactive, nor were the basophil bodies associated with them. When cirrhotic changes were present the radio-sulphate image corresponded closely with the pattern of fibrous tissue in the liver (fig. 3,11). This correspondence has already been noted in experimental cirrhosis (fig. 1,36).

In kidney the incorporation of radio-sulphate in glomerular

tuft amyloid gave a characteristic image in autoradiographs (fig. 3,21). The image was clearly confined to the glomerular tufts but was diffuse over endothelial cells and amyloid (fig. 3,2). It was not possible to exclude epithelial activity in the tufts, but there was clearly no evidence of epithelial sulphate-utilisation in the lining cells of Bowman's capsule. A sulphate-image was also usually seen over the colloid casts in tubules. This was not apparent at 4 hours after isotope injection but frequently seen at 24 hours and later. In animals with advanced glomerular amyloid there was occasional evidence of radioactivity in capillary endothelium and basement membranes in the renal papilla. In the caseinate group the aggregates of pyroninophilic cells related to the larger vessels showed a diffuse image, with occasional more active cells, as in the hepatic cellular foci. Adrenal amyloid deposits also showed a radio-sulphate image.

In spleen the usual sulphate-image was perifollicular, corresponded with the appearance of amyloid in this site, and was diffuse over cells and amyloid (fig. 3,13). On the periphery of such deposits a diffuse lighter image was apparent, related to groups of pyroninophilic cells some of which showed a punctate heavier image. As in liver it was possible in favourable cases to identify such more active cells with reasonable certainty as immature plasma cells. In some animals with splenic congestion there was a perifollicular grouping of dilated vascular spaces, and under these circumstances endothelial cell activity in relation to the amyloid deposits was

demonstrable (fig. 3,15). When amyloid deposits were minimal the sulphate-image was confined to the red pulp and related diffusely to endothelial cells and pyroninophilic cells in this site (fig. 3,17). In the caseinate and Freund-type adjuvant groups the splenic giant cells consistently showed sulphate-utilisation, whether amyloid was present or not, and these cells had no definite topographical relation to amyloid. The parasitised groups of phagocytes in the hamster spleens consistently showed no evidence of radio-sulphate metabolism.

Sulphur-35 methionine. This amino acid had a diffuse distribution in the tissues examined but was concentrated in amyloid. There was evidence of utilisation by the plasma cell series, by parasitised phagocytes, but not significantly by endothelial cells.

In kidney the amyloid was in glomerular tufts as before and the methionine-image was more sharply confined to the amyloid than the sulphate-image. This was particularly obvious in those glomeruli with nodules of amyloid. In such cases, and also in the more diffuse type of glomerular amyloidosis, the endothelial cells were not selectively active (fig. 3,4). The image was not diffuse over cells and amyloid as it was with radio-sulphate. Radio-methionine was also present diffusely in colloid casts and in a recent thrombus in renal vein in one animal. A heavy image was seen over convoluted tubular epithelium in experimental and normal control animals. There was no evidence of radioactivity in

capillary endothelium in the renal papilla.

In liver the most striking negative finding was the absence of the endothelial ring-image already described as characteristic of radio-sulphate distribution in hepatic amyloid. Although there was evidence of incorporation of labelled methionine in perivascular amyloid, this image was not sharply delineated by a more intense image over endothelial cells (fig. 3,22). The radio-methionine image was uniformly diffuse over parenchymatous cells, but there was evidence of rather greater uptake by some of the pyroninophilic cells related to vessels, and again it was possible to identify such cells with reasonable certainty as immature plasma cells. The difference in image intensity between such cells and others in the focus was not as marked as with radio-sulphate because of the less specific utilisation of radio-methionine. The clumps of phagocytes containing L. donovani were also more active with respect to methionine uptake than hepatic parenchyma cells.

In spleen there was again evidence of concentration of radio-methionine in amyloid but in contrast to the radio-sulphate pattern there was no suggestion of an endothelial ring-image (figs. 3,23-3,24). The general cellular image in red and white pulp was diffuse and fairly uniform, but it was apparent that there was a rather greater image intensity over immature plasma cells in the pyroninophilic groups of cells and also over the discrete clumps of parasitised phagocytes in the red pulp (fig. 3,19).

Carbon-14 labelled glycine. The findings were similar to those for radio-methionine. The amino acid was incorporated in amyloid deposits, and there was evidence of utilisation by the plasma cell series and by parasitised phagocytes. There was no evidence in liver of the ring-image indicative of active endothelial uptake.

DISCUSSION

The plasma cell series utilised both radio-sulphate and the labelled amino acids. The synthesis of a glycoprotein may be postulated. The polysaccharide moiety of this glycoprotein is a sulphated mucopolysaccharide. Vascular endothelial cells utilised radio-sulphate but not the labelled amino acids. Endothelial synthesis of a sulphated mucopolysaccharide may be postulated. Amyloid deposits contained both radio-sulphate and the labelled amino acids. Amyloid may be regarded as a glycoprotein - sulphated mucopolysaccharide complex.

Methods of inducing amyloidosis

Amyloid occurring as a complication of leishmaniasis in the hamster is in many ways a better experimental model of human secondary amyloidosis than that given by repeated injections of foreign protein in animals. The cause of death in the infected hamsters is advanced renal amyloidosis and it is of interest that there is occasional renal vein thrombosis. In human kala-azar however, although there is a characteristic alteration in the plasma

albumin/globulin ratio, and generalised oedema is described, there is no report of amyloidosis (Gellhorn et al, 1946). Occasional cirrhotic changes in the hamsters provided radio-sulphate distribution patterns which were very similar to those obtained in experimental cirrhosis in mice (fig. 1,36). This supports the view discussed above (p. 42) that the cirrhotic lesion must involve the activity of connective tissue cells, and cannot result entirely from passive condensation of pre-formed fibres (Popper, 1954; Popper and Elias, 1955). Caseinate and Freund-type adjuvant injections have not given a very high incidence of amyloid in mice and, although the régime has given useful information concerning the early stages of amyloidosis, it has proved itself time consuming and frustrating when well-established amyloid is required in the laboratory animal. Fruhling et al, (1960) found it necessary to combine both caseinate and Freund-type adjuvant injections to achieve amyloidosis more easily in C3H mice. Hass et al, (1943) reported failure to produce amyloid with 48 injections of sodium caseinate whereas Perāsalo and Latvalahti (1954) found amyloid in 4 out of 10 mice which had received 28 injections.

Pathogenesis

There are two schools of thought on the pathogenesis of amyloid. One is based on the hypothesis stated by Virchow (1860) that a precursor of amyloid in the circulating plasma is transformed into a gel after crossing the vessel walls. The other holds that amyloid is a local cellular secretion of polysaccharide-containing

globulins and is not a precipitation from the blood (Teilum, 1956). A common factor to both hypotheses is the possibility that an immune reaction is involved in the elaboration of the protein-carbohydrate complex.

Neither theory is wholly satisfactory and the present work suggests a consolidating hypothesis integrating both. There is a circulating soluble precursor - a glycoprotein produced by cells of the plasma series as a result of chronic infection by L. donovani or of repeated foreign protein injections. This crosses the capillary walls and becomes the insoluble compound amyloid because the capillary endothelial cells elaborate locally a further polysaccharide fraction which forms a complex with the circulating glycoprotein to give an insoluble precipitate. The endothelial contribution is a sulphated mucopolysaccharide and the stimulus to its production may be increased capillary permeability caused by the circulating globulin-containing complex. The characteristic subendothelial deposition of amyloid requires the movement of protein through or between endothelial cells if the protein is derived from the plasma. The abnormal circulating glycoprotein may or may not be the result of an immune process.

In the following sections the chemistry of amyloid, the related blood chemistry, the current hypotheses, and the present hypothesis will be examined in detail.

Chemistry of amyloid

Table 3,1 summarises some published analyses of human amyloid.

The present work supports the views of Hass (1942), Meyer (1947) and Bassiouni (1955) that amyloid contains a sulphated mucopolysaccharide. The suggestion that this compound is akin to heparin is of interest because, as A.C. Kennedy tells me, to achieve the same anti-coagulant effect, a much larger dose of heparin is required in a patient with amyloidosis than in one without. Calkins et al, (1960) however, consider that the carbohydrate part of amyloid is not sulphated.

The present distribution studies with labelled amino acids give no direct information on the nature of the protein fraction of amyloid, but are consistent with the evidence that it is mainly globulin (table 3,1).

The protein and carbohydrate fractions of amyloid most probably exist in the tissues as a complex, and the nature of the bond between the fractions is the subject of considerable controversy (Bettelheim-Jevons, 1958). Larsen (1957), however, was successful in obtaining two fractions from amyloid tissues by alcohol fractionation. The larger fraction did not contain glucuronic acid, was not metachromatic, but was PAS positive. It migrated as alpha 2-beta globulin on paper electrophoresis. Larsen interpreted this finding as supporting the theory of Teilum (1956) that PAS positive glycoproteins, secreted locally by cells of the reticulo-endothelial system, play a major part in the pathogenesis of amyloid. It is obviously also quite consistent with the view that the fraction is derived from the plasma although originally synthesised by reticulo-

endothelial cells. Larsen commented that the fraction had the properties of a serum glycoprotein. The smaller fraction was described as probably of ground-substance origin and was metachromatic. On electrophoresis it showed a diffuse pattern around the starting line. Larsen stated that it had been prepared by a procedure known to yield acid mucopolysaccharide and dismissed it as less important than the larger glycoprotein fraction. He concluded that amyloid is a mixture of different glycoproteins, and that the larger fraction is not of ground-substance origin but similar to serum glycoprotein. The smaller fraction might well be the sulphated mucopolysaccharide described in the present work as of endothelial origin. In a histochemical investigation of the properties of amyloid Braunstein and Buerger (1959) described three components namely protein, carbohydrate, and acid mucopolysaccharide. This again would fit the hypothesis of a circulating glycoprotein precipitated by the addition of an acid mucopolysaccharide of endothelial origin. They pointed out, and I have confirmed, that metachromasia with toluidine blue is revealed after peptic digestion, and that therefore the anionic dye binding sites of acidic mucopolysaccharide are probably blocked by combination with glycoprotein. The autoradiographic findings however are unequivocal in the demonstration of a sulphated mucopolysaccharide.

Plasma proteins

Changes in plasma proteins in human and experimental amyloidosis are set out in table 3,2. The results quoted are fairly representative

of a general survey of the literature.

In hamsters infected with L. donovani Gellhorn et al, (1946) explained the altered albumin/globulin ratio by a decrease in albumin caused by the proteinuria accompanying amyloidosis. Plasma globulin was increased before amyloid deposition, and decreased with amyloidosis, but the authors claimed no significance for the data. Ada and Fulton (1948) reported an increase in alpha-globulin 7-9 weeks after infection and a decrease in albumin. This is the time of onset of amyloidosis.

Caseinate-induced amyloidosis is also associated with a hyperglobulinaemia in a variety of animals. Perasalo and Latvalahti (1954) noted a decrease in the globulin level as amyloid formed. The decrease was mainly in gamma-globulin although the other fractions were involved in the original increase. Teilum (1956) considered that amyloid formation generally is associated with changes in the electrophoretic pattern of the plasma proteins, and related the elevated level of gamma-globulin to the active phase of proliferation of pyroninophilic cells of the reticulo-endothelial system. Caseinate was again used by Giles and Calkins (1958) in experimental amyloidosis in rabbits. They found that hyperglobulinaemia was a frequent antecedent of amyloidosis, but that in all rabbits that developed amyloid the hexosamine level was increased in the serum and in the amyloid tissues. They concluded that the plasma hexosamine was probably protein-bound, was mainly glucosamine, but was probably not in the

form of an acidic mucopolysaccharide. These findings support the present hypothesis of a circulating glycoprotein synthesised by cells of the plasma series. A point of divergence is in the present evidence that the polysaccharide is sulphated, but the implications are the same - that the circulating hexosamine is directly related to the hexosamine content of amyloid.

In human amyloid disease Wagner (1955) described combined plasma and amyloid protein studies which suggested a relationship between a circulating abnormal protein-polysaccharide complex and the composition of amyloid. In a case of amyloidosis associated with the nephrotic syndrome the plasma showed decreased albumin and elevated alpha 2, beta 1 and beta 2 globulins. Parallel with these findings the acid mucopolysaccharide fractions were elevated. Analysis of amyloid in this case showed alpha 2 and beta-globulins and mucopolysaccharide in the same zones. Faber (1948) pointed out that in suppurative infections and in secondary amyloidosis the glucosamine level was increased in plasma and in amyloid tissues. Further evidence was presented by Gilliland et al, (1954) who indicated that in health the maximum protein-bound polysaccharide concentration occurs in the alpha 2 fraction of serum globulin, and that in amyloid disease, and in the Kimmelstiel-Wilson syndrome, there is a significant increase in the alpha 2 and beta fractions.

Amyloidosis as a feature of rheumatoid arthritis is of current interest. Missen and Taylor (1956) commented that the plasma globulins in rheumatoid arthritis commonly show an absolute increase,

with and without amyloid. They quoted Gilliland and Stanton (1954) who showed that in rheumatoid arthritis and in untreated tuberculosis there is an increase in plasma protein-bound polysaccharide. In the diagnosis of amyloidosis a low albumin/alpha 2 globulin ratio was found to be of value. Missen and Taylor concluded that, if a high concentration of plasma globulin and its associated polysaccharide is a prerequisite for the development of secondary amyloidosis, then it is evidently satisfied in most cases of active rheumatoid arthritis. In a review paper Symmers (1956) noted that hyperglobulinaemia had been found least regularly in cases of secondary amyloidosis but suggested, from experimental evidence, the possibility of at least a transitory hyperglobulinaemia in human amyloidosis generally. He also made the interesting comment that in those cases of myelomatosis in which amyloidosis had developed, hyperglobulinaemia was usually absent, and quoted Eisen (1946) as having observed a fall in the degree of hyperglobulinaemia in myelomatosis as amyloid developed.

Symmers stated that primary amyloidosis is also not uncommonly associated with hyperglobulinaemia. Block et al, (1955) showed an atypical electrophoretic peak in the serum of patients with familial primary systemic amyloidosis, and Mulligan (1958), in a review of cardiac amyloidosis made out a case for amyloid being secondary to a disturbance in blood proteins in which hyperglobulinaemia is paramount. He pointed out that cardiac amyloid was commoner in men and could be related to senility, malnutrition, a decrease in

plasma albumin, an increase in plasma globulin, and, in men, a decrease in blood ascorbic acid.

There is then evidence that in both experimental and naturally occurring amyloidosis the level of plasma globulin is increased before the deposition of amyloid, and may decrease with deposition. The fraction of globulin involved varies, but this is consistent with the view that as far as permeability-increasing properties go there is also a significant species variation in the globulin fraction involved, (Spector, 1959).

Theory of local secretion

Teilum (1954, 1956) has presented evidence that amyloidosis is the result of a perversion of the protein-synthesising function of the reticulo-endothelial system caused by a breakdown of control by ascorbic acid and adrenal steroids. He has described a chain of events following prolonged antigenic stimulation. Initially there is proliferation of reticulo-endothelial cells including plasma cells, reticulum cells, Kupffer cells and vascular endothelial and adventitial cells. Such cells are pyroninophilic. The pyroninophilia is maintained by ascorbic acid and depressed by cortisone. Later, just before and accompanying amyloid deposition, there is a depression of pyroninophilia and an increase in the number of reticulo-endothelial cells containing PAS positive material. Such cells are linked directly with the local synthesis of amyloid and related substances which are considered to be polysaccharide-containing globulins.

This is an attractive hypothesis but it is open to criticism on several points. The present work shows that the endothelial cell is topographically most closely and constantly related to amyloid deposits. Such endothelial cells show evidence of synthesis of sulphated mucopolysaccharide, but evidence of active protein synthesis is lacking. Christensen (1960) supported Teilum's views and made the point that metachromatic material is added to glycoprotein in amyloid formation. He suggested that the metachromatic material comes from reticulum cells, which react in a fibroblastic manner to continued stimulation. The present evidence for the origin of this metachromatic material in endothelial cells is perhaps stronger. Reticulum cells, Kupffer cells, immature and mature plasma cells, and vascular adventitial cells have an inconstant topographical relationship to amyloid deposits. It is confirmed that cells of the plasma series produce glycoprotein, but local deposition at the site of production would not explain amyloid in renal glomeruli. The Kupffer cells of the liver are not related topographically or functionally to amyloid deposition. Moreover there is evidence that globulin synthesis is not a function of cells with phagocytic properties (Roberts, 1955, Marshall, 1956). There is no indication of a proliferation and transition of Kupffer cells generally to immature or mature plasma cells. The earliest hepatic amyloid is perivascular and is as often as not unrelated to groups of pyroninophilic plasma cells. In kidney there is no evidence of transition of endothelial cells to immature or mature plasma cells

and there is strong evidence that these are predominantly the cells responsible for gamma-globulin synthesis (Fagraeus, 1948; Oakley, 1959). Similarly, in adrenal, there is no evidence of proliferation at any time before or during amyloid deposition of cells of the plasma series.

Undoubtedly in splenic red pulp there is a proliferation of pyroninophilic cells but this is just as consistent with a release of globulin into the circulation as with a mechanism of local formation of amyloid. Pirani et al, (1959) have shown that casein-induced amyloidosis in mice may be prevented by splenectomy and that there is a significant depression of total plasma protein including alpha 2 globulin; but Calkins et al, (1960) stated that amyloidosis in rabbits is not inhibited by splenectomy. Cohen et al, (1959) reported that in experimental amyloidosis pyroninophilia bore no clear cut chronological or quantitative relation to the amyloid; PAS-positive cells were occasionally observed but were not necessarily related to the evolution of the disease. In the present work also there is no evidence of a progression from pyroninophilic to PAS-positive cells as the lesions developed, and I have been unable to confirm Teilum's findings that amyloid deposition is potentiated by corticotrophin, cortisone and nitrogen mustard. Calkins et al, (1960) also found that amyloidosis in rabbits is not accentuated by cortisone. The effect of ascorbic acid has not been investigated in the present work, but deficiency of the compound is associated with an altered pattern of muco-

polysaccharide production (Friberg, 1958; Williams, 1959) and with an altered plasma globulin pattern (Banerjee and Rohatgi, 1958).

Circulating precursor theory

This is probably the most widely held view and the parallelism between plasma protein findings and the chemistry of amyloid is convincing, as is the decrease in plasma globulin noted as amyloid deposition occurs. The major difficulty is to explain the mechanism of deposition of a soluble intravascular precursor as an insoluble compound outside the vessels. Bywaters and Glynn (1957) make this point in their review and suggest some significant change, possibly a polymerisation at the site of deposition. I suggest that the significant change is the formation of an insoluble complex of the soluble precursor with a sulphated mucopolysaccharide of endothelial origin.

Immune reactions have been thought to play a part in amyloidosis. Letterer (1934) suggested that amyloid is the result of an antigen-antibody precipitation. Hass et al, (1943) considered that persistent or repeated stimulation of immune mechanisms is a fundamental factor in the genesis of amyloid disease. Vazquez and Dixon (1956) described gamma-globulin as a major component of amyloid and discussed the implications. They reduced the possible mechanisms to a local non-immunological accumulation of circulating globulin, or an immune reaction which might be combination of circulating antibody with the tissue antigen, or antigen-antibody reaction occurring at the site of antibody

formation. There is no evidence that endothelial cells are concerned with antibody formation. The other possibilities remain, and may involve a permeability factor. It is of interest that the parasitised phagocytes in hamster spleen and liver showed a quite marked amino acid uptake (fig. 3,19). This lends some support to the sequence of events in antibody production described by Marshall (1956) and adapted from Burnet and Fenner (1949). The presence of antigen in a phagocytic cell stimulates production of protein which is not antibody but may be transferred to primitive reticular cells. Proliferation of the plasma cell series follows, with glycoprotein synthesis.

The present theory

If it is accepted that a globulin-carbohydrate fraction of amyloid is derived from the plasma then it is obviously a truism that capillary endothelial permeability to protein must be increased to permit the circulating protein-polysaccharide complex to assume its characteristic subendothelial distribution. As a result of increased permeability endothelial cells are stimulated to produce a sulphated mucopolysaccharide. This might be regarded as a stop-gap reaction. Amyloid is then deposited because the acidic mucopolysaccharide forms an insoluble complex with the glycoprotein. However the stop-gap reaction fails because the amyloid deposits involve the basement membrane and proteinuria results. As a consequence of this chain of events there is a tendency in

established amyloidosis to a decrease in plasma albumin. The plasma globulin level may or may not be affected depending on the balance between globulin production and globulin loss to amyloid. The over-all tendency will be to a decrease in globulin, because of the continued incorporation of the protein in amyloid and also because amyloid deposits in spleen and liver will reduce the functional capacity of these organs as sources of globulin. The plasma albumin/globulin ratio may thus vary within wide limits.

The evidence for a circulating precursor of amyloid has been surveyed. The present work shows that cells of the plasma series, mainly in spleen but also in lymph nodes, are most probably the source of the glycoprotein. It has been shown that the carbohydrate moiety is sulphated. It has not been established that the protein is globulin, but there is strong circumstantial evidence that this is so.

The mechanism of increased capillary permeability is unknown. Spector (1958) has reviewed the subject and it is apparent that globulin fractions may play an important part. It is just possible that the globulin-carbohydrate complex of amyloid is itself responsible for the increase in capillary permeability. Spector quotes Miles and Wilhelm (1955) who pointed out that there is a considerable species variation in the globulin fraction that possesses permeability-increasing powers. More recently Davies and Lowe (1960) have found that gamma-globulin isolated from guinea-pig serum by diethylaminoethyl cellulose chromatography has a high permeability increasing

effect. The alpha 2 fraction also caused increased permeability. They pointed out that permeability factors may be induced by antigen-antibody complexes under certain conditions, for example when solubilised by excess antigen, and referred to the work of Ishizaka and Campbell (1958) and others. It is tempting to speculate that the variation in the protein fractions of amyloid is a function of the variation in globulin fractions that are involved in the permeability-increasing mechanism.

The present work focuses attention on the endothelial cell as a major factor in experimental amyloidosis. Curran (1957) has claimed the elaboration of mucopolysaccharides by normal vascular endothelium, and suggested that the sulphated compounds may be concerned with regulating the passage of materials from the blood-stream to the neighbouring tissues. In the present work there is no clear evidence that normal endothelium utilises radio-sulphate, and it has been pointed out by Stehbens (1962) that it is not yet certain that vascular endothelium is one of the tissues with a marked capacity to concentrate sulphur-35 given as sulphate. The deposition of amyloid involves the conversion of a soluble haematogenous precursor to insoluble form in the subendothelial site. It is of interest in this connection that Curtain (1955), in an investigation of the nature of the protein in the hyaluronic complex of synovial fluid, found that at ionic strength of 0.1 hyaluronic acid bound globulins rather than albumin over a wide pH range. The endothelial secretion of a sulphated mucopolysaccharide

may achieve a precipitating complex with a globulin in such a fashion in amyloidosis.

Electron-microscope studies lend considerable support to the view that the endothelial cell is intimately related to amyloid deposition. Cohen et al, (1960) examined caseinate-induced amyloid in rabbit spleen. They found that amyloid was most often in contact with endothelial cells in place of, or in addition to, basement membrane. Where amyloid was scanty it was consistently separated from the sinus lumen by endothelial cytoplasm. They concluded that amyloid, on the evidence to date, is deposited in the area of the basement membrane, but, at least in part, is a distinct structural entity. In rabbit kidney with caseinate-induced amyloid Cohen and Calkins (1960) found that the amyloid first appeared as small amounts of fibrillar material subjacent to the endothelial cell cytoplasm. Initially the basement membrane was not altered but later amyloid deposits appeared to merge with it. There was never any great amount of amyloid on the epithelial side of the basement membrane and endothelial cells invariably separated the amyloid from the capillary lumen. Before amyloid deposition they described the appearance of blebs in the endothelial cells. Fruhling et al, (1960) also described the fibrillar structure of caseinate-induced amyloid in mouse kidney and suggested a resemblance to long polymerised chains of glycoprotein.

In the electron microscopy of human amyloidosis Farquhar et al,

(1959) pointed out that the renal lesions were similar to experimentally induced lesions in mice and consisted of an irregular focal thickening of the basement membrane. Spiro (1959) examined renal biopsy specimens from patients with amyloidosis and found the earliest glomerular deposits beneath the endothelium. In more extensive lesions the basement membrane was focally involved. He occasionally observed in early lesions cellular organelles embedded in a matrix of amyloid suggesting that the material was synthesised by the endothelial cells.

SUMMARY

Leishmaniasis in the golden hamster yields a good experimental model of secondary amyloidosis.

Autoradiographic distribution studies with radio-sulphate and labelled amino acids have suggested a hypothesis for the pathogenesis of amyloid.

A glycoprotein, the carbohydrate part of which is sulphated, is formed by proliferating cells of the plasma cell series mainly in the red pulp of spleen. This glycoprotein circulates and is a soluble precursor of amyloid. Endothelial cells form another sulphated mucopolysaccharide which gives an insoluble complex with the glycoprotein. This complex is amyloid. Amyloid has a characteristic subendothelial distribution and therefore there is increased capillary endothelial permeability for which the glycoprotein may be responsible. The endothelial production of sulphated mucopolysaccharide may then be considered as reactive.

P A R T IV

1. The Distribution of Organically Bound Iodine in Goitre

1. THE DISTRIBUTION OF ORGANICALLY BOUND IODINE IN GOITRE

When iodine-131 is given to a patient before thyroidectomy it is metabolised as ordinary iodine and takes part in the synthesis of thyroid hormone. Autoradiographs prepared from sections of the resected gland show the distribution pattern of organically bound iodine-131. Inorganic iodine is washed out in the usual histological processing of the tissue.

This study was undertaken for several reasons. Firstly, in the problem of goitre in childhood it was thought that it would add useful information to chromatographic studies carried out concurrently by McGirr and his colleagues, and I have contributed autoradiographic data to 2 reports on dyshormonogenetic goitre which have been published (McGirr et al, 1959, 1960). Secondly, in cases of thyroid carcinoma, the question of therapeutic use of iodine-131 required knowledge of the utilisation of the isotope by the tumour. Thirdly, it was hoped that if a distinctive distribution pattern was observed it might help in diagnosis. And lastly, the pattern of distribution might give some information on the site of thyroid hormone synthesis.

MATERIALS AND METHODS

Iodine-131 was given orally by clinical colleagues to patients usually 24 hours before thyroidectomy. The range was 30 minutes to 10 days. The dose range varied from 100 μc. to 1 mc. and was usually 100-200 μc. The series of surgically resected glands comprises 159 cases of goitre classified by disease in table

4,1. The resected tissues were fixed in 4 per cent neutral or alcoholic formaldehyde, embedded in paraffin and cut at 5-6 μ. Autoradiographs were prepared by the stripping film method with Kodak AR10, AR50, and V1042 plates. Ilford G5 nuclear emulsion as gel was also used. Sections adjacent to those used for autoradiography were stained variously with haematoxylin and eosin, periodic acid-Schiff, Weigert's elastic tissue stain and van Gieson.

RESULTS

Thyroid Adenoma (30 Cases)

The classification used in tables 4,2-4,3 is that of Warren and Meissner (1953). Type classification is often difficult in tumours showing more than one pattern of differentiation, and in such cases the dominant pattern has determined the category chosen. In 3 cases multiple nodules were considered to be examples of adenomata rather than non-toxic nodular goitres. The decision was again made on the criteria suggested by Warren and Meissner. Particular note was taken of uniformity of structure in the tumours, and the difference in growth pattern between the tumours and the adjacent gland, but it is acknowledged that there is a considerable overlap with appearances seen in non-toxic nodular goitre.

The radioactivity in the adenoma group was predominantly in colloid within follicles. In 3 cases extra-follicular labelled colloid was demonstrable. This was present in the extracapsular

compressed tissue and associated with a lymphocytic infiltrate in 2 cases. In the third case, where no cellular reaction was demonstrable, it is not possible to exclude operative trauma. Focal thyroiditis of Hashimoto type (Lindsay et al, 1952), was not present in those cases, although it was observed in 3 other cases, one of which was an example of multiple tumours including a Hürthle (Askanazy)-cell adenoma of mainly solid type. The distribution-pattern of radioactivity in the focal thyroiditis of Hashimoto type resembled that in diffuse Hashimoto's disease which is described below. In particular, it was apparent that small follicles lined by Askanazy-type epithelium often contained intensely radioactive colloid. Less specific degrees of chronic inflammatory change were seen frequently, most often in the form of lymphocytic infiltration with or without lymphoid follicles.

The autoradiographic image intensities in the tumours shown in table 4,3 are related to image intensities observed in normal thyroid tissue resected with the tumours, and the comparison was made in autoradiographs showing both neoplastic and normal tissue in the same preparation. The microfollicular group was the most active (figs. 4,1-4,2). One case was clinically toxic. The simple follicular group contained rather fewer active tumours (figs. 4,3-4,4). In the macrofollicular group the activity where present was less intense than in normal thyroid tissue, and the trabecular group contained no active tumours at all (figs. 4,5-4,6). The solitary papillary tumour showed only occasional

activity in microfollicular areas. In the multiple tumour glands the activity-pattern followed the structural correlation given above for solitary tumours. In the single case (table 4,3), showing activity greater than normal the neoplasms concerned were microfollicular. Among the inactive multiple tumours was a mainly solid Hürthle (Askanazy)-cell adenoma. There was generally no strict correlation between the usual histological appearances and the autoradiographic appearances of functional activity.

Thyroid Carcinoma (24 Cases)

Details of the cases and classification by type (Warren and Meissner, 1953) are shown in tables 4,4-4,5. Again, as in the adenoma series, there was often considerable difficulty in deciding on histological classification. In the differentiated group of tumours mixed patterns were often seen and it was apparent that a decision on dominant pattern could be a function of the number of blocks of tissue examined.

Only 3 tumours showed evidence of iodine-131 utilisation. They were all in the differentiated group and the radio-iodine was detected in colloid in follicular areas. Papillary and solid areas were inactive in this respect. This was particularly evident in the single case in the series in which it was possible to examine primary and secondary growth. Here only the follicular areas of primary and secondary tumour showed bound iodine-131 (figs. 4,7-4,10). Other follicular tumours, apparently as well differentiated

as those showing bound iodine-131, did not utilise the isotope (figs. 4,11-4,12). The single case of a Hürthle (Askanazy)-cell carcinoma was of predominantly solid type and showed no evidence of radioactivity. An inactive papillary carcinoma is shown in figs. 4,13-4,14. Extrafollicular labelled colloid was seen in thyroid tissue outwith the neoplastic area in 3 cases. In one of these there was clear evidence of crushing. In the other 2 trauma was not evident. Hashimoto's thyroiditis was present in one, and a focal lymphocytic reaction in the other, without Askanazy-change in epithelium. All the undifferentiated tumours failed to utilise iodine-131 (figs. 4,15-4,16).

As has just been indicated Hashimoto's thyroiditis was a concomitant lesion in a thyroid gland showing an undifferentiated giant-cell carcinoma (figs. 4,17-4,19). The diffuse nature of the thyroiditis was clearly demonstrable in the lobe unaffected by the tumour, and the pattern of iodine-131 utilisation was identical with that described below in uncomplicated Hashimoto's thyroiditis. Another case of a small cell carcinoma associated with Hashimoto's thyroiditis was observed. Again the diffuse nature of the thyroiditis was evident in parts of the gland unaffected by the tumour. Focal thyroiditis of Hashimoto type (Lindsay et al, 1952) was seen in 3 cases of undifferentiated carcinoma (figs. 4,20-4,21). A less specific degree of focal thyroiditis was common in the form of lymphocytic infiltration with or without lymphoid follicles.

Other conditions observed associated with thyroid carcinoma were nodular goitre in one case, adenoma in 3 cases, and a defect in thyroid hormone synthesis in one case. There was no evidence of transition from follicular adenoma to carcinoma in 2 cases where the tumours coexisted. The benign tumours appeared as discrete nodules uninvolved in the malignant process. In the third case the adenoma which showed a mixed papillary and follicular structure also showed evidence of either invasion by, or transition to undifferentiated giant cell carcinoma. The case of a defect in thyroid hormone synthesis with malignant change is described below in the section on dyshormonogenetic goitre.

Non-Toxic Nodular Goitre (31 Cases)

Details of the cases are given in table 4,6. In most it was comparatively easy to decide on the diagnosis of nodular hyperplasia rather than multiple adenomata taking the differential points suggested by Warren and Meissner (1953) but admitting the possibility of multiple benign tumours. However, for any given nodule a differentiation between hyperplasia and neoplasia could be extremely difficult, if indeed possible. Most nodules in the group showed a rather similar pattern of peripheral microfollicles, intermediate medium sized follicles and central macrofollicles. There was often evidence of central degeneration or haemorrhage. Encapsulation was quite commonly a feature, and there was fairly often evidence of expansile growth in compression of extracapsular follicles. In 5 cases there was a papillary element in the

nodular hyperplasia. These formed a group of average age 36 years which was less than the 48 years average age of the whole group. In one case of recurrent nodular goitre there was a greater nodule to nodule variation in structure than was usually seen in the group. The internodular tissue generally showed a pattern of normal sized follicles with only occasional small foci of microfollicular hyperplasia.

A fairly consistent distribution-pattern of iodine-131 emerged from the group. The radio-iodine image was most intense in the colloid of microfollicles whether these occurred in the nodules or in the internodular tissue. There was quite frequently a peripheral rim of radioactive microfollicles in a nodule, and a further extranodular rim of compressed radioactive microfollicles. The medium sized follicles showed a lower level of colloid activity, and the macrofollicles were usually either inert or showed just detectable radioactivity (figs. 4,22-4,23). This was the general picture, but occasionally nodules clearly much more or much less active than the internodular tissue were seen (figs. 4,24-4,26). Such active nodules did not usually differ much in structure from the typical nodule described above although in 2 instances a microfollicular-papillary pattern was evident (figs. 4,27-4,28). Inactive nodules were usually macrofollicular. In all 12 active nodules and 3 inactive nodules were seen in the group.

In the internodular tissue there was generally a low level colloid-image in the medium sized follicles. The occasional focal

areas of microfollicular hyperplasia showed greater colloid radio-activity.

Iodine-131 was given 10 days before thyroidectomy in one case and after operation there was no evidence of its presence in colloid in the gland. In all the other cases the dose of radio-iodine was given 1-2 days before operation.

Concomitant lesions observed in the non-toxic nodular goitre group were focal thyroiditis of Hashimoto type in 2 cases, lympho-cytic infiltration with lymphoid follicles in 7 cases, and a discrete microfollicular adenoma in one case. The adenoma satisfied all the differential points of Warren and Meissner (1953) except, of course, its existence as a solitary nodule.

Toxic Nodular Goitre (5 Cases)

On the usual histological criteria only one case showed evidence of hyperfunction (table 4,7) but thiouracil and Lugol's iodine had been administered pre-operatively in all. Compared with the non-toxic group above the nodular pattern was very similar. In the internodular tissue in one case there was considerably more focal microfollicular hyperplasia.

The distribution-pattern of bound iodine-131 differed from that in the non-toxic group in only the one case which showed marked internodular focal microfollicular hyperplasia. Here the maximum image intensities and apparently the greatest amount of organically bound iodine-131 were in the colloid in the small follicles in the internodular tissue (fig. 4,29). In the other cases the distribution

of radio-iodine was much as in the non-toxic group. There was no evidence that hyperactive nodules dominated the picture. In fact no such nodules were demonstrable at all, and in one case a clearly inactive macrofollicular nodule was seen. Iodine-131 was given only 30 minutes before operation in one instance and was demonstrable in the colloid in the resected gland, but not in thyroid epithelium.

No focal thyroiditis of Hashimoto type was seen. One gland showed areas of lymphocytic infiltration with lymphoid follicles.

Diffuse Colloid Goitre (3 Cases)

The cases are listed in table 4,8. The histological picture was one of monotonous uniformity with medium sized to large follicles containing abundant colloid. Areas of focal hyperplasia were not seen. There was no evidence of focal thyroiditis in the group.

The iodine-131 images were generally of very low intensity and were present diffusely over all colloid. The intensity appeared slightly greater over the colloid in the small follicles, and occasionally an isolated random active follicle stood out from the surrounding almost inert structures (fig. 4,30). There was no evidence of increased lobular activity in many autoradiographs examined. Again, as for the histology, the picture was one of monotonous uniformity.

Thyroid Cyst (10 Cases)

As shown in table 4,9 the group is made up of 9 degenerative

cysts and a solitary case of thyroglossal cyst. In most of the degenerative cases it was not possible to decide on the exact pathogenesis of the lesion although an adenoma seemed a probable precursor in one. In 3 others the cyst appeared to be the dominant lesion in non-toxic nodular goitre, since a nodular pattern was apparent in thyroid tissue adherent to the cyst wall (fig. 4,31). Generally examination of the peripheral thyroid tissue resected with the cyst was not informative because of its limited mass. In the case of the thyroglossal cyst in a male aged 17 years it is probable that the portion of thyroid gland removed with the cyst is as near normal as obtained in the whole series.

The autoradiographs mainly showed utilisation of iodine-131 by thyroid tissue attached externally to the cyst walls. In the normal tissue resected with the thyroglossal cyst all colloid present was labelled, and the image was most intense in the smaller follicles (fig. 4,32). It is probable that this represents the physiological distribution pattern. One cyst contained remanants of functional thyroid tissue and the morphology and distribution-pattern suggested a cystic follicular adenoma.

Dyshormonogenetic Goitre (20 Cases)

The case details are set out in table 4,10. Four types of defect in hormone synthesis, whether in sporadic goitrous cretinism or in sporadic goitre without marked hypothyroidism are represented (McGirr, 1960a, b). The nature of the defect in any given case was determined by McGirr and his colleagues by testing the effect of anion

block on the iodine-131 content of the thyroid gland, by study of dehalogenase activity, by chromatography of serum, urine and thyroid tissue, and by electrophoresis of thyroid proteins (McGirr, 1960b). I prepared autoradiographs from sections of thyroid in every case and assessed the distribution-patterns with knowledge of the chromatographic and other findings.

The goitres were diffuse (3 cases) or nodular (17 cases). In those showing a diffuse hyperplasia there was usually a marked degree of cellular pleomorphism at the trabecular to microfollicular stage of differentiation (fig. 4,33). Colloid was very scanty in 2 cases with formation of an abnormal iodoprotein. In the single example of a coupling defect colloid was abundant and pleomorphism was not obvious. In this case the follicles were mainly of average size with areas of microfollicular hyperplasia.

In the nodular group there were 6 cases with formation of an abnormal iodoprotein, 5 cases with impaired ability to utilise trapped iodide, and 4 cases with impaired dehalogenase activity. In 2 cases of nodular goitre the nature of the defect was not fully established. In the nodular group as a whole there was a similarity in histological structure. The nodules varied in size from microscopic to about 6 cm. in diameter. A wide variety of nodular structure was seen including trabecular, microfollicular, simple follicular, papillary, macrofollicular, haemorrhagic and cystic types. Occasional bizarre nodules, not seen in any other condition studied, were observed. They showed a lung-like structure

of poorly formed follicles lined by flat cells and containing no colloid. There was frequently quite marked variation in intranodular structure. Some nodules were encapsulated, others not. Some nodules showed evidence of expansile growth, others did not. Cellular pleomorphism was seen in occasional trabecular nodules. There was usually very little colloid. Some cases, particularly those with the largest goitres, showed the whole range of nodular structure. In others trabecular and micro-follicular nodules were dominant.

The internodular tissue was pleomorphic in 7 of the nodular goitres. This appearance was most striking in the cases with impaired dehalogenase activity (fig. 4,33). The pattern of hyperplasia was similar to that in the diffuse goitres but more marked. Where pleomorphism was not a feature there was often a papillary type of internodular proliferation with medium size and small follicles and in some cases both pleomorphism and papillary hyperplasia were noted. In addition to the tumour-like pleomorphism epithelial cells were quite frequently seen in vessels, and in many fields the thyroid capsule or nodular capsules were infiltrated by pleomorphic epithelium (fig. 4,34).

A histological diagnosis of carcinoma was made in one case, a girl aged 11 years with impaired dehalogenase activity, because of the appearances described above and evidence of mural invasion of vessels (fig. 4,35). Epithelial cells were found under the endothelium of large veins and there was invasion of small vascular

channels in the adventitia of a large artery with disruption of the external elastic lamina.

The autoradiographs in the dyshormonogenetic group showed distribution-patterns of iodine-131 which were not seen in any other thyroid disorder studied. Two diffuse goitres with formation of an abnormal iodoprotein showed evidence of epithelial utilisation of iodine-131 and rather more intense images over colloid in occasional microfollicles (fig. 4,36). Colloid generally was scanty in the cases of diffuse hyperplasia, but in the example of a coupling defect it was abundant and uniformly contained small amounts of bound iodine-131. Here there was no epithelial activity.

There were 5 cases in the nodular group with a defect in organic binding of iodide. No image at all was obtained in 2, a just detectable colloid-image in nodular microfollicles in one, in internodular microfollicles in another, and in the last a low level colloid-image was seen in nodules and internodular tissue.

In the remainder of the nodular group, made up of cases of formation of an abnormal iodoprotein and of impaired dehalogenase activity, the distribution-pattern was often strikingly variable from nodule to nodule and even in different parts of the same nodule. This degree of variation was unique to this group of goitres (figs. 4,37-4,42). Images were often most intense over colloid in microfollicles; trabecular nodules were often inert. In one case with formation of an abnormal iodoprotein only 5 out of 12 structurally

similar nodules showed utilisation of iodine-131. The bizarre lung-like nodules were always inactive. Epithelial images were seen in some nodules and were most intense over cells lining medium size follicles containing labelled colloid (fig. 4,43).

The internodular tissue usually showed evidence of epithelial utilisation of iodine-131 with the exception of the cases showing a defect in organic binding. Occasionally in a follicle an epithelial image, a ring-type image, and a more diffuse colloid image were all present (fig. 4,44). However, the more usual picture was one of low level epithelial activity in fields of trabecular to microfollicular hyperplasia with little or no colloid (figs. 4,37-4,38). Epithelial cells and labelled colloid were observed in the lumen of a vessel in a gland showing internodular pleomorphism (figs. 4,45-4,46).

The carcinoma associated with impaired dehalogenase activity was differentiated of mixed follicular-papillary structure. Bound iodine-131 was detected in colloid but the amount was small and the distribution irregular (fig. 4,47).

Graves' Disease (9 Cases)

As shown in table 4,11 two of the cases are of special interest having received therapeutic doses of iodine-131 5 days and 5 weeks before death from other causes. The thyroid glands were obtained at autopsy. The others received the usual small dose of radioiodine 1-2 days before thyroidectomy and histologically presented the well known picture of treated thyrotoxicosis. Many trabecular

and microfollicular areas of hyperplasia were present in one and 2 showed focal thyroiditis of Hashimoto type. This was so marked in one instance that a concomitant Hashimoto's disease was considered.

The gland examined 5 days after 12 mc. iodine-131 was devoid of lymphoid tissue and follicular disruption was obvious. Epithelial Askanazy-change was seen in cells in intact follicles, sometimes involving only part of the epithelial lining. This change was also apparent in isolated clumps of epithelial cells not arranged in follicular form. Nuclear pyknosis was common. Very little colloid was present. There was no evidence of thrombosis or of fibrinoid cuffing of vessels.

Five weeks after a therapeutic dose of iodine-131 the picture was considerably different. Askanazy-change was again seen. Areas of focal hyperplasia, often of microfollicular pattern with little colloid, were a feature and nuclear pleomorphism was quite marked. There were also regenerated lobules of larger follicles with more abundant colloid. Thrombosis was associated with areas of infarction, and there was some evidence of fibrinoid cuffing of vessels.

The distribution-pattern of iodine-131 in the non-irradiated glands was of labelled colloid in every follicle in which colloid was present (fig. 4,48). The image intensity was greatest over the smaller follicles. A point of interest was the variable response to the thiouracil type of drug. In one case a pre-operative period of 3 weeks anti-thyroid medication was enough to block

binding of any detectable amount of iodine-131. In another a period of 4 months on thiouracil led to an autoradiographic picture of abundant labelled colloid. In both cases Lugol's iodine had also been given. In glands which histologically looked active there was frequently a ring-type image in the autoradiographs. Trabecular areas of hyperplasia were usually negative in respect of iodine-131 utilisation. On the other hand radioactivity in colloid could be correlated with Askanazy-change in the follicular cells in the cases showing focal thyroiditis of Hashimoto type. Epithelial papillary structures containing microfollicles often showed a definite colloid-image.

In the irradiated gland examined 5 days after administration of a large dose of iodine-131 the most striking feature was evidence of epithelial radioactivity (fig. 4,49). This was apparent not only in epithelium lining more or less intact follicles but also in epithelial cells lying within the lumen of disrupted follicles. It was also clear that the intensity of the epithelial image was fairly uniformly less than that of the small amount of colloid occasionally seen. It was not clear that Askanazy-type epithelium was more active than epithelium not showing the change, but there was no evidence that the Askanazy-epithelium was inactive. In the gland examined 5 weeks after a therapeutic dose of radio-iodine activity was still detectable but was confined to the colloid wherever it occurred, and some extrafollicular labelled colloid was seen.

Hashimoto's Thyroiditis (27 Cases)

Some details of the group are presented in table 4,12. Two cases with concomitant carcinoma are excluded and described in the carcinoma group. One case, described under Graves' disease, showed such a marked focal thyroiditis of Hashimoto type that an association of Graves' disease and Hashimoto's thyroiditis was considered probable. Fields of squamous metaplasia were seen in one case and an adenoma was a concurrent lesion in 2. A clinical history of preceding thyrotoxicosis was given in one instance.

The autoradiographs of the group gave a consistent distribution-pattern of bound iodine-131. This pattern was lobular. The image was confined to colloid. There was no clear evidence of epithelial radioactivity. The most intense images were seen over colloid in microfollicular areas of regeneration. The follicular epithelium in these areas was cuboidal and showed no evidence of Askanazy-change. Foci of normal size uninvolved follicles were only occasionally seen, but where present showed again an intense colloid-image and again no evidence of Askanazy-change (figs. 4,50-4,53). Fields of degenerating follicles were a feature of one case and here an extrafollicular colloid-image was present on the periphery of, and diffusely in the affected lobules. There was a light plasma cell infiltrate in the region, but no Askanazy-change (figs. 4,54-4,55).

Askanazy-type epithelium occurred in two forms, follicular and non-follicular. The follicles were usually small and contained

colloid. An iodine-131 image was consistently obtained over such colloid (figs. 4,56-4,59) and was somewhat less intense than the image seen in the microfollicular areas of regeneration. When Askanazy-type follicles were the predominant structural units, and this was so in many cases, the total bound iodine seen in the autoradiographs was in the colloid in such follicles. Non-follicular clumps of Askanazy-cells showed no evidence of radio-activity. When an image was present in such areas it was not possible to exclude the presence of a minute amount of colloid.

DISCUSSION

Thyroid Adenoma

There is considerable difficulty in differentiating between hyperplasia and neoplasia in the thyroid. Evans (1956) states that there can be no acceptable histological definition as to the structural constitution of an essentially neoplastic adenoma. Willis (1953) believes that in the thyroid gland structural distinction between adenoma and carcinoma is often impossible.

The distribution-pattern of organically bound iodine-131 does not resolve the difficulty, because it is similar in papillary adenoma and papillary carcinoma, in simple follicular adenoma and in well differentiated follicular carcinoma, in follicular adenoma and in the nodular hyperplasia of non-toxic nodular goitre.

The pattern described is essentially similar to that described by Dobyns and Lennon (1948). In the present series there is a rather greater preponderance of active microfollicular tumours, but

the general conclusion that utilisation of iodine-131 is proportional to degree of differentiation is supported. Fitzgerald (1955) described only a minimal image in micro-follicular adenoma and 2 of the tumours in the present group showed this pattern.

The presence occasionally of labelled extrafollicular colloid in the compressed tissue just outside the capsule is of interest, particularly when associated with a focal cellular reaction. It is not possible however to draw any conclusion about a causal relationship, because in some cases there was clear evidence of tissue crushing. However, a focal thyroiditis of Hashimoto type was a concomitant lesion in 3 glands with adenoma.

The Hürthle (Askanazy)-cell tumour showed no evidence of iodine-131 utilisation, but was of solid type devoid of colloid. This point is discussed further in the section on Hashimoto's thyroiditis below.

Thyroid Carcinoma

From the standpoint of the possible therapeutic use of iodine-131 the distribution-patterns obtained are as disappointingly meagre as in other series. Fitzgerald and Foote (1949) found that undifferentiated and papillary tumours failed to utilise the isotope. They described only rare focal images in Hürthle (Askanazy)-cell tumours, and such a tumour in the present group showed no evidence of radioactivity. The tumours described which did concentrate radio-iodine fell into the follicular or mixed

papillary-follicular categories, and this is true of the present series. It is also true that even in this well differentiated group there is a considerable proportion of tumours which did not show any uptake of iodine-131. A further important factor is that the image in colloid in the functional tumours is of patchy irregular distribution, and the possibility of achieving a therapeutic concentration of radio-iodine is to that extent more unlikely. The conclusion reached by Pochin (1958) that iodine-131 therapy is best in follicular tumours, not of much value in papillary tumours, and unsatisfactory in undifferentiated and Hürthle (Askanazy)-cell types is supported.

The occurrence together in 2 cases of thyroid carcinoma and Hashimoto's thyroiditis make it essential that the limitations of needle biopsy of thyroid be appreciated. It is often to differentiate between neoplasia and thyroiditis that such a biopsy is taken. Focal thyroiditis of Hashimoto type could easily provide another pitfall in small diagnostic biopsies. Dailey et al, (1955) described 35 cases of thyroid carcinoma, usually papillary, associated with Hashimoto's thyroiditis.

The association of carcinoma and dyshormonogenesis is discussed below in the section on dyshormonogenetic goitre.

Non-Toxic Nodular Goitre, Diffuse Colloid Goitre

The dominant pattern of a spectrum of functional activity from a maximum at the microfollicular stage of differentiation to a minimum at the macrofollicular stage of degeneration is in accord

with the scheme of pathogenesis for non-toxic nodular goitre suggested by Taylor (1953). The first stage of diffuse colloid goitre is one of uniform low level colloid radioactivity as seen in the present 3 cases. Next, small foci of microfollicular hyperplasia stand out as more active than the surrounding medium-sized follicles. Nodules develop showing a gradual increase in follicular size, and degenerate by central haemorrhage and necrosis. The distribution of bound iodine-131 is constantly related to colloid, and the image intensity varies inversely with follicular diameter. This is the general pattern. In rather more than a third of the nodular goitres there are nodules considerably more active than the internodular tissue. Fitzgerald (1955) noted that about 35 per cent of hyperplastic nodules showed more activity than the internodular tissue. The distribution-pattern is also consistent with the observation by Pitt-Rivers *et al*, (1957) that there is generally a low concentration of bound iodine-131 in the nodules and in the internodular tissue. They suggested that in non-toxic nodular goitre there is either a high turnover of iodine-131, or faulty or delayed coupling.

Compared with dyshormonogenetic goitre it is apparent that the distribution-pattern in non-toxic nodular goitre differs considerably. It is more uniformly related to structure; it is consistently related to colloid, and not to epithelium; it is generally of lower intensity; and it is more an example of minor variation in pattern of a standard nodule than a bizarre mixture of

structurally and functionally divergent nodules.

Toxic Nodular Goitre

In this small group there was surprisingly little structural or functional difference when compared with the non-toxic group above, but thiouracil had been administered, and in some of the cases the clinical degree of toxicity was slight. In one of the types of toxic nodular goitre the internodular tissue is hyperactive (Puppel et al, 1946), and in the other there is a single hyperactive nodule (Cope et al, 1947). There was no evidence in the present group of solitary hyperactive nodules. Small hyperactive nodules did occur in the non-toxic group, but were not big enough to produce hyperthyroidism. In one toxic case, however, the internodular tissue did appear to be more active than the nodular, but there was no evidence of epithelial activity. The image was confined to colloid, and this was so when the iodine-131 had been given only 30 minutes before thyroidectomy. Wollman and Wodinsky (1955) reported bound radio-iodine in the colloid of mouse thyroid only 11 seconds after intravenous injection of the inorganic isotope.

Thyroid Cyst

The main point of interest here is in the portion of normal thyroid resected with a thyroglossal cyst in a male aged 17 years. It was small but consisted of several lobules, taking a lobule as 20-40 follicles (Taylor, 1960). There was no significant image

variation from lobule to lobule, and within a lobule all the colloid was labelled. The image was most intense over the colloid in the smaller follicles. Fitzgerald et al, (1950) also found that the smaller follicles showed the highest concentration of iodine-131 in portions of thyroid resected with thyroid tumours. They described a marked variation in colloid-activity from follicle to follicle, and particularly a striking variation in uptake of iodine-131 in morphologically similar follicles. This follicle to follicle variation was not a feature of the present case. It is debateable whether the uninvaded portion of a thyroid gland with carcinoma can be regarded as normal. Certainly in all the pathological glands of the present series fields of follicle to follicle variation in image can be found. It is also true, of course, that some doubt must exist about the state of normality of a thyroid gland which is associated with a congenital defect in development, and that a firm base line of distribution-pattern in normal human thyroid is still to be established.

Dyshormonogenetic Goitre

The distribution-patterns of iodine-131 described are distinctive and generally consistent with the established defects in hormone synthesis. The apparent paradox of finding evidence of bound radio-iodine in 3 of the cases with a defect in organic binding is explicable on the hypothesis that the defect is not absolute. This view was sustained by the chromatography by McGirr and his colleagues which also showed some evidence of binding

of iodine-131 in these cases. This was encouraging because it had been thought that comparison between the distribution-patterns and the chromatography-patterns might be subject to error due to sampling different parts of the thyroid. Dobyns and Lennon (1948) had suggested that, because uniformity of distribution of labelled iodinated compounds is seldom seen, random chemical sampling may give misleading results. The variable distribution-patterns obtained in the present group emphasise this cautionary note, and so far as possible, in current work, autoradiography and chromatography are done on as similar tissue as possible. For example, a nodule may be divided for this purpose, or neighbouring portions of internodular tissue taken. Chromatography and autoradiography cannot both be done on the same tissue.

Epithelial activity has been noted as characteristic of the internodular tissue in cases other than those with a coupling or binding defect. For reasons discussed in the next section on Graves' disease it is not considered justifiable to argue from these findings that intracellular iodination of thyroid hormone normally occurs. It is perhaps significant that in the single example of a coupling defect the bound iodine-131 was all in colloid.

Although the autoradiographic distribution-patterns in dyshormonogenetic goitre have not been described before in any detail there are a few references to the characteristic histology. Milles (1955) regarded the histology in sporadic goitrous cretinism

as diagnostic. He described, in 2 cases, a kaleidoscopic pattern of hyperplastic, foetal, embryonal and bizarre nodules, and remarked that the internodular tissue, composed of angular lobules of pleomorphic cells, was unique. Hayles et al, (1956) described 5 cases of goitrous cretinism showing trabecular and microfollicular nodules. They also noted that the internodular tissue was abnormal, showing small follicles with little colloid. More recently Fraser et al, (1961) found that in 31 out of 113 cases with a fault in organic binding the goitre was initially diffuse and later nodular. The average age of the diffuse goitre cases in the present series was 7 years and that of the nodular goitre cases 15 years.

In a considerable proportion of thyroid glands in the dyshormonogenetic goitre group there were histological features suggesting neoplasia viz. cellular pleomorphism in the diffuse goitres and in the internodular tissue of the nodular goitres; papillary proliferation in nodules and in internodular tissue; mitotic activity and the presence of epithelial cells in the lumen of vessels; infiltration of capsule of gland or nodule by pleomorphic epithelium. However, local invasion is an unreliable index of malignancy in endocrine tumours generally. Halley (1961) has suggested that proliferation of endocrine epithelium occurs along the only available capillaries regardless of conventional anatomical boundaries. This point is discussed again in Part V in reference to malignancy in phaeochromocytoma.

The diagnosis of carcinoma in one case with a dehalogenase defect was based on evidence of mural invasion of vessels together with the other features already noted. It is speculative but of interest to consider the possibility that in this case the enzymic defect caused the neoplasia. A diagnosis of carcinoma without metastases or recurrence was made in one case of sporadic goitrous cretinism reported by Wilkins et al, (1954). In 3 of their series of cases with organic binding defects Fraser et al, (1961) diagnosed carcinoma, but so far without report of secondary spread. In the present case 4 years after thyroidectomy there is no indication of recurrence or secondary spread.

Graves' Disease

The general findings described of a diffuse colloid-image in the thyrotoxic glands are in accord with the results of Hamilton et al, (1938-41) quoted by Kelsey et al, (1949). No evidence of a patchy distribution of iodine-131 was seen as reported by Saylor and Kelsey (1949) again quoted by the same authors. Fitzgerald (1955) noted a heavy concentration of radio-iodine as characteristic of Graves' disease, and observed that in thiouracil treated cases often the only trace of isotope present was in macro-follicles. In the present group one such case showed no concentration of radio-iodine at all.

The changes caused by therapeutic doses of iodine-131 have been described by Curran et al, (1958). The appearances in the present 2 cases fall into their acute group and are very similar.

The distribution-pattern of radio-iodine however differs in some respects, although in accord with the main findings of maximum patchy activity in colloid, occasional ring-images, and irregular stromal activity due to extrafollicular labelled colloid. Epithelial activity was not described in any of their material, but was a prominent feature of the case in the present group in which the gland was examined 5 days after the therapeutic dose.

There is considerable controversy about where iodination of protein occurs in the thyroid. Leblond and Gross (1948, 1949) interpreted the ring-type image as indicating that radio-iodine was organically bound in the apex of the epithelial cell. Later, Doniach et al, (1953) interpreted the ring-type image to represent a slow rate of diffusion of newly iodinated thyroglobulin. They considered that iodination occurred entirely in the colloid. Nadler and Leblond (1955) also supported this view, and held that iodination of tyrosyl radicles of thyroglobulin occurred at or near the colloid-cell interface of every follicle. After intravenous injection of iodine-131 Wollman and Wodinsky (1955) found that the bound iodine was in the colloid in mouse thyroid glands at all time intervals studied from 11 seconds upwards. Ring-images were obtained first, and later the image was diffuse over the colloid. Nandi et al, (1956), from grain counts over cells and colloid, concluded that thyroglobulin was formed in the cells and secreted into the colloid. The ring-type image was again taken to represent a slow rate of mixing of newly iodinated

thyroglobulin.

About this time Gross (1957) reviewed the problem and compromised by suggesting that iodination up to the diiodotyrosine stage was intracellular, and that the later stages occurred in the colloid. More recently Pulvertaft et al, (1959) studied tissue cultures of human pathological thyroids. They demonstrated labelled monoiodotyrosine in the cells, and in one toxic goitre gland found intracellular triiodothyronine and thyroxine. They admitted the possibility that the absence of colloid in the tissue culture studies may have stimulated the cells to assume functions for which they may not normally be responsible.

This is also an acceptable interpretation of the present findings of epithelial activity, both in the irradiated gland and in the examples of dyshormonogenetic goitre discussed above. A common factor is certainly a deficiency of colloid, and this is particularly obvious in the internodular tissue of many of the glands with defects in hormone synthesis. It was this type of hyperplasia which was most often associated with epithelial utilisation of iodine-131. Another factor common to the present work and to that of Pulvertaft et al, is that the thyroid epithelium studied is in pathological material. Any conclusions about site of iodination under normal circumstances are unwarranted. The possibility also of species variation must be considered in any comparison of findings in human and animal thyroid glands.

Hashimoto's Thyroiditis

It was apparent that in many glands of this group, with extensive Askanazy-change in epithelium, the organic binding of iodine-131 occurred predominantly in small follicles lined by this type of epithelium. There was, however, no evidence of utilisation of radio-iodine by the non-follicular clumps and strands of disorganised Askanazy-type epithelial cells also usually present. This is consistent with the failure of the non-follicular Hürthle (Askanazy)-cell adenoma and carcinoma to bind iodine. The colloid-images obtained in follicles lined by Askanazy-cells were intense and reflected an active process of hormone synthesis.

Lennox (1948) did much to clarify the confused terminology applied to the epithelial change, but considered that the Askanazy-cells must be without endocrine function. In an autoradiographic study of a single Hashimoto gland Dempsey et al, (1949) described activity only in normal follicles. No iodine-131 was detected in zones of acidophilic (Askanazy) epithelium. On the other hand Statland et al, (1951) gave an account of autoradiography in 3 cases of Hashimoto's thyroiditis. Zones composed primarily of degenerating follicles sometimes showed a small amount of darkening of the autoradiographic film where, from the histological appearance alone, one would suppose that the seeming degree of atrophy would preclude any functional activity. It is not certain that this is a reference to Askanazy-cell zones, but it probably is, because of

the commonly held belief that the epithelial change is degenerative or involutionary. Fitzgerald (1955) reported that in 2 cases of Hashimoto's thyroiditis autoradiography showed relatively little iodine-131. It is not clear from the report how this activity was distributed and how it was related to Askanazy-cells.

Tremblay and Pearse (1960) applied histochemical techniques for oxidative enzymes to a series of pathological thyroid glands from human subjects. They found that Askanazy-cells contained an abundance of actively functioning mitochondria which are capable of carrying out the metabolic functions of the tricarboxylic acid cycle, glycolysis, fatty acid oxidation and amino acid synthesis. They observed that while no proof was offered that the cells are actually performing any of these functions in vivo, possession of the capacity to perform them makes it unlikely that the cells are degenerated or involuted. In the present work the demonstration of labelled colloid in follicles lined by Askanazy-cells is proof that the cells are actually performing some of these functions in vivo, and supports the view that the cells are more probably hyperactive than degenerated or involuted. I agree with Tremblay and Pearse that this hyperactive phase may lead to ultimate collapse and involution. At this stage the epithelium is hyaline rather than granular.

Another point of interest in the Hashimoto's thyroiditis group is the occurrence of 2 male cases in the series of 27 cases.

Statland et al, (1951) reviewed 51 cases all of which were women. In a series of 170 cases Lindsay et al, (1952) described the disease in 2 men. Fisher and Creed (1956) recorded a further 2 male cases and found only 9 previous cases in the literature.

SUMMARY

Autoradiographs have been prepared and examined from 159 pathological thyroid glands after administration of iodine-131.

The distribution patterns of organically bound iodine-131 do not help to differentiate between hyperplasia and benign and malignant neoplasia when this is most difficult histologically.

Nodular goitre in the adult shows a characteristic distribution-pattern of iodine-131 which is quite different from that seen in dyshormonogenetic nodular goitre in the young. The histology also differs in a distinctive way.

A case of thyroid carcinoma associated with impaired dehalogenase activity is described with autoradiographic findings. Askanazy-type epithelium is shown to be metabolically active in Hashimoto's thyroiditis and in other conditions.

Epithelial utilisation of iodine-131 is demonstrated in an irradiated gland and in some cases of dyshormonogenetic goitre. It cannot be concluded that normal iodination of thyroid protein occurs within the thyroid epithelial cells.

P A R T V

1. The Chromaffin and Iodate Reactions for Catechol Amines

1. THE CHROMAFFIN AND IODATE REACTIONS FOR CATECHOL AMINES

In this section I used radio-chromate, radio-iodate, and radio-iodide in an examination of the histochemistry of chromaffin tissue generally and of a group of chromaffin tumours. The work started with the discovery at an autopsy I performed of a malignant phaeochromocytoma, and the appreciation during the detailed examination of this tumour of what Willis (1953) has called the fickle nature of the chromaffin reaction. In the published report (Kennedy et al, 1961) a full account of the whole range of the investigation has been given. In what follows here I have given my personal contribution.

The diagnosis of malignancy in chromaffin tumours is often difficult. Symington and Goodall (1953) reviewed 280 cases of phaeochromocytoma of which 31 had been reported as malignant. In 19 of them they found insufficient evidence of malignancy; in 5 the tumours could have been multicentric in origin, and in the remaining 7 the chromaffin reaction had not been done or was unsatisfactory. Since then further malignant tumours have been reported, but in only one was secondary tumour assayed for catechol amines (Davis et al, 1955). The tumour described by Andreassen (1954) had metastases in the liver, but no assay was attempted. Belkin et al, (1954) reported a phaeochromocytoma that recurred in the right adrenal region with secondaries in para-aortic lymph nodes, liver, lung and rib: total catechol amines were estimated in the adrenal tumour, but the secondaries were not

assayed. Manger et al, (1954) described a malignant phaeochromocytoma with extensive secondary spread found at operation, but no tissue was removed for assay. McMillan (1956) reported hydroxytyramine as well as noradrenaline in the biopsy of an adrenal tumour and classified the tumour as probably malignant on the grounds of local invasiveness. In a case described by Kimberley (1956), removal of the tumour resulted in a fall of blood pressure from 200-260/100-160 to 170/100 mm.Hg. Tumour nodules were observed in the liver at operation: one year later the blood pressure rose to 190/100 and on this evidence a vasopressive effect was deduced for the metastases. Phaeochromocytomas of the organs of Zuckerkandl have been reviewed by Cook et al, (1960) who described two such malignant tumours: the chromaffin reaction was positive in both and catechol amines were assayed in one primary tumour. Pugh et al, (1960) reported a malignant chromaffin tumour of the urinary bladder with metastases in liver and lymph glands: the chromaffin reaction was positive but no assay was performed. They also remarked on the vagaries of the chromaffin reaction.

MATERIALS AND METHODS

Tissues. The tissues studied fall into two groups: (1) Phaeochromocytoma: 6 tumours, 4 necropsy and 2 resected. The malignant tumour is one of the post-mortem cases (table 5,1). (2) Normal adrenal glands obtained from patients undergoing adrenalectomy for breast cancer or at autopsy in man, ox, rat, mouse and guinea-pig;

this group of tissues was used particularly in the investigation of histochemical methods.

Chromaffin reaction. A variety of dichromate solutions was employed, including Müller's fluid, Régaud's fluid without acetic acid, equal parts of 5 per cent potassium dichromate and 10 per cent neutral formalin, and a mixture of 100 parts of 5 per cent potassium dichromate and 7 parts of 5 per cent potassium chromate. The mixtures were used with and without addition of phosphate or acetate buffer and the pH range investigated was from 3.5 to 7.5. Paraffin and frozen sections were cut and examined with and without further staining. Sevki's modification of Schmorl's Giemsa method was used on some sections; others were stained with methylene blue.

Blocks of tissue from case 5 and from a variety of normal human and animal adrenal glands were fixed in dichromate solutions containing radioactive sodium chromate-51. The pH range was from 5 to 6.8. The specific activity of the solutions was 20 μc. per ml. The tissues were kept in the radio-chromate solutions for 18-24 hours and were then processed in the usual way for frozen and paraffin sections. Autoradiographs were prepared for comparison with neighbouring sections. Kodak AR10 and AR50 stripping films were used with exposure times of 80-90 days. The radio-chromate was not immediately available and the method could not be employed routinely, particularly in cases coming to autopsy. This applied also to the radio-iodate method.

Iodate reaction. This method demonstrates noradrenaline only. Hillarp and Hökfelt (1953), the originators, used saturated potassium iodate buffered to pH 5.4-6.0. Tissue from cases 3-5 and animal adrenals were placed in solutions containing iodine-131 as iodide (Eränkö, 1957) or as iodate. The labelled iodide solution was mixed with 9 times its volume of unlabelled saturated iodate: the specific activity of the mixture was 200 µc. per ml. The labelled iodate was usually added to a saturated solution of unlabelled potassium iodate and occasionally to a 10 per cent solution to give specific activities ranging from 12.5 to 100 µc. per ml. The pH of all the radio-iodine solutions was in the range 5.4-6.0 and the tissues were kept in the solutions from 7 to 25 hours. Thereafter they were fixed in 10 per cent neutral formalin and autoradiographs were prepared as before on frozen and paraffin sections. Exposure times were from 30 to 147 days.

CASE REPORTS

The malignant phaeochromocytoma (case 1) was discovered at autopsy, performed 5½ hours after death, in a 56 year old woman who clinically had presented as a case of diabetes mellitus. The growth involved the left adrenal region, measured 12 x 10 x 10 cm. and weighed 388 g. The tumour (fig. 5,1) was well encapsulated and, on section, consisted of flesh-coloured tissue resembling normal adrenal medulla. Large areas of haemorrhage and necrosis were present.

Microscopically, the structure is indistinguishable from that

seen in benign tumours. The cells are mature phaeochromocytes grouped together to form large and small alveoli (figs. 5,2-5,3). There is slight nuclear and cellular pleomorphism with only a few giant-cells. Mitotic figures are scanty. There is no invasion of capsule, but groups of tumour cells are present in sinusoids and thin-walled veins.

The liver (1,720 g.) was enlarged and numerous tumour nodules (0.25 - 2.5 cm. in diameter) similar in colour to the primary growth were present. Histologically the cells of the secondaries have an alveolar arrangement and are composed of mature phaeochromocytes. Clumps of similar tumour cells are present in hepatic vessels (fig. 5,4). Secondary tumour deposits were found also in the left lateral aortic lymph glands, in the left ilium, in the first and fourth left ribs and in the second lumbar vertebra. The histological pattern in all secondary sites is similar to that in the primary tumour and liver (fig. 5,5). No tumour was seen in the lungs.

The only other significant findings were enlargement of the heart (430 g.), due mainly to left ventricular hypertrophy, and evidence of hypertensive nephrosclerosis.

The benign tumours (cases 2-6) varied in size from 1.25 to 8 cm. in diameter, were composed of mature phaeochromocytes and were similar in appearance to those described by Symington and Goodall (1953).

RESULTS

Catechol amines were assayed (von Euler and Hamberg, 1949) in representative portions of the tumours by B.A. Woodger and the results are listed in tables 5,2 and 5,3. Both adrenaline and noradrenaline were present in the primary tumour in case 1, but only noradrenaline was demonstrable in the hepatic secondaries.

Chromaffin reaction (table 5,4). In cases 1-4, where no attempt was made to measure or control the pH of the various dichromate and chromate solutions used, the reaction was unsatisfactory as a diagnostic method. In cases 5 and 6, where the dichromate solutions were adjusted to pH 5.8, a satisfactory chromaffin reaction was obtained even in case 6 where the autopsy was performed 21 hours after death. The effect of pH variation was investigated more fully with normal human, ox, mouse, rat and guinea-pig adrenals. It was found that for pH values below 4 the reaction failed, and for pH values above 7 the reaction again tailed off. With pH about 6 a positive chromaffin reaction was obtained regularly in paraffin or frozen sections of adrenal medullary tissue. A weak positive chromaffin reaction was obtained with human adrenals removed as long as 31 hours post-mortem.

In case 5 chromium-51 was detected in the autoradiographs of sections showing a positive chromaffin reaction (pH 5-6), and the radioactivity corresponded with the distribution of the brown pigment. The effect of pH control was also investigated in animal adrenals, and a constant association of pigment and radio-

active chromium was found with labelled dichromate mixtures in the pH range 5–6 (fig. 5,6). Outside this range the results were variable.

Iodate reaction (table 5,4). In cases 3 and 4 the iodate reaction was negative, but it should be noted that in both tumours the predominant catechol amine present was adrenaline. Noradrenaline, however, was detectable chemically. In case 5, where the ratio of adrenaline to noradrenaline estimated chemically was about 1, the iodate reaction was successful. The reaction was also consistently successful in the normal animal adrenals used (fig. 5,7), with the exception of those of the guinea-pig which has little or no noradrenaline in its adrenal medulla. A saturated solution of potassium iodate gave rather better results generally than a 10 per cent solution.

In case 3 where the iodate reaction was negative no radioactivity was demonstrable in autoradiographs of tissue treated in a labelled-iodide/unlabelled-iodate mixture (Eränkö, 1957). This mixture gave a good colour reaction in groups of medullary cells in the mouse adrenal, but the corresponding autoradiographs were uniformly negative. In cases 4-5 labelled iodate was used in a saturated solution of unlabelled potassium iodate. Though the colour reaction was negative in case 4, a positive image was obtained on autoradiography over some groups of cells. In case 5, where the colour reaction was positive, an image was also obtained in the autoradiographs. A rate-meter count on the open face of the

blocks gave 2.9 x 10^7 c.p.m. (counts per minute). When the blocks had been deparaffinised, taken down to water, washed in running water for 24 hours and activity again estimated with a similar geometry the count rate had decreased to 10^3 c.p.m., corrected for natural decay. The brown pigment was, however, still clearly present. Further washing reduced the radioactivity and autoradiographic images were not obtainable although the brown pigment was still present. Mouse and rat adrenals treated in the labelled iodate solutions gave the usual brown pigment in groups of medullary cells, but corresponding autoradiographs were negative. The sections were well washed before autoradiography.

DISCUSSION

Most chromaffin tumours are unilateral, but bilateral growths do occur, and it should be appreciated that growths that appear to involve one adrenal and the para-aortic lymph glands may be examples of tumours of multicentric origin, and not malignant. Local invasion is an unreliable index of malignancy in endocrine tumours. In the normal testis (Halley, 1961) Leydig cells may be found adjacent to blood vessels outwith the capsule, and I have observed parathyroid cells in a similar extracapsular site where there is no question of malignancy. In the normal adrenal, cells may be present near blood vessels outwith the capsule.

In the dyshormonogenetic goitre group (Part IV) reference has already been made to the presence of epithelial cells in the

thyroid capsule giving an appearance indistinguishable from that of neoplastic infiltration. Malignancy in a phaeochromocytoma can be accepted as proved only if it metastasises to sites where there is no chromaffin tissue, and the tumour reported here (case 1) illustrates this point. Histologically the primary growth was composed of mature phaeochromocytes indistinguishable from the cells of benign tumours. No immature cells were seen, pleomorphism was not a prominent feature, and mitotic activity was scanty. The secondary deposits in liver and bones are clearly outside the normal distribution of chromaffin tissue and the possibility of multicentric origin can be excluded. The maturity of the cells and the ability of the liver secondaries to form noradrenaline is proof that a malignant phaeochromocytoma can be functionally active.

Chromaffin reaction. Hillarp and Hökfelt (1953) reported that little or no brown pigment appeared in adrenal medullary cells treated with dichromate solutions at pH 4. They suggested that solution of catechol amines from the cells occurred at pH levels below 5 and recommended dichromate solutions at pH 5-6 for routine use. Present results bear this out. The mixture of 100 ml. of 5 per cent potassium dichromate and 7 ml. of 5 per cent potassium chromate, giving a pH of 5.8 is particularly reliable. With this technique a reaction was obtained in the tumour from case 6 removed 21 hours post-mortem. It is suggested that the most important cause of failure in the chromaffin reaction is

failure to control pH.

The nature of the brown pigment formed in the chromaffin reaction is not known with certainty. Ogata and Ogata, (1922-23) considered it to be an oxide of chromium. Gerard et al, (1930) suggested that the chromaffin reaction is an oxidative phenomenon independent of the presence of chromium and Bennett (1941) reaffirmed this hypothesis. Coupland (1954) however, concluded that after formol-dichromate fixation chromium is fixed by the cells of the adrenal medulla, and he suggested that a chrome-adrenochrome is produced. Hale (1958) used an X-ray micro-technique to show that chromium was deposited in the cytoplasm and nuclei of adrenal medullary cells as a result of potassium dichromate fixation. The pH of the dichromate solutions used is not discussed in the above papers, but Coupland informed me in a personal communication in 1958 that he had found that pH levels below 4 were of no value for staining the catechols. The auto-radiographic findings support Coupland's conclusion of 1954, but I agree with Hale's proviso that although chromium is demonstrable in the brown-stained medullary cells it is still not possible to decide whether this brown colour is due to formation of an adreno-chrome-chromium complex, or to deposition of chromium, or to an organic oxidation product of adrenaline.

Harley-Mason (1948) proposed a formula for adrenochrome as a zwitterion and later (1950) described the oxidation of solutions of adrenaline, giving first a red colour and then a

brown or black precipitate of adrenaline black. The formation of adrenaline black proceeded via adrenochrome and appeared to be a polymerisation. Adrenaline black is soluble in alkalies. The brown colour of the chromaffin reaction may be due partly to the presence of this compound and partly to the presence of a chromium-adrenochrome complex, and the failure of the reaction at alkaline pH may be explicable on the hypothesis that the pigment itself is soluble in this pH range. Even less is known of the oxidation products of noradrenaline with dichromate, but since all the medullary cells are stained in a similar way, and since chromium is demonstrable in them all, it is probable that the chemistry is similar. Hillarp and Hökfelt (1953) stated that both noradrenaline and adrenaline could be converted by oxidation into brownish-black insoluble products of a melanin-like character, but pointed out that little is known of this transformation of noradrenaline.

Iodate reaction. Again much of the basic work is due to Hillarp and Hökfelt (1953), who concluded that oxidation with potassium iodate in acetate buffer at pH 6.0 or in phosphate buffer at pH 5.4 effected such a rapid and selective pigment formation from noradrenaline that the reaction might be applied to the cytological demonstration of noradrenaline in the adrenal medulla. They pointed out that the pigment was confined to certain groups of medullary cells whose number varied more or less directly with the content of extractable noradrenaline. I have confirmed that in

the normal animal adrenal medulla only certain clearly delineated groups of cells are stained, and this observation was also valid in a phaeochromocytoma known by assay to contain a substantial amount of noradrenaline (case 5). In a later paper (Hillarp and Hökfelt, 1955), they gave a warning that dopamine, DOPA, and possibly other related substances could form dark pigments on oxidation with potassium iodate. These compounds, however, were absent from the adrenal medulla or were present only in very small quantities. They stated that paraffin embedding could not be used in the iodate reaction because this procedure dissolved the pigments. I have not found this to be so, and in fact some of the most satisfactory iodate reactions were seen in the paraffin sections. Frozen sections of the same material did not give such clear-cut results.

As in the case of the chrome reaction, the chemistry of the pigment formed is not known accurately. It differs obviously from that formed by dichromate fixation in that chromium is not associated with it in the cells, and that it is present only in clearly delineated groups of medullary cells. Hillarp and Hökfelt (1953) found that in-vitro iodate oxidation at pH 5.4 transformed noradrenaline within minutes to a dark pigment; adrenaline, on the other hand, did not undergo such a transformation until after 24 hours. In each case there is also presumably formation of iodonoradrenochrome and iodoadrenochrome, but iodoadrenochrome is not retained in the cells, and in solution

gives a reddish colour. The cells that stain brown may contain a mixture of pigment and iodonoradrenochrome. Eränkö (1957) suggested that the brown colour produced by treatment with iodate was due to an iodine-containing compound which on solubility considerations was probably not iodonoradrenochrome. My finding that radio-activity could be washed out of the material though the brown colour remained suggested that the positive autoradiographs obtained by Eränkö might have been due to the presence of iodo-noradrenochrome and pigment together. The pigment is possibly a non-iodinated polymeric oxidation product of noradrenochrome. The more soluble iodonoradrenochrome could, on this hypothesis, be washed out leaving the almost insoluble non-iodinated pigment.

SUMMARY

The main cause of failure in the chromaffin reaction is failure to control the pH of the dichromate solution used. The pigment formed in the chromaffin reaction may be a mixture of adrenaline black and a chrome-adrenochrome complex.

The iodate reaction does not stain all adrenal medullary cells nor all chromaffin tumour cells. The pigment formed is probably a non-iodinated oxidation product of noradrenaline.

The following methods are recommended in the investigation of a chromaffin tumour:

(a) Catechol amine assay

(b) Chromaffin reaction using a dichromate mixture at pH

between 5 and 6.

(c) Iodate reaction using a saturated solution of potassium iodate at pH between 5 and 6.

P A R T VI

1. Interstitial Injection of Radioactive Colloidal Gold in Cancer.

1. INTERSTITIAL INJECTION OF RADIOACTIVE COLLOIDAL GOLD IN CANCER

During my tenure of a cancer research scholarship there was considerable interest in the use of radioactive colloids in the treatment of cancer. I decided to carry out an autoradiographic study of the distribution of gold-198, which was available in colloidal form, and sought the co-operation of two surgical colleagues J.D. Thomson and F.C. Walker. They agreed to inject small doses of the radio-gold in a series of cases prior to operation for removal of malignant tumours. I received the resected tissues and determined the distribution of the isotope in them by autoradiography. The results were published (Kennedy et al, 1957), and I have composed the present account personally.

A colloid injected into the interstitial tissue near a tumour is removed in the usual way to the lymph nodes draining the site of injection. The purpose of the experiment was to determine how many regional lymph nodes took up colloidal radio-gold, and how this distribution was affected by the presence of secondary tumour in the nodes. In this way an assessment could be made of the probable field of irradiation in a therapeutic application. Allen et al, (1954, 1955) reported good results in carcinoma of cervix treated with colloidal radio-gold followed 19 to 36 days later by panhysterectomy, and Hahn et al, (1953) used radioactive colloids in bronchial carcinoma. The effect of ionising radiation on lymphoid tissue is well known. External irradiation caused a

marked decrease in lymphocytes (Bloom, 1948). Therapeutic doses of colloidal radio-gold caused necrosis (Sherman and Ter-Pogossian, 1953; Berg et al, 1955; Wheeler et al, 1956).

MATERIALS AND METHODS

The radio-gold was obtained from the United Kingdom Atomic Energy Authority as a colloidal suspension of metallic gold-198 stabilised with gelatin. The particle size is 20-70 mμ and specific activity is 100 mc./ml. The half-life of the isotope is 2.7 days; it emits beta particles in the energy spectrum 0.29 to 1.37 MeV, and gamma rays 96 per cent of which have an energy of 0.41 MeV. The k factor is of the same order as that for iodine-131. From these data it is apparent that if the colloidal radio-gold is concentrated in regional lymph nodes, then at therapeutic levels of dosage it should be as effective as large doses of iodine-131 in the thyroid gland.

The surgical cases comprising the series are listed in table 6,1. In the 2 non-neoplastic cases there was clinical suspicion of malignancy, and biopsy of the lesions with removal of lymph nodes was carried out after injection of the colloidal gold. In the other cases of established tumour the radio-gold was injected interstitially near the primary growth, and 12 hours to 7 days later the tumour and regional lymph nodes were removed. In each case of mammary, rectal and bronchial carcinoma 0.5-1 mc. of the radioactive colloid was infiltrated around the tumour. In the 3 cases of carcinoma of cervix the injection was made bilaterally into

the parametrium.

I dissected the tissues removed at operation and identified each lymph node by a serial number giving its position in relation to the tumour. Blocks were taken from all lymph nodes and, in some cases, from the injection site and from the region between the injection site and the nodes. Alcoholic formalin or a mixture of alcohol and acetone were used as fixatives, and autoradiographs prepared from sections cut at 5-6 μ with Kodak AR10, AR50, and V1001 plates. Neighbouring sections to those used for autoradiography were stained with haematoxylin and eosin. At least 4 autoradiographs were prepared from each block. I estimated the level of radioactivity in blood and urine samples in 2 cases, in faeces and rectal washings in one case, and on instruments, gloves and swabs in one case.

Larger doses (5-10 mc.) of colloidal radio-gold were injected interstitially in a group of 12 rabbits and 6 guinea-pigs. I examined liver, spleen, kidney, bone marrow, and regional lymph nodes by autoradiography and by rate meter counting at intervals up to 11 weeks after administration of the isotope.

RESULTS

The distribution of the radioactive colloid in lymph nodes is summarised in tables 6,1 and 6,2. A significant finding is that in the 2 cases found by biopsy to be inflammatory rather than neoplastic only one-third of the lymph nodes examined contained radio-gold. Histologically the lymph nodes removed at the time

of biopsy showed non-specific reactive hyperplasia.

Carcinoma of breast. Seventy-three lymph nodes were auto-radiographed from 6 cases of mammary carcinoma. In 44 there was complete replacement by secondary tumour and no radio-gold was detected. The remainder were either partly replaced by tumour, or were free of tumour. In the former group 11 per cent contained some radio-gold, and in the latter group 36 per cent gave positive autoradiographs. The radioactive colloid was found occasionally in lymph nodes anatomically distal to completely replaced nodes. In one case radio-gold was demonstrated in lymph nodes, free of tumour, removed from the internal mammary group. There was considerable variation in the amount of colloid present from node to node (figs. 6,1-6,2).

The lymph nodes were removed 2-6 days after injection in this group of tumours, and it was apparent that there was no marked increase in the amount of colloid present in the nodes with time. It was also apparent, in a comparison of individual cases, that with increased intervals between injection and operation there was no clear pattern of an increase in number of nodes containing radio-gold. Six days after injection radioactive colloid was still demonstrable at the injection site (fig. 6,3). The tumour cells showed no evidence of uptake but the radio-gold was present in stromal macrophages. Estimation of radioactivity in blood samples in 2 cases one day after injection showed a level of about 0.007 per cent of the injected activity, and daily urinary levels fell

from 0.02 per cent of the injected amount at one day. to 0.001 per cent at 10 days after injection. No significant contamination of instruments, gloves or swabs was demonstrable by monitoring during one mastectomy.

Carcinoma of rectum. No radioactive colloid was demonstrable at all in 64 lymph nodes from 8 cases of rectal carcinoma, and 47 of the nodes were free of secondary deposits. The tissues were removed 12 hours to 7 days after injection of the radio-gold, and in every case the isotope was demonstrable in the region of the primary tumour. In one case 5.3 per cent of the injected dose was present in faeces and rectal washings one day after injection.

Carcinoma of cervix. Three panhysterectomies in this condition led to the autoradiography of 69 lymph nodes 63 of which did not contain secondary carcinoma. Seventy per cent of the tumour-free nodes gave positive autoradiographs, but again the amount present varied greatly from node to node and the distribution-pattern in any node was not uniform (fig. 6,4). Four lymph nodes showed partial replacement by secondary tumour and they all contained some radioactive colloid in the surviving lymphoid tissue. Two lymph nodes were wholly replaced by tumour and no radio-gold was demonstrable in them. The tissues were examined 4-5 days after injection of the colloid and it was present in each case at the injection sites.

Carcinoma of bronchus. There were 6 cases of bronchial carcinoma

yielding 41 lymph nodes for autoradiography. Twenty-seven of the nodes had no secondary deposits, and 33 per cent of this group contained a variable amount of the colloid. Six nodes were partially replaced by tumour and they all gave positive autoradiographs (fig. 6,5). Again all nodes wholly replaced by tumour showed no evidence of radioactivity. The tissues were examined 2-7 days after injection of the colloid.

In the group of animals given larger doses of radio-gold no significant amount of radioactivity was demonstrable by counting or by autoradiography of liver, spleen, kidney and bone marrow. The lymph nodes draining the injection sites did contain large amounts of the colloid.

DISCUSSION

In favour of possible therapeutic application is the sinus distribution of the colloidal radio-gold (figs. 6,1 and 6,4): that is the colloid lodges where metastatic tumour emboli lodge. Also, particularly in the cases of carcinoma of bronchus and cervix, the colloid is consistently demonstrable in lymph nodes containing fairly early secondary deposits. It has been suggested by Jentzer and Wenger (1950) that such nodes have a greater affinity than normal nodes for colloidal gold, but the number of nodes examined in the present series is inadequate to substantiate this view. In the mammary carcinoma cases only 11 per cent of nodes partially replaced by tumour contained radio-gold, but generally when the colloid was present it was in close apposition to the secondary carcinoma (fig.

6,6). Another favourable point is that nodes completely replaced by tumour do not stop the colloid reaching more distal partially involved nodes, or nodes which are free from tumour, probably by collateral lymphatics.

The dispersal rate from the injection site seems to be adequately fast. Bryant et al, (1953) found that after submucosal injection into bronchus in dogs an appreciable concentration of radioactive colloid was present in the regional lymph nodes within 4 hours. In the present cases at 2-7 days after injection the concentration in the regional nodes did not vary appreciably with time, nor was the residue at the injection site considerable. However it should be noted that Hultborn et al, (1955) reported that in mammary carcinoma more than 85 per cent of the injected amount of radioactive colloid remained at the site of injection. They also described the distribution in the regional nodes as irregular.

In the group of experimental animals there was no evidence of appreciable transport of the colloidal gold by blood in that liver, spleen, kidney and bone marrow were consistently free of radioactivity by counting and by autoradiography. Small amounts of radioactivity were detected in blood and urine in 2 cases of mammary carcinoma. Andrews et al, (1953) found that after injection of colloidal radio-gold into serous cavities in the therapy of malignant effusions a small but appreciable quantity of the isotope found its way, probably through lymphatic channels, into the blood stream from which it was deposited in liver, spleen and bone marrow. In dogs,

on the other hand, after injection of radioactive colloid into the bronchial wall Berg et al, (1955) found that radiation damage was confined to the injection site and to the regional lymph nodes. Meneely et al, (1953) instilled radioactive colloidal gold in lung and found that little, if any, entered the blood stream. It is probable that the view of McCormick et al, (1954), that the small particle size of the colloid favours transport to liver and spleen, is too cautious.

Kottmeier and Moberger (1955) in 8 cases of carcinoma of cervix described an ample but uneven dispersion of large doses of colloidal radio-gold in the regional lymph nodes. They recorded a good palliative effect. The present distribution results in carcinoma of cervix and of bronchus are also encouraging, but the results, as a whole, indicate that it is improbable that a therapeutic concentration of radioactivity can be achieved in all regional lymph nodes following interstitial injection of colloidal radio-gold. It is clear that carcinoma cells have no affinity for the colloid, and that lymph nodes replaced by tumour do not concentrate any radioactivity. If such nodes were not resected following the isotope injection there could be no chance of arrest of tumour spread by the effect of radiation from colloidal gold.

Of greater importance however is the fact that not all tumour-free nodes take up the colloid. This finding has also been reported by Mellgren et al, (1954) and by Knutson and Norin (1956) in cases of mammary carcinoma. It is therefore probable that even if only

microscopic secondary deposits were present in a lymph node they might escape irradiation. Hahn and Carothers (1953) reported a non-uniform distribution of radioactive colloids in normal lymph nodes in dogs. It is not clear that the presence of secondary carcinoma in a lymphatic region enhances the tendency to a haphazard distribution of the colloid, but it certainly does not reduce it. The results in rectal carcinoma in the present series are difficult to explain without further data. The total activity was not excreted in the case in which faeces and rectal washings were monitored. The total activity was not injected into the peritoneum because positive autoradiographs were obtained from the interstitial tissue near the tumours. It is possible that the colloid was absorbed and filtered out in the liver, spleen and bone marrow. Unfortunately in these cases estimations of blood radioactivity were not undertaken.

SUMMARY

The distribution of colloidal radio-gold in regional lymph nodes has been studied by autoradiography following interstitial injection of the colloid near carcinomas of breast, cervix, rectum and bronchus.

Lymph nodes replaced by tumour do not concentrate the colloid. There is a non-uniform distribution in other lymph nodes. The colloid is largely confined to the site of injection and its regional lymphatic system. Very small amounts were detected in blood and urine.

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REFERENCES

REFERENCES

PART I

ASBOE-HANSEN, G.	1950.	Ann. Rheum. Dis., 9, 149.
" " "	1953.	Cancer Res., 13, 587.
" " "	1954.	In Connective tissue in health and disease, ed., by the author, Copenhagen, p. 291.
" " "	1957.	In Connective tissue, C.I.O.M.S. symposium, ed. by R.E. Tunbridge, Oxford, p. 12.
BÉLANGER, L.F.	1954.	Anat. Rec., 118, 755.
BETTELHEIM-JEVONS, F.R.	1958.	Advanc. Protein Chem., 13, 46.
BOSTRÖM, H.	1952.	J. Biol. Chem., 196, 477.
BOSTRÖM, H., AND ÅQVIST, S.	1953.	Acta chem. Scand., 6, 1551.
BOSTRÖM, H., AND GARDELL, S.	1953.	Ibid., 7, 216.
BOSTRÖM, H., AND ODEBLAD, E.	1953.	Anat. Rec., 115, 505.
BOWES, J.H., ELLIOTT, R.G., AND MOSS, J.A.	1956.	Biochem. J., 63, 1P.
BRYANT, J.H., LEDER, I.G., AND STETTEN, De W., JR.	1958.	Arch. Biochem. Biophys., 76, 122.
BUNTING, H., AND WHITE, R.F.	1950.	Arch. Path., 49, 590.
CAMPANI, M.	1951.	Lancet, 1, 802.
COLLINS, D.H.	1949.	The pathology of articular and spinal diseases, London, p. 8.

CRAMER, W., AND SIMPSON, W.L.	1944.	Cancer Res., 4, 601.
CURRAN, R.C.	1952.	Brit. J. Exp. Path., 33, 82.
" " "	1953.	J. Path. Bact., 66, 271.
" " "	1957.	Ibid., 74, 347.
CURRAN, R.C., AND KENNEDY, J.S.	1955a.	Ibid., 70, 449.
" " " " "	1955b.	Nature, Lond., 175, 435.
DENKO, C.W., AND PRIEST, R.E.	1959.	Arch. Biochem. Biophys., 79, 252.
DONIACH, I., AND PELC, S.R.	1950.	Brit. J. Radiol., 23, 184.
DRENNAN, J.M., AND BEARE, J.M.	1954.	J. Path. Bact., 68, 345.
DZIEWIATKOWSKI, D.D.	1949.	J. Biol. Chem., 178, 197.
" " "	1951.	Ibid., 189, 187.
" " "	1954.	Ibid., 207, 181.
ENGFELDT, B., AND WESTERBORN, O.	1960.	Acta path. microbiol. scand., 49, 73.
EVERETT, N.B., AND SIMMONS, BARBARA S.	1952.	Arch. Biochem., 35, 152.
FAWCETT, D.W.	1954.	J. Exp. Med., 100, 217.
GIBSON, T., CURRAN, R.C., AND KENNEDY, J.S.	1955.	Transplant. Bull., 2, 44.
GROSSFIELD, H., MEYER, K., AND GODMAN, G.C.	1955.	Proc. Soc. Exp. Biol. Med., 88, 31.
HALMI, N.S., AND DAVIES, J.	1953.	J. Histochem. Cytochem., 1, 447.
HEILBRUNN, L.V., AND WILSON	1949.	Proc. Soc. Exp. Biol. Med., 70, 179.

HOLMGREN, H., AND WILANDER, O. 1937. Z. Mikr.-anat. Forsch., 42, 242.

HOLMGREN, H., AND WOHLFART, G. 1947. Cancer Res., 7, 686.

JACKSON, D.S. 1954. Biochem. J., 56, 699.

" " " 1957. Ibid., 65, 277.

JACKSON, SYLVIA F. 1957. In Connective tissue, C.I.O.M.S. symposium, ed. by R.E. Tunbridge, Oxford, p. 77.

JORPES, E. 1935. Biochem. J., 29, 1817.

JORPES, E., ODEBLAD, E., AND BOSTRÖM, H. 1953. Acta. haem., 9, 273.

KENNEDY, J.S. 1960. J. Path. Bact., 80, 359.

KENNEDY, J.S., AND KENNEDY, G.D.C. 1957. J. Anat., 91, 398.

KOWALEWSKI, K. 1959. Acta endocrinol., 32, 19.

KRAMER, H., AND WINDRUM, G.M. 1953. J. Clin. Path., 6, 239.

LAIDLAW, J.C., AND YOUNG, L. 1948. Biochem. J., 42, L.

LAYTON, L.L. 1950a. Proc. Soc. Exp. Biol. Med., 73, 570.

" " " 1950b. Cancer, 3, 725.

" " " 1951. Arch. Biochem. Biophys., 32, 224.

LAYTON, L.L., FRANKEL, DORIS R., AND SCAPA, SYLVIA. 1950. Arch. Biochem., 28, 142.

LAYTON, L.L., AND DENKO, C.W. 1952. Cancer, 5, 405.

LISON, L. 1954. Stain Technol., 29, 131.

LOEVEN, W.A.	1955.	Acta anat., 24, 217.
MEYER, K.	1951.	In Connective tissues, Trans. 1st Conference, Josiah Macy Jr. Foundation, New York, p. 32.
MEYER, K., AND CHAFFEE, ELEANOR.	1940.	Amer. J. Ophthalmol., 23, 1320.
MEYER, K., HOFFMAN, P., AND LINKER, A.	1957.	In Connective tissue C.I.O.M.S. symposium, ed. by R.E. Tunbridge, Oxford, p. 86.
MUIR, HELEN.	1961.	In The biochemistry of mucopolysaccharides of connective tissue, Biochem. Soc. symposium no. 20, ed. by F. Clark and J.K. Grant, Cambridge, p. 18.
NORDLIE, R.C., AND FROMM, H.J.	1958.	Proc. Soc. Exp. Biol. Med., 97, 246.
OLIVER, J., BLOOM, F., AND MANGIERI, CARMEN.	1947.	J. Exp. Med., 86, 107.
PAFF, G.H., BLOOM, F., AND REILLY, C.	1947.	Ibid., 86, 117.
PAFF, G.H., SUGIURA, H.T., BOCHER, C.A., AND ROTH, J.S.	1952.	Anat. Rec., 114, 499.
PARTRIDGE, S.M.	1948.	Biochem. J., 43, 387.
PARTRIDGE, S.M., DAVIS, H.F., AND ADAIR, G.S.	1961.	Ibid., 79, 15.
PEARSE, A.G.E.	1953.	Histochemistry, London, p. 436.
PELC, S.R., COOMBES, J.D., AND BUDD, G.L.	1961.	Exp. Cell Research, 24, 192.
POPPER, H.	1954.	Amer. J. Med., 16, 98.

POPPER, H., AND ELIAS, H.	1955.	Amer. J. Path., 31, 405.
PORTER, K.R., AND VANAMEE, P.	1949.	Proc. Soc. Exp. Biol Med., 71, 513.
PRODI, G.	1955.	Nature, Lond., 175, 1130.
PRZYBYLSKI, R.J.	1961.	Exp. Cell Research, 24, 181.
PULLAR, P., AND COCHRANE, T.	1957.	Scot. Med. J., 2, 189.
RILEY, J.F., SHEPHERD, D.M., WEST, G.B., AND STROUD, S.W.	1955.	Nature, Lond., 176, 1123.
RILEY, J.F., AND WEST, G.B.	1955.	J. Path. Bact., 69, 269.
ROBB-SMITH, A.H.T.	1954.	In Connective tissue in health and disease, ed. by G. Asboe-Hansen, Copenhagen, pp. 28–29.
" " " " "	1958.	In British Gelatine and Glue Research Association, Recent advances in Gelatin and glue research, ed. by G. Stainsby, London, pp. 38–44.
ROBERTSON, W. van B., AND SCHWARTZ, B.	1953.	J. Biol. Chem., 201, 689.
SCHILLER, S., MATHEWS, M.B., CIFONELLI, J.A., AND DORFMAN, A.	1958.	Ibid., 218, 139.
SINGHER, H.O., AND MARINELLI, L.	1945.	Science, 101, 414.
SLACK, H.G.B.	1956.	Biochem. J., 64, 7P.
STEHBENS, W.E.	1962.	J. Path. Bact., 83, 337.
SYLVÉN, B.	1945.	Acta Radiol., suppl. 59.
" "	1950.	Exp. Cell Research, 1, 582.

TARVER, H., AND SCHMIDT, C.L.A. 1939. J. Biol. Chem., 130, 67.

" " " " 1942. Ibid., 146, 69.

TAYLOR, H.E., AND SAUNDERS, A.M. 1957. Amer. J. Path., 33, 525.

VANAMEE, P., AND PORTER, K.R. 1951. J. Exp. Med., 94, 255.

WATSON, W.C. 1958. Brit. J. Exp. Path., 39, 540.

WATSON, W.C., AND KENNEDY, J.S. 1960. Ibid., 41, 385.

WILANDER, O. 1939. Skand. Arch. Physiol., 81, suppl. 15.

WILLIAMS, G. 1957. J. Path. Bact., 73, 557.

WILLIS, R.A. 1953. Pathology of tumours, 2nd ed., London, p. 335.

ZACHARIAE, F., AND DYRBYE, M.O. 1959. Acta rheum. Scand., 5, 190.

REFERENCES

PART II

ANGEVINE, D.M.	1959.	In Modern trends in pathology, ed. by D.H. Collins, London, pp. 55-59.
BACHRA, B.N., AND SOBEL, A.E.	1959.	Arch. Biochem. Biophys., 85, 9.
BÉLANGER, L.F.	1954.	Canad. J. Biochem., 32, 161.
" " "	1955.	J. Dent. Res., 34, 20.
BEVELANDER, G., AND JOHNSON, P.L.	1955.	Ibid., 34, 123.
BOSTRÖM, H., AND ÅQVIST, S.E.G.	1952.	Acta chem. Scand., 6, 1557.
BRAY, H.G., GREGORY, J.E., AND STACEY, M.	1944.	Biochem. J., 38, 142.
CASTELLANI, A.A., AND CASTELLANI-BISI, CARLA.	1958.	Proc. Soc. Exp. Biol. Med., 98, 318.
CLEMMONS, J.J., AND ANGEVINE, D.M.	1957.	Amer. J. Path., 33, 175.
COLLINS, D.H.	1949.	The pathology of articular and spinal diseases, London, p. 8.
CURRAN, R.C.	1957.	J. Path. Bact., 74, 347.
CURRAN, R.C., AND COLLINS, D.H.	1957.	Ibid., 74, 207.
DASLER, W.	1957.	Chicago Med. School Quart., 18, 1.
DASLER, W., AND MILLISER, R.V.	1958.	Proc. Soc. Exp. Biol. Med., 98, 759.
DZIEWIATKOWSKI, D.D.	1952.	J. Exp. Med., 95, 489.

DZIEWIATKOWSKI, D.D.	1954.	Ibid., 100, 11.
ENGFELDT, B., ENGSTRÖM, A., AND BOSTRÖM, H.	1954.	Exp. Cell. Res., 6, 251.
ENGFELDT, B., TEGNER, BIRGIT, AND BERGQUIST, E.	1961.	Acta path. microbiol. scand., 51, Suppl. 144, 47.
ENZINGER, F.M., AND WARNER, E.D.	1957.	Lab. Invest., 6, 251.
FOLLIS, R.H., JR., AND TOUSIMIS, A.J.	1958.	Proc. Soc. Exp. Biol. Med., 98, 843.
FREEMAN, D.J.	1956.	Arch. Path., 61, 219.
GEIGER, BEATRICE, J., STEENBOCK, H., AND PARSONS, HELEN T.	1933.	J. Nutr., 6, 427.
GIBSON, T., CURRAN, R.C., AND KENNEDY, J.S.	1955.	Transplant. Bull., 2, 44.
GIBSON, T., DAVIS, W.B., AND CURRAN, R.C.	1958.	Brit. J. plast. Surg., 11, 177.
GRANT, R.A., HATHORN, M., AND GILLMAN, T.	1960.	Nature, Lond., 186, 164.
GROSS, J., LEVENE, C.I., AND ORLOFF, S.	1960.	Proc. Soc. Exp. Biol. Med., 105, 148.
HAM, KATHYRYN, N.	1960.	J. Path. Bact., 79, 175.
HESS, W.C., AND LEE, C.	1952.	J. Dent. Res., 31, 793.
HURLEY, J.V., AND HAM, KATHRYN, N.	1959.	Brit. J. Exp. Path., 40, 216.
IRVING, J.T.	1957.	Calcium metabolism, London, p. 141.

IRVING, J.T., AND WEINMANN, J.P. 1948. J. Dent. Res., 27, 669.

IRVING, J.T., WEINMANN, J.P., SCHOUR, I., AND TWEEDY, W.R. 1949. Ibid., 28, 362.

KENNEDY, J.S. 1960. J. Path. Bact., 80, 359.

KENNEDY, G.D.C., AND KENNEDY, J.S. 1962. Arch. oral Biol., in press.

KENNEDY, J.S., AND KENNEDY, G.D.C. 1957. J. Anat., 91, 398.

" " " " " 1959. J. Dent. Belge, 40, 63.

" " " " " 1962. J. Path. Bact., 84, 123.

KENT, P.W., JOWSEY, JENIFER, STEDDON, L.M., OLIVER, R., AND VAUGHAN, JANET. 1956. Biochem. J., 62, 470.

KOWALEWSKI, K., AND EMERY, M.A. 1960. Acta endocrinol., 34, 317.

KRIKOS, G.A., AND ORBISON, J.L. 1960. Arch. Path., 70, 188.

LACROIX, P. 1954. Proc. Radioisotope Conference, London, pp. 134-137.

LALICH, J.J. 1956. Arch. Path., 61, 520.

LAYTON, L.L. 1950. Cancer, 3, 725.

LEBLOND, C.P., BÉLANGER, L.F., AND GREULICH, R.C. 1955. Ann. N.Y. Acad. Sci., 60, 630.

LEBLOND, C.P., AND GREULICH, R.C. 1956. In The biochemistry and physiology of bone, ed. by G.H. Bourne, New York, p. 333.

LEVENE, C.I. 1961. Brit. J. Exp. Path., 42, 89.

LEVENE, C.I., AND GROSS, J. 1959. J. Exp. Med., 110, 771.

LISON, L. 1954. Stain Technol., 29, 131.

MARSLAND, E.A. 1951. Brit. Dent. J., 91, 251.

" " " 1952. Ibid., 92, 110.

MATHEWS, M.B. 1959. Biochim. Biophys. Acta. 35, 9.

MENZIES, D.W., AND MILLS, K.W. 1957. J. Path. Bact., 73, 223.

MEYER, K. 1955. Ann. N.Y. Acad. Sci., 60, 803.

" " 1956. In Ciba Foundation symposium on bone structure and metabolism, ed. by G.E.W. Wolstenholme and Cecilia M. O'Connor, London, p. 66.

" " 1959. In Polysaccharides in biology, Trans. 4th Conference, Josiah Macy, Jr. Foundation, New York, p. 25.

MIKKONEN, L., TUOMINEN, T., AND KULONEN, E. 1959-1960. Biochem. Pharmacol., 3, 181.

MUIR, HELEN. 1961. In The biochemistry of mucopolysaccharides of connective tissue, Biochem. Soc. symposium no. 20, ed. by F. Clark and J.K. Grant, Cambridge, p. 20.

PARTRIDGE, S.M., DAVIS, H.F., AND ADAIR, G.S. 1961. Biochem. J., 79, 15.

PEARSE, A.G.E. 1953. Histochemistry, London, p. 436.

PINCUS, P. 1949. Brit. Med. J., 2, 358.

PONSETI, I.V., AND BAIRD, W.A. 1952. Amer. J. Path., 28, 1059.

PONSETI, I.V., AND SHEPARD, R.S.	1954.	J. Bone and Jt. Surg., 36A, 1031.
PONSETI, I.V., WAWZONEK, S., SHEPARD, R.S., EVANS, T.C., AND STEARNS, GENEVIEVE.	1956.	Proc. Soc. Exp. Biol. Med., 92, 366.
ROBINSON, R.A., AND WATSON, M.L.	1953.	In Metabolic interrelations, Trans. 5th Conference, Josiah Macy, Jr., Foundation, New York, p. 104.
ROGERS, H.J.	1951.	Biochem. J., 49, xii.
RUBIN, P.S., AND HOWARD, J.E.	1950.	In Metabolic interrelations, Trans. 2nd Conference, Josiah Macy, Jr. Foundation, New York, pp. 155-166.
SCHILLER, S., MATHEWS, M.B., CIFONELLI, J.A., AND DORFMAN, A.	1958.	J. Biol. Chem., 218, 139.
SCHWARTZ, C.J.	1959.	Brit. J. Exp. Path., 40, 44.
SELYE, H.	1957a.	Ibid., 38, 186.
" "	1957b.	Rev. Canad. Biol., 16, 1.
SELYE, H., AND BOIS, P.	1957.	Endocrinology, 60, 507.
SOBEL, A.E.	1955.	Ann. N.Y. Acad. Sci., 60, 713.
SOGNNAES, R.F.	1955.	Ibid., 60, 545.
STACK, M.V.	1951.	Brit. Dent. J., 90, 173.
" " "	1954.	J. Amer. Dent. Ass., 48, 297.
STEHBENS, W.E.	1962.	J. Path. Bact., 83, 337.
STOREY, E., AND VARASDI, G.	1958.	Brit. J. Exp. Path., 39, 376.
TARVER, H., AND SCHMIDT, C.L.A.	1939.	J. Biol. Chem., 130, 67.

WAWZONEK, S., PONSETI, I.V., SHEPARD, R.S., AND WIEDENMANN, L.G. 1955. Science, 121, 63.

WEATHERELL, J.A., AND WEIDMANN, S.M. 1959. J. Path. Bact., 78, 233.

WISLOCKI, G.B., AND SOGNNAES, R.F. 1950. Amer. J. Anat., 87, 239.

REFERENCES

PART III

ADA, G., AND FULTON, J.D.	1948.	Brit. J. Exp. Path., 29, 524.
BANERJEE, S., AND ROHATGI, K.	1958.	Proc. Soc. Exp. Biol. Med., 97, 234.
BASSIOUNI, M.	1955.	Ann. Rheum. Dis., 14, 288.
BETTELHEIM-JEVONS, F.R.	1958.	Advanc. Protein Chem., 13, 35.
BLOCK, W.D., RUKAVINA, J.G., AND CURTIS, A.C.	1955.	Proc. Soc. Exp. Biol. Med., 89, 175.
BOHLE, A., HARTMANN, F., AND POLA, W.	1950-51.	Arch. path. Anat., 319, 231.
BRAUNSTEIN, H., AND BUERGER, L.	1959.	Amer. J. Path., 35, 791.
BURNET, F.M., AND FENNER, F.J.	1949.	The production of antibodies. Monograph no. 1, Walter and Eliza Hall Institute, Melbourne, 2nd ed.
BYWATERS, E.G.L., AND GLYNN, L.E.	1957.	In Biochemical disorders in human disease, ed. by R.H.S. Thompson and E.J. King, London, p. 648.
CALKINS, E., COHEN, A.S., AND LARSEN, B.	1960.	Ann. N.Y. Acad. Sci., 86, 1033.
CHRISTENSEN, H.E.	1960.	Acta path. microbiol. scand., 50, 29.
COHEN, A.S., AND CALKINS, E.	1960.	J. Exp. Med., 112, 479.
COHEN, A.S., CALKINS, E., AND LEVENE, C.I.	1959.	Amer. J. Path., 35, 971.

COHEN, A.S., WEISS, L., AND CALKINS, E. 1960. Ibid., 37, 413.

CURRAN, R.C. 1957. J. Path. Bact., 74, 347.

CURTAIN, C.C. 1955. Biochem. J., 61, 688.

DAVIES, G.E., AND LOWE, J.S. 1960. Brit. J. Exp. Path., 41, 335.

DICK, G.F., AND LEITER, L. 1941. Amer. J. Path., 17, 741.

EKLUND, C.M., AND REIMANN, H.A. 1936. Arch. Path., 21, 1.

FABER, M. 1948. Acta med. scand., suppl. 206, p. 351.

FAGRAEUS, ASTRID. 1948. J. Immunol., 58, 1.

FARQUHAR, MARYLIN G., HOPPER, J., JR., AND MOON, H.D. 1959. Amer. J. Path., 35, 721.

FRIBERG, U. 1958. Arkiv Kemi Min. Geol., 12, 481.

FRUHLING, L., KEMPF, J., AND PORTE, A. 1960. C.R. Acad. Sci., 250, 1385.

FULTON, J.D., AND NIVEN, JANET S.F. 1950-51. Trans. Roy. Soc. Trop. Med. Hyg., 44, 717.

GELLHORN, A., VAN DYKE, H.B., PYLES, W.J., AND TUPIKOVA, N.A. 1946. Proc. Soc. Exp. Biol. Med., 61, 25.

GILES, R.B., JR., AND CALKINS, E. 1958. J. Clin. Invest., 37, 846.

GILLILAND, I.C., HANNO, M.G., AND STRUDWICK, J.I. 1954. Biochem. J., 56, xxxii.

GILLILAND, I.C., AND STANTON, E. 1954. J. Clin. Path., 7, 172.

HASS, G. 1942. Arch. Path., 34, 92.

HASS, G.M., HUNTINGTON, R., AND KRUMDIECK, N. 1943. Ibid., 35, 226.

HASS, G., AND SCHULZ, R.Z. 1940. Ibid., 30, 240.

KENNEDY, J.S. 1962. J. Path. Bact., 83, 165.

LARSEN, B. 1957. Acta. rheum. scand., 3, 30.

LETTERER, E. 1934. Arch. path. Anat., 293, 34.

MARSHALL, A.H.E. 1956. An outline of the cytology and pathology of the reticular tissue, Edinburgh and London, p. 107.

MELLORS, R.C., AND ORTEGA, L.G. 1956. Amer. J. Path., 32, 455.

MESSIER, B., AND LEBLOND, C.P. 1957. Proc. Soc. Exp. Biol. Med., 96, 7.

MEYER, K. 1947. Physiol. Rev., 27, 335.

MISSEN, G.A.K., AND TAYLOR, JEAN D. 1956. J. Path. Bact., 71, 179.

MULLIGAN, R.M. 1958. Arch. Path., 65, 615.

OAKLEY, C.L. 1959. In Modern trends in pathology, ed. by D.H. Collins, London, p. 61.

PERÄSALO, O., AND LATVALAHTI, J. 1954. Acta path. microbiol. scand., 34, 208.

PIRANI, C.L., CATCHPOLE, H.R., AND MOORE, O. 1959. Fed. Proc., 18, 500.

POPPER, H. 1954. Amer. J. Med., 16, 98.

POPPER, H., AND ELIAS, H.	1955.	Amer. J. Path., 31, 405.
ROBERTS, K.B.	1955.	Brit. J. Exp. Path., 36, 199.
ROTHBARD, S., AND WATSON, R.F.	1954.	Proc. Soc. Exp. Biol. Med., 85, 133.
SPECTOR, W.G.	1958.	Pharmacol. Rev., 10, 475.
" " "	1959.	In Modern trends in pathology, ed. by D.H. Collins, London, p. 73.
SPIRO, D.	1959.	Amer. J. Path., 35, 47.
STEHBENS, W.E.	1962.	J. Path. Bact., 83, 337.
SYMMERS, W. ST. C.	1956.	J. Clin. Path., 9, 187.
TEILUM, G.	1952.	Ann. Rheum. Dis., 11, 119.
" "	1954.	J. Lab. Clin. Med., 43, 367.
" "	1956.	Amer. J. Path., 32, 945.
VAZQUEZ, J.J., AND DIXON, F.J.	1956.	J. Exp. Med., 104, 727.
VIRCHOW, R.	1860.	Cellular pathology, 2nd ed., London, p. 371.
WAGNER, B.M.	1955.	Arch. Path., 60, 221.
WILLIAMS, G.	1959.	Brit. J. Exp. Path., 40, 176.

REFERENCES

PART IV

COPE, O., RAWSON, R.W., AND McARTHUR, J.W. 1947. Surg. Gynec. Obstet., 84, 415.

CURRAN, R.C., ECKERT, H., AND WILSON, G.M. 1958. J. Path. Bact., 76, 541.

DAILEY, M.E., LINDSAY, S., AND SKAHEN, R. 1955. Arch. Surg., Chicago, 70, 291.

DEMPSEY, W.S., DINSMORE, R.S., AND HAZARD, J.B. 1949. Cleveland Clin. Quart., 16, 132.

DOBYNS, B.M., AND LENNON, BEATRICE. 1948. J. clin. Endocrin., 8, 732.

DONIACH, I., HOWARD, ALMA, AND PELC, S.R. 1953. Progr. Biophys. biophys. Chem., 3, 1.

EVANS, R.W. 1956. Histological appearances of tumours, Edinburgh, p. 555.

FISHER, E.R., AND CREED, D.L. 1956. Amer. J. Surg., 91, 60.

FITZGERALD, P.J. 1955. In The thyroid, Brookhaven Symposia in Biology, no. 7, Upton, New York, p. 220.

FITZGERALD, P.J., AND FOOTE, F.W. 1949. J. clin. Endocrin., 9, 1153.

FITZGERALD, P.J., FOOTE, F.W., AND HILL, R.F. 1950. Cancer, 3, 86.

FRASER, G.R., MORGANS, M.E., AND TROTTER, W.R. 1961. In Advances in thyroid research, ed. by Rosalind Pitt-Rivers, London, p. 19.

GROSS, J. 1957. Int. Rev. Cytol., 6, 265.

HALLEY, J.B.W. 1961. J. Path. Bact., 81, 347.

HAYLES, A.B., KENNEDY, R.L.J., WOOLNER, L.B., AND BLACK, B.M. 1956. J. clin. Endocrin. Metab., 16, 1580.

KELSEY, M.P., HAINES, S.F., AND KEATING, F.R. JR. 1949. J. clin. Endocrin., 9, 171.

LEBLOND, C.P., AND GROSS, J. 1948. Endocrinology, 43, 306.

" " " " " 1949. J. clin. Endocrin., 9, 149.

LENNOX, B. 1948. J. Path. Bact., 60, 295.

LINDSAY, S., DAILEY, M.E., FRIEDLANDER, J., YEE, G., AND SOLEY, M.H. 1952. J. clin. Endocrin., 12, 1578.

McGIRR, E.M. 1960a. Brit. Med. Bull., 16, 113.

" " " 1960b. In Progress in clinical endocrinology, ed. by E.B. Astwood, New York, 1, 133.

McGIRR, E.M., CLEMENT, W. ELSPETH, CURRIE, A.R., AND KENNEDY, J.S. 1959. Scot. Med. J., 4, 232.

McGIRR, E.M., HUTCHISON, J.H., CLEMENT, W. ELSPETH, KENNEDY, J.S., AND CURRIE, A.R. 1960. Ibid., 5, 189.

MILLES, G. 1955. Amer. J. Path., 31, 997.

NADLER, N.J., AND LEBLOND, C.P. 1955. In The thyroid, Brookhaven Symposia in Biology, no. 7, Upton, New York, p. 40.

NANDI, S., PODDAR, R.K., AND PYNE, C.K. 1956. J. Endocrin., 13, 125.

PITT-RIVERS, ROSALIND, HUBBLE, D., AND HOATHER, W.H. 1957. J. clin. Endocrin., 17, 1313.

POCHIN, E.E. 1958. In Modern trends in endocrinology, ed. by H. Gardiner-Hill, London, p. 46.

PULVERTAFT, R.J.V., DAVIES, JOAN R., WEISS, L., AND WILKINSON, U.H. 1959. J. Path. Bact., 77, 19.

PUPPEL, I.D., LEBLOND, C.P., AND CURTIS, G.M. 1946. Trans. Amer. Ass. Goiter, 175.

STATLAND, H., WASSERMAN, M.M., AND VICKERY, A.L. 1951. Arch. intern. Med., 88, 659.

TAYLOR, S. 1953. J. clin. Endocrin. Metab., 13, 1232.

" " 1960. Brit. Med. Bull., 16, 102.

TREMBLAY, G., AND PEARSE, A.G.E. 1960. J. Path. Bact., 80, 353.

WARREN, S., AND MEISSNER, W.A. 1953. Atlas of tumour pathology, Washington, D.C., section 4, fascicle 14.

WILKINS, L., CLAYTON, G.W., AND BERTHRONG, M. 1954. Pediatrics, 13, 235.

WILLIS, R.A. 1953. Pathology of tumours, 2nd ed., London, p. 601.

WOLLMAN, S.H., AND WODINSKY, I. 1955. Endocrinology, 56, 9.

REFERENCES

PART V

ANDREASSEN, A.K.	1954.	Acta chir. scand., 107, 214.
BELKIN, A., MACQUEEN, D.G., AND DUFFIN, J.D.	1954.	Canad. Med. Assoc. J., 71, 59.
BENNETT, H.S.	1941.	Amer. J. Anat., 69, 333.
COCK, J.E., URICH, R.W., SAMPLE, H.G., JR., AND FAWCETT, N.W.	1960.	Ann. Int. Med., 52, 126.
COUPLAND, R.E.	1954.	J. Anat., 88, 142.
DAVIS P., PEART, W.S., AND VAN'T HOFF, W.	1955.	Lancet, 2, 274.
ERÄNKÖ, O.	1957.	J. Histochem. Cytochem., 5, 408.
VON EULER, U.S., AND HAMBERG, U.	1949.	Science, 110, 561.
GERARD, P., CORDIER, R., AND LISON, L.	1930.	Bull. Histol. Tech. Micr., 7, 133.
HALE, A.J.	1958.	J. Physiol., 141, 193.
HALLEY, J.W.B.	1961.	J. Path. Bact. 81, 347.
HARLEY-MASON, J.	1948.	Experientia, 4, 307.
" " "	1950.	J. Chem. Soc., 2, 1276.
HILLARP, N.Å., AND HÖKFELT, B.	1953.	Acta physiol. scand., 30, 55.
" " " " "	1955.	J. Histochem. Cytochem., 3, 1.
KENNEDY, J.S., SYMINGTON, T., AND WOODGER, B.	1961.	J. Path. Bact., 81, 409.

KIMBERLEY, R.C. 1956. Arch. Surg., 73, 369.

McMILLAN, MARY. 1956. Lancet, 2, 284.

MANGER, W.M., FLOCK, E.V., BERKSON, J., BOLLMAN, J.L., ROTH, G.M., BALDES, E.J., AND JACOBS, H. 1954. Circulation, 10, 641.

OGATA, T., AND OGATA, A. 1922-23. Beitr. path. Anat., 71, 376.

PUGH, R.C.B., GRESHAM, G.A., AND MULLANEY, JOAN. 1960. J. Path. Bact., 79, 89.

SYMINGTON, T., AND GOODALL, A.L. 1953. Glasg. Med. J., 34, 75.

WILLIS, R.A. 1953. Pathology of tumours, 2nd ed., London, p. 873.

REFERENCES

PART VI

ALLEN, W.M., SHERMAN, A.I., AND ARNESON, A.N.	1954.	Amer. J. Obstet. Gynec., 68, 1433.
ALLEN, W.M., SHERMAN, A.I., AND ARNESON, A.N.	1955.	Amer. J. Obstet. Gynec., 70, 786.
ANDREWS, G.A., ROOT, S.W., AND KNISELEY, R.M.	1953.	Cancer, 6, 294.
BERG, H.F., CHRISTOPHERSEN, W.M., AND BRYANT, J.R.	1955.	J. thorac. Surg., 29, 497.
BLOOM, W.	1948.	Histopathology of irradiation. New York, p. 352.
BRYANT, J.R., BERG, H.F., AND CHRISTOPHERSEN, W.M.	1953.	J. thorac. Surg., 26, 221.
HAHN, P.E., AND CAROTHERS, E.L.	1953.	Ibid., 25, 265.
HAHN, P.E., HILLIARD, G.W., AND CAROTHERS, E.L.	1953.	Brit. J. Radiol., 26, 595.
HULTBORN, K.A., LARSSON, L.G., AND RAGNHULT, I.	1955.	Acta radiol. Stockh., 43, 52.
JENTZER, A., AND WENGER, P.	1950.	Rev. méd. Liège, 5, 425.
KENNEDY, J.S., THOMSON, J.D., AND WALKER, F.C.	1957.	Scot. Med. J., 2, 105.
KNUTSON, F., AND NORIN, T.	1956.	Acta. path. microbiol. scand., 38, 447.
KOTTMEIER, H.L., AND MOBERGER, G.	1955.	Acta. obstet. gynec. scand., 34, 1.
McCORMICK, J.B., MILLES, G., JAFFE, B., AND SEED, L.	1954.	Arch. Path., 58, 187.

MELLGREN, J., KNUTSON, F., AND NORIN, T.	1954.	Acta. path. microbiol. scand., 34, 393.
MENEELY, G.R., AUERBACH, S.H., WOODCOCK, C.C., KORY, R.C., AND HAHN, P.F.	1953.	Amer. J. med. Sci., 225, 172.
SHERMAN, A.I., AND TER-POGOSSIAN, M.	1953.	Cancer, 6, 1238.
WHEELER, H.B., JAQUES, W.E., ALLEN, M.B., SOLTES, M., O'CONOR, V.J., AND BLACK, H.	1956.	Surg. Gynec. Obstet., 102, 166.

AUTORADIOGRAPHIC STUDIES WITH RADIOACTIVE ISOTOPES

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF MEDICINE

OF THE UNIVERSITY OF GLASGOW

BY

JOHN STEWART KENNEDY, M.A., M.B., CH.B.

VOLUME II

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VOLUME II

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P A R T I

Tables 1,1 - 1,8

TABLE 1,1

INTENSITY OF SULPHUR-35 IN AUTORADIOGRAPHS COMPARED WITH THE DENSITY OF ALCIAN BLUE STAINING IN COMPARABLE SECTIONS OF TISSUES FROM THE NEW-BORN MOUSE

Tissue	Intensity of	
	sulphur-35 in autoradiograph	alcian-blue staining
SKIN		
Mast cells	+++	+
Reticular layer of dermis	+	+
Hair bulb and papilla	++	++
Hair (whisker): hyaline and dermal layers of the follicle	++	++
SKELETAL SYSTEM		
Cartilage cell: cytoplasm	+++	+ to ++
Matrix of cartilage	++	++ to +++
Bone lamellae	++	+++
EYE		
Cornea	++	++
Sclera	+	+
Lens	0	+
Lens capsule	+	+
Ciliary body	+	+
Retina: inner plexiform layer	+	+
Lachrymal gland: stroma	+	++
Lachrymal gland: acinar cells	0	0
CARDIOVASCULAR SYSTEM		
Valve cusps	++	++
Aorta and venae cavae: tunica media	++	+
Arteries and veins, including coronary vessels: tunica media	++	+
Endocardium: connective tissue	+	+
ALIMENTARY SYSTEM		
Unerupted tooth: cells and matrix of dental papilla	++	+
Unerupted tooth: predentine	++	+++
Unerupted tooth: odonto-blasts; cytoplasm	+	?

Intensity of image and staining indicated on scale 0 to +++

continued on next page

TABLE 1,1 (continued)

INTENSITY OF SULPHUR-35 IN AUTORADIOGRAPHS COMPARED WITH THE DENSITY OF ALCIAN BLUE STAINING IN COMPARABLE SECTIONS OF TISSUES FROM THE NEW-BORN MOUSE

Tissue	Intensity of	
	sulphur-35 in autoradiograph	alcian-blue staining
ALIMENTARY SYSTEM		
Salivary gland: acinar cells	++	+ to ++
Salivary gland: peri-ductal connective tissue	++	++
Salivary gland: stroma	+	+
Stomach: mucus-secreting glands	+++	+++
Stomach: proventriculus; submucosa	+	+
Stomach: micro-organisms on mucosal surface	+	++
Small and large intestines: goblet-cell cytoplasm and mucus	+++	+++
Pancreas: acinar cells	+	+
Pancreas: islets of Langerhans	0	0
Liver: giant cells (?mega-karyocytes)	++	++
Liver: bile-duct epithelium	+	+
RETICULO-ENDOTHELIAL AND HAEMATOPOIETIC SYSTEMS		
Spleen: giant cells (?mega-karyocytes)	+++	+
Spleen: fibrous septa	+	+
Marrow: megakaryocytes	++	++
RESPIRATORY SYSTEM		
Connective tissue of lung	+	+
Respiratory epithelium	0	+
GENITO-URINARY SYSTEM		
Convoluted tubules of kidney	?	+
Granulosa and thecal cells in ovary	+	0

Intensity of image and staining indicated on scale 0 to +++

continued on next page

TABLE 1,1 (continued)

INTENSITY OF SULPHUR-35 IN AUTORADIOGRAPHS COMPARED WITH THE DENSITY OF ALCIAN BLUE STAINING IN COMPARABLE SECTIONS OF TISSUES FROM THE NEW-BORN MOUSE

Tissue	Intensity of	
	sulphur-35 in autoradiograph	alcian-blue staining
GENITO-URINARY SYSTEM		
Liquor folliculi	+	++
Vaginal wall: connective tissue	+	+
Vas deferens: connective tissue	+	+
NERVOUS SYSTEM		
Brain	+	+
Peripheral nerves	++	+
ENDOCRINE SYSTEM		
X zone of adrenal	0	+++

Intensity of image and staining indicated on scale 0 to +++

TABLE 1,2

CONCENTRATION OF SULPHUR-35 IN AUTORADIOGRAPHS

+++	++	+
Mast cells	Megakaryocytes of marrow	Connective tissues of salivary and lachyymal glands, proventriculus, eye, spleen, lung, vagina, skin and heart
Cartilage cells	Liver: giant-cells (?megakaryocytes)	Retina: inner plexiform layer
Mucin glands of stomach	Bone: lamellae	Peripheral nerves and brain
Goblet cells of intestine: mucigen and cytoplasm	Salivary gland: acinar cells	Lens capsule
Mucin in lumen of intestine	Hair: hyaline layer of the follicle and connective tissues of bulb and papilla	Bacteria of proventriculus
Giant-cells (?megakaryocytes) of spleen	Unerupted tooth: papilla and predentine	Acinar cells of pancreas
	Aorta, venae cavae, and small arteries and veins: tunica media	Odontoblasts
	Valve cusps	
	Brain: superficial acellular zone	
	Cartilage: matrix	
	Cornea	

Intensity of image is indicated on scale + to +++

TABLE 1,3

GROUP I - NORMAL FIBROGENESIS

EXPERIMENTAL DETAILS AND SUMMARY OF RESULTS

<table>
<tr><th>Animals</th><th>Age</th><th>Sulphate-35</th><th>Results</th></tr>
<tr><td>11 mice</td><td>Foetal</td><td>1-5µc/g to parent</td><td rowspan="5">1. Compact mast cell image (Storage).

2. Diffuse fibroblast-fibre image (Secretion). Maximum at young collagen stage.

3. Fibrogenesis in absence of mast cells (Tendon, cartilage, compact connective tissue).

4. Fibrogenesis in presence of mast cells (Dermis, loose connective tissue).</td></tr>
<tr><td>2 chicks</td><td>Embryonic</td><td>100 µc to C.A.M.</td></tr>
<tr><td>18 mice</td><td rowspan="2">Neo-natal</td><td rowspan="2">1-5µc/g to parent or direct</td></tr>
<tr><td>10 rats</td></tr>
<tr><td colspan="3">Animals killed 12-48 hours after isotope injection.</td></tr>
</table>

C.A.M. = chorio-allantoic membrane

TABLE 1,4

GROUP 2 - EXPERIMENTAL TUMOURS

EXPERIMENTAL DETAILS AND SUMMARY OF RESULTS

Animals	Carcinogen	Tumours	Results
7 rats	20-methyl cholanthrene. Subcutaneous injection.	Sarcoma (Locally invasive)	Sarcoma 1. Mast cell population varies indirectly with degree of cellular pleomorphism. 2. Mast cell population low in fields of active fibrogenesis. 3. Fibroblast-fibre image maximum at young collagen stage.
13 mice	20-methyl cholanthrene. Repeated cutaneous application.	Papilloma Carcinoma (Locally invasive)	
Sulphate-35 given in dose 1-5μc/g 12-48 hours before sacrifice.			Epithelial tumours 1. Stromal activity mainly (Fibroblasts + fibres). 2. Grouping of mast cells on periphery.

TABLE 1,5

GROUP 3 - GRANULATION TISSUE

EXPERIMENTAL DETAILS AND SUMMARY OF RESULTS

Animals	Lesions	Agent	Results
14 guinea-pigs 10 mice	Granuloma	Carrageenin	1. Most fields of fibrogenesis unrelated to mast cells. 2. S35 utilisation begins at fine argyrophilic fibre stage. Image diffuse. 3. S35 utilisation a/ maximum about same time as total new collagen maximal. 4. S35 utilisation decreases thereafter.
12 mice	Abscess	Sodium Caseinate	
10 rats	Ulcer	Sodium Fluoride	
1 mouse	Peritonitis	Freund-Type Adjuvant	
2 chicks	Human skin graft on C.A.M.		
Sulphate-35 given in dose 1-5µc/g or 100 µc to yolk sac 24-48 hours before sacrifice.			

C.A.M. = chorio-allantoic membrane

TABLE 1,6

GROUP 4 - EXPERIMENTAL CIRRHOSIS

EXPERIMENTAL DETAILS AND SUMMARY OF RESULTS

Animals	Lesion	Cortisone	Results
9 mice	Cirrhosis	None	1. Sulphur-35 utilisation in cirrhotic lesions. 2. Sulphur-35 utilisation in catgut lesions. 3. Cortisone does not inhibit sulphur-35 utilisation in fibrogenesis.
9 mice	Catgut implant	Daily	
9 mice	Catgut implant	None	
25 mice	Cirrhosis and catgut implant	None	
1. Sulphate-35 dose 100 μc 24 hours before sacrifice. 2. Daily cortisone dose 0.25 mg. 3. Carbon tetrachloride given orally in dose 0.1 ml. of mixture 4 parts CCl_4 and 6 parts liquid paraffin.			

TABLE 1,7

GROUP 5 - HUMAN BIOPSY MATERIAL

EXPERIMENTAL DETAILS AND SUMMARY OF RESULTS

Lesion	Cases	Results
Pleomorphic Adenoma	10	Pleomorphic adenoma 1. No active fibrogenesis and no mast cells: occasional stromal fibroblast shows sulphate-35 uptake.
Urticaria Pigmentosa	1	
Tissue maintained 48 hours in nutrient medium containing 1 µc/ml sulphate-35.		Urticaria pigmentosa 1. Mast cell utilisation of sulphate-35 as *in vivo*. 2. No activity in argyrophilic fibres between mast cells.

TABLE 1,8

RADIO-SULPHATE UPTAKE IN MAST CELLS AT DIFFERENT STAGES OF RECOVERY AFTER THE ADMINISTRATION OF COMPOUND 48/80

Rat	Estimated sulphur-35 uptake (coarse grain)	Number of mast cells showing radioactivity (50 counted)	
		Fine grain I	Fine grain II
R0	+++	9	47
R1	++++	26	44
R2	+++	50	46
R3	0	0	9
R4	++	10	43
R7	0	1	5
R9	++++	50	49
R11	+	8	9
R14	+++	41	44
R16	+	47	35
R22	0	36	9
Normal	++++	17	19

Image intensity indicated on scale 0 to ++++

P A R T I

Figs. 1,1 - 1,42

Fig. 1,1. - Skin showing sulphate-image over mast cells in the dermis. Mouse. Autoradiograph, Kodak AR10. X 750.

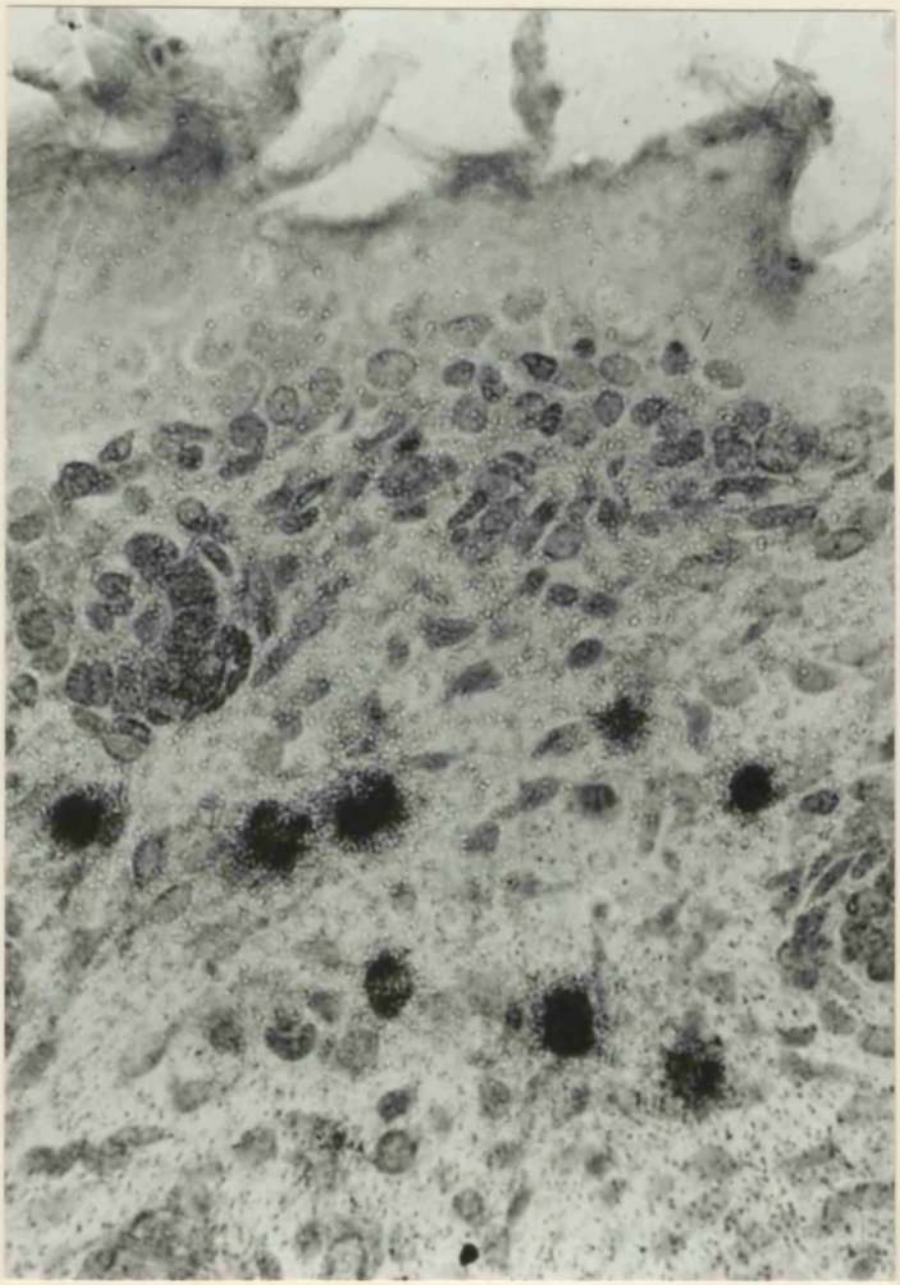

FIG.1,1

Fig. 1,2. - Tactile hair. Radio-sulphate is present in the hyaline and dermal layers of the follicle. The hair shaft and external sheath are inactive. Mouse. Autoradiograph, Kodak AR50. X 50.

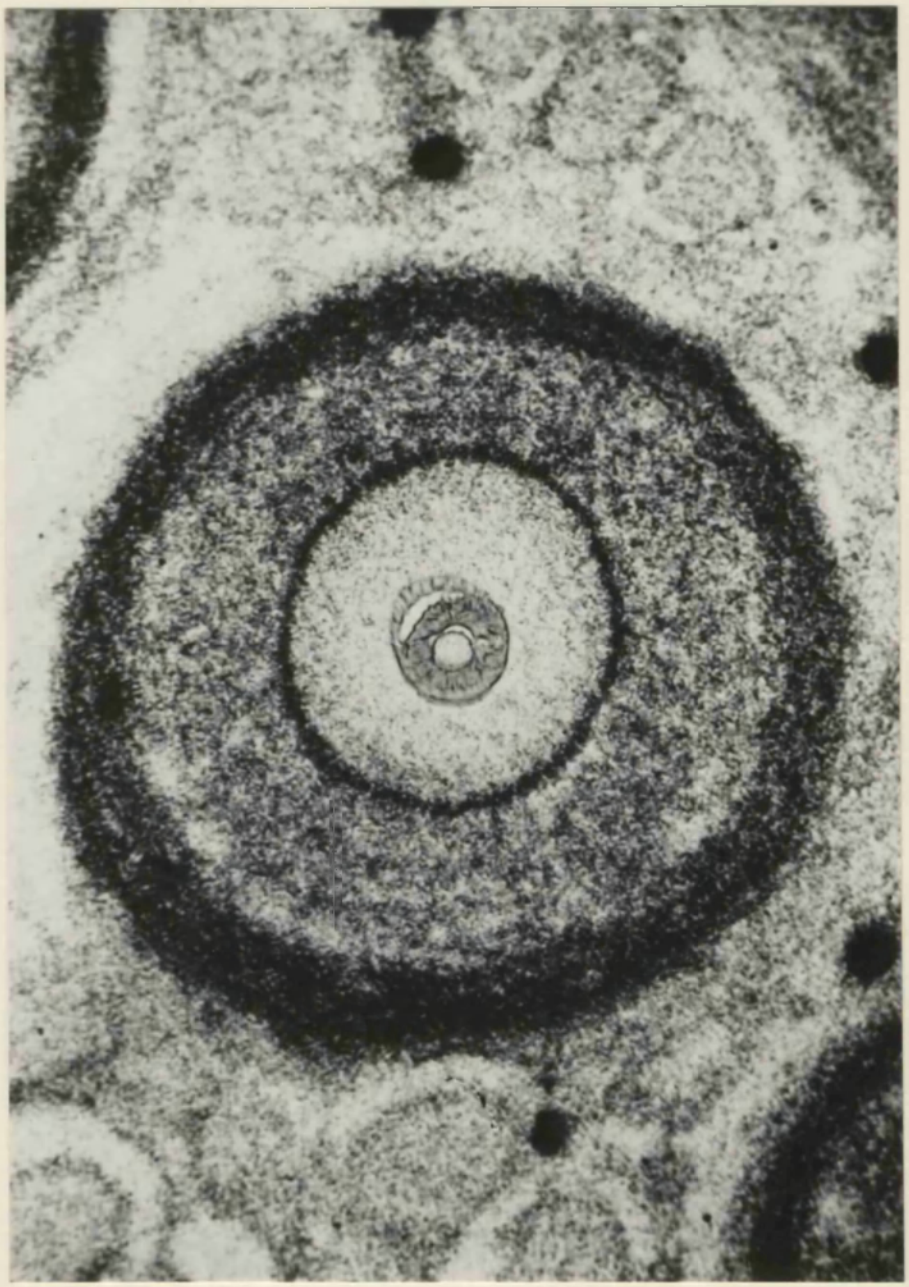

FIG.1,2

Fig. 1,3. - Rib. Intense sulphate-image over cartilage matrix and cells. Mouse. Autoradiograph, Kodak AR10. X 225.

Fig. 1,4. - Rib. The sulphate-image after hyaluronidase incubation of the section is mainly cellular. The matrix is almost unlabelled. Mouse. Autoradiograph, Kodak AR10. X 225.

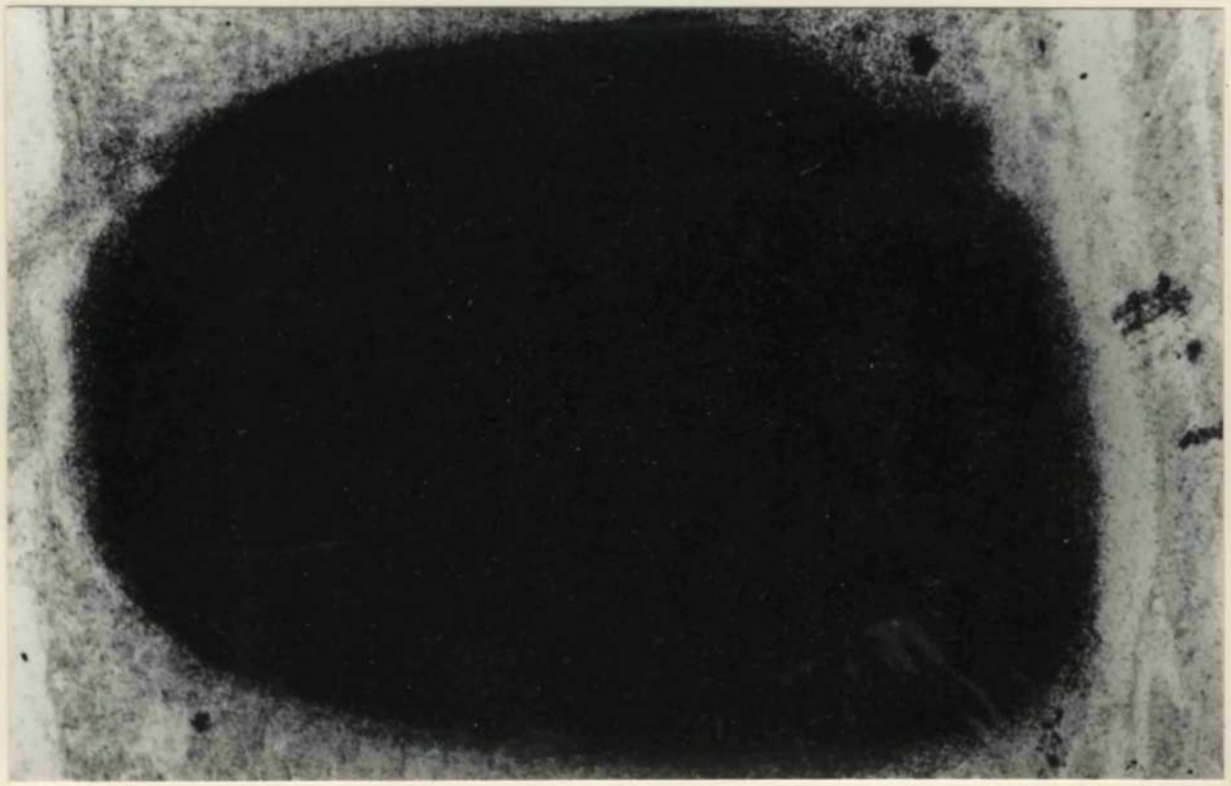

FIG.1,3

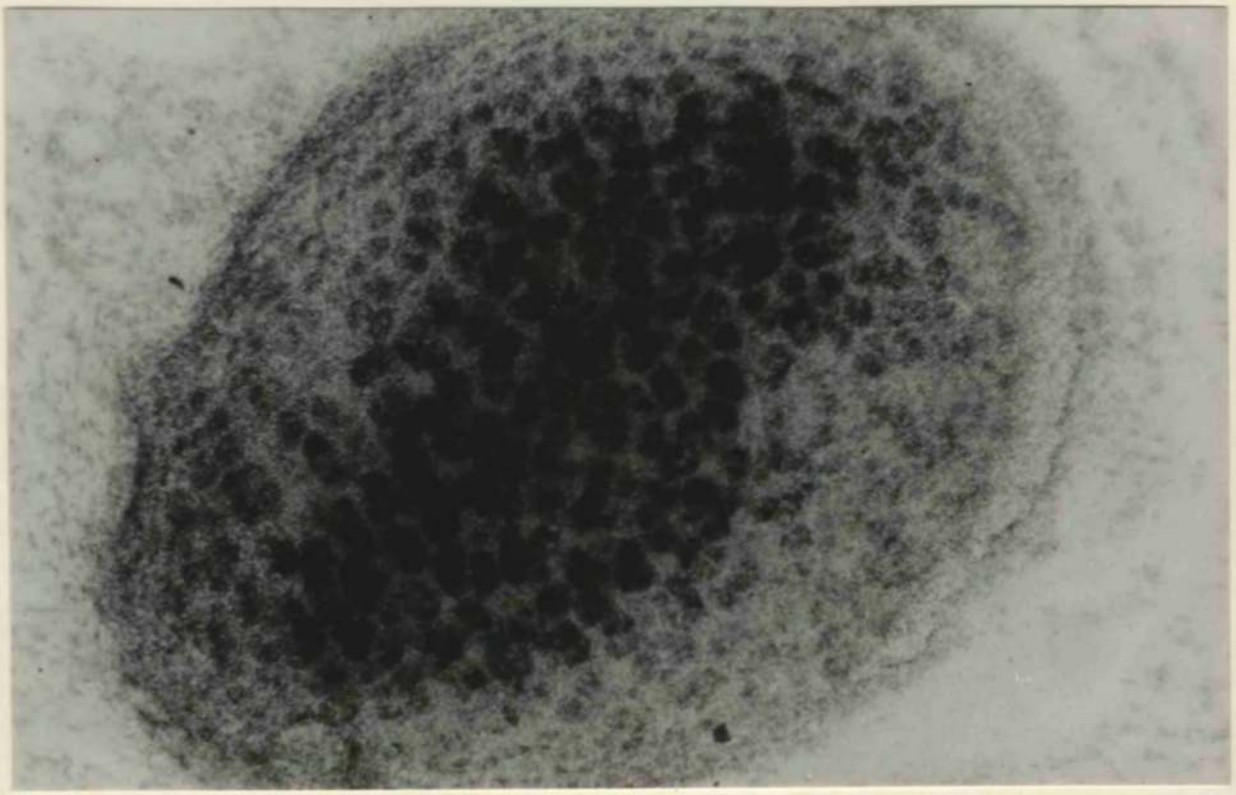

FIG.1,4

Fig. 1,5. - Vertebral bodies. Bone lamellae show a dense sulphate-image. The epiphyseal cartilage also contains radio-sulphate. Mouse. Autoradiograph, Kodak AR10. X 90.

Fig. 1,6. - Eye. The cornea and lens capsule contain sulphur-35. Mouse. Autoradiograph, Kodak AR10. X 75.

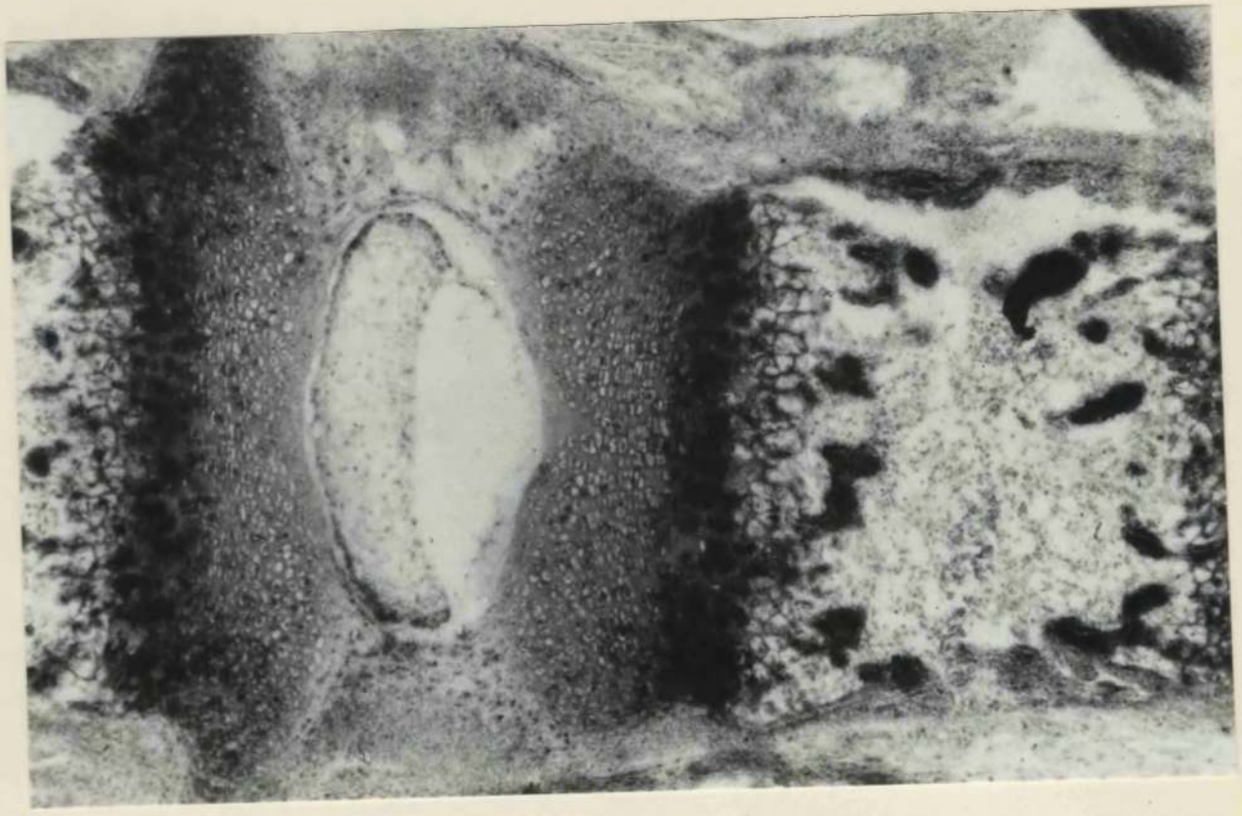

FIG.1,5

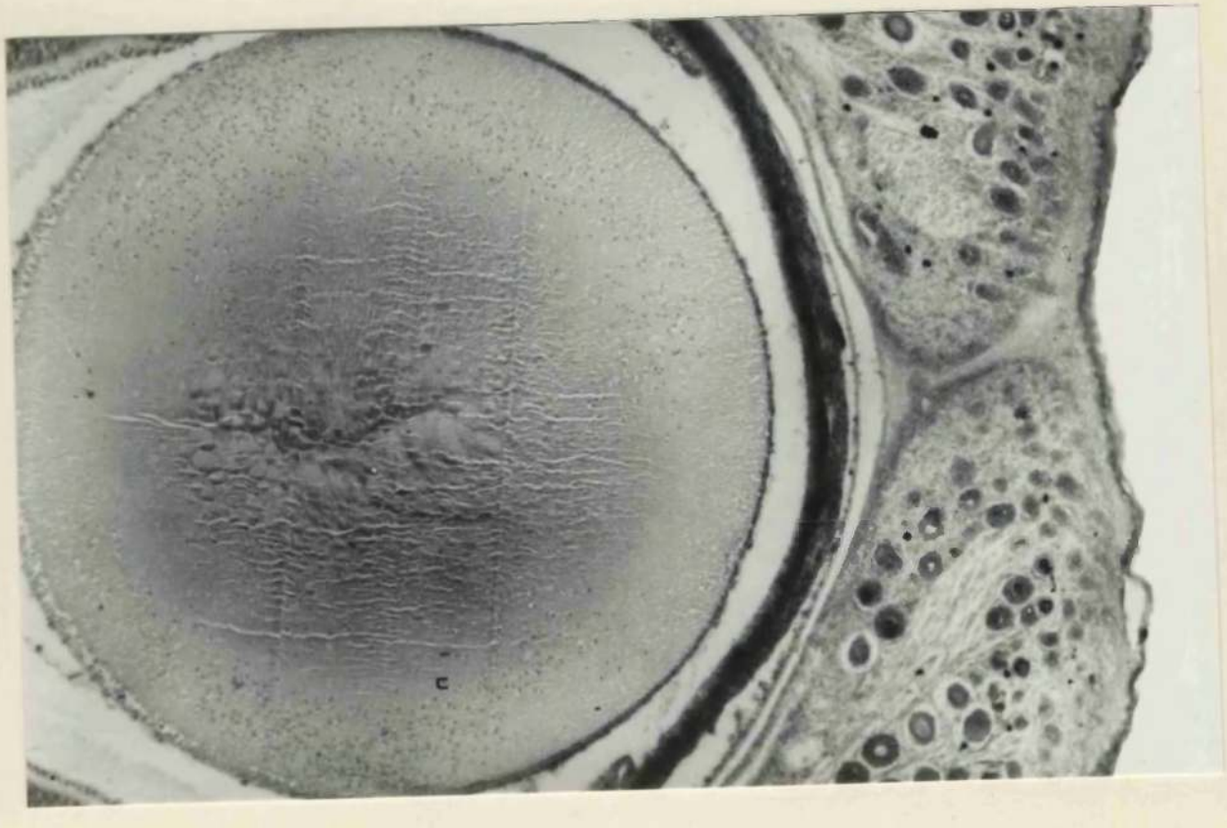

FIG.1,6

Fig. 1,7. - Heart. The valve rings and cusps are clearly outlined by their sulphate-images. Mouse. Autoradiograph, Kodak AR10. X 90.

Fig. 1,8. - Blood vessel. Sulphur-35 is present in the media. Mouse. Autoradiograph, Kodak AR10. X 90.

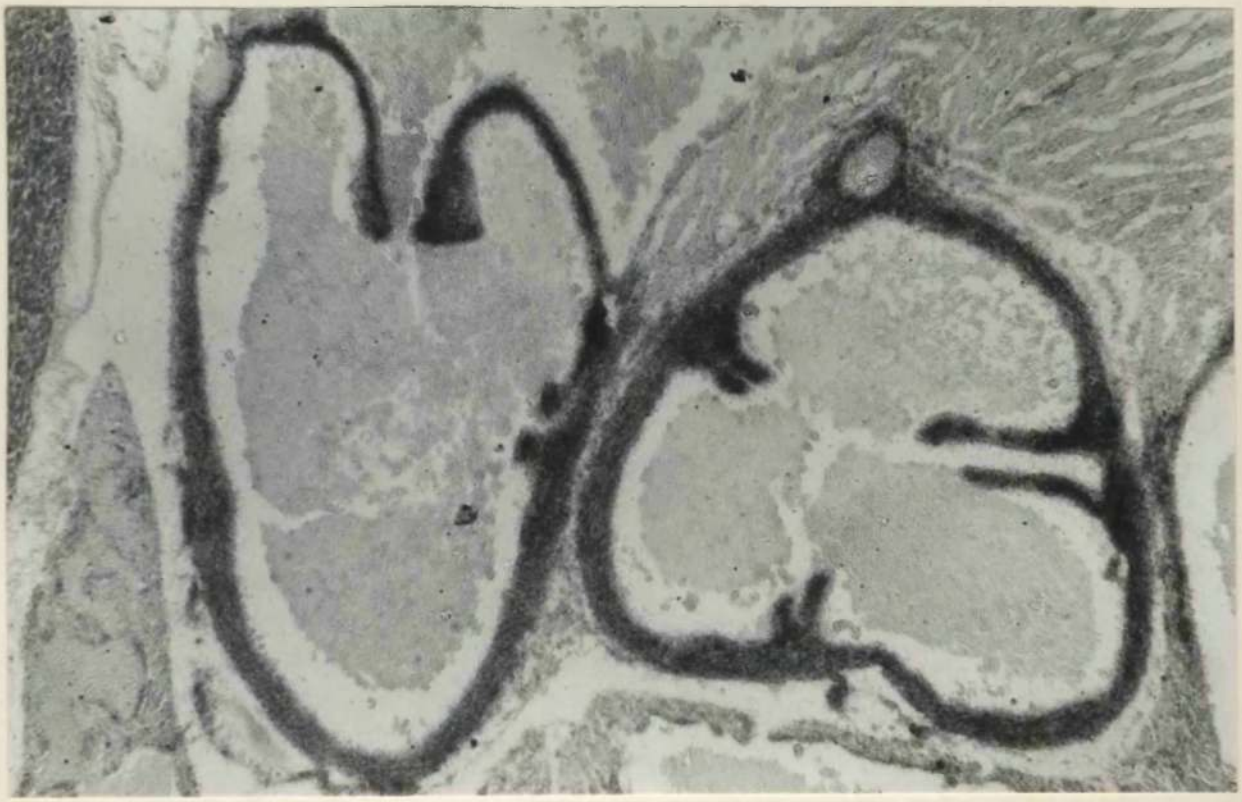

FIG.1,7

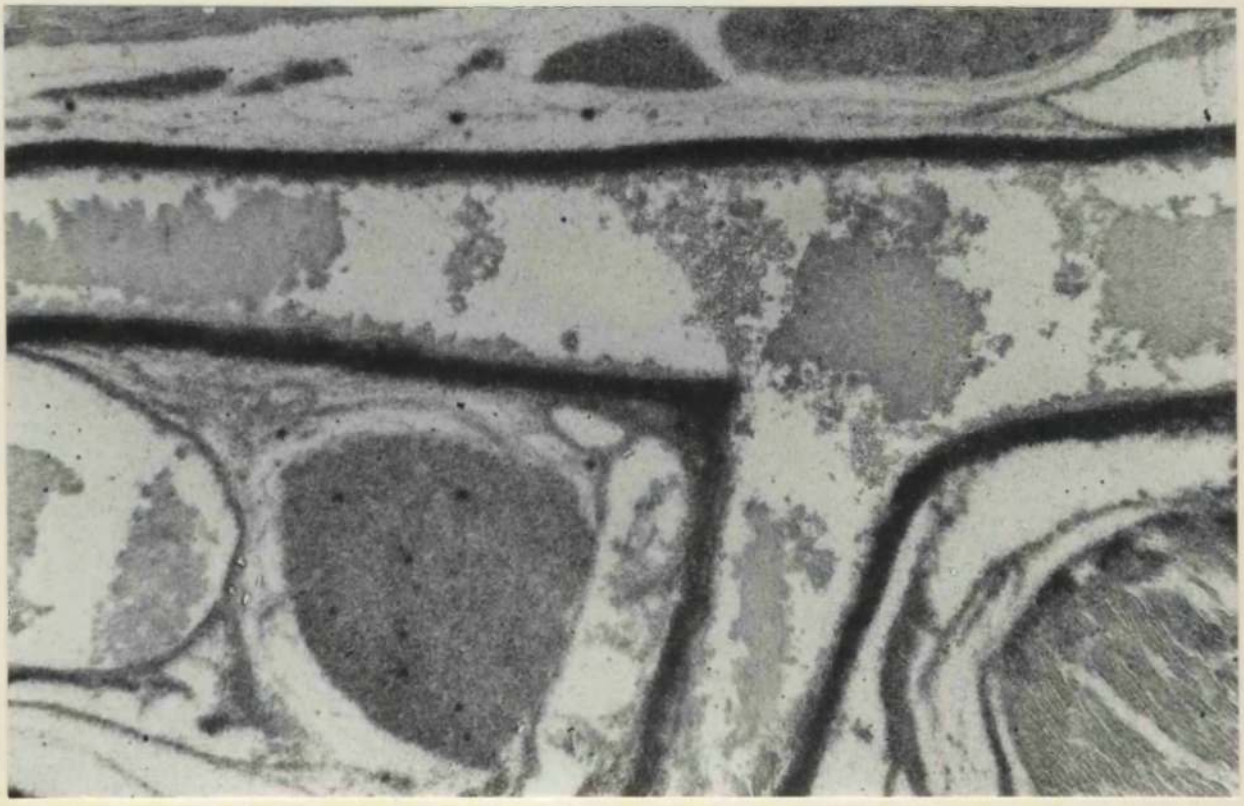

FIG.1,8

Fig. 1,9. - Pharyngeal gland. An intense sulphate-image is present over the acini and labelled mucus is seen in the duct. Mouse. Autoradiograph, Kodak AR10. X 140.

Fig. 1,10. - Intestine and pancreas. Goblet cells, mucus and pancreatic acinar cells show intense sulphate-images. The islet cells stand out clearly as inactive. Mouse. Autoradiograph, Kodak AR10. X 90.

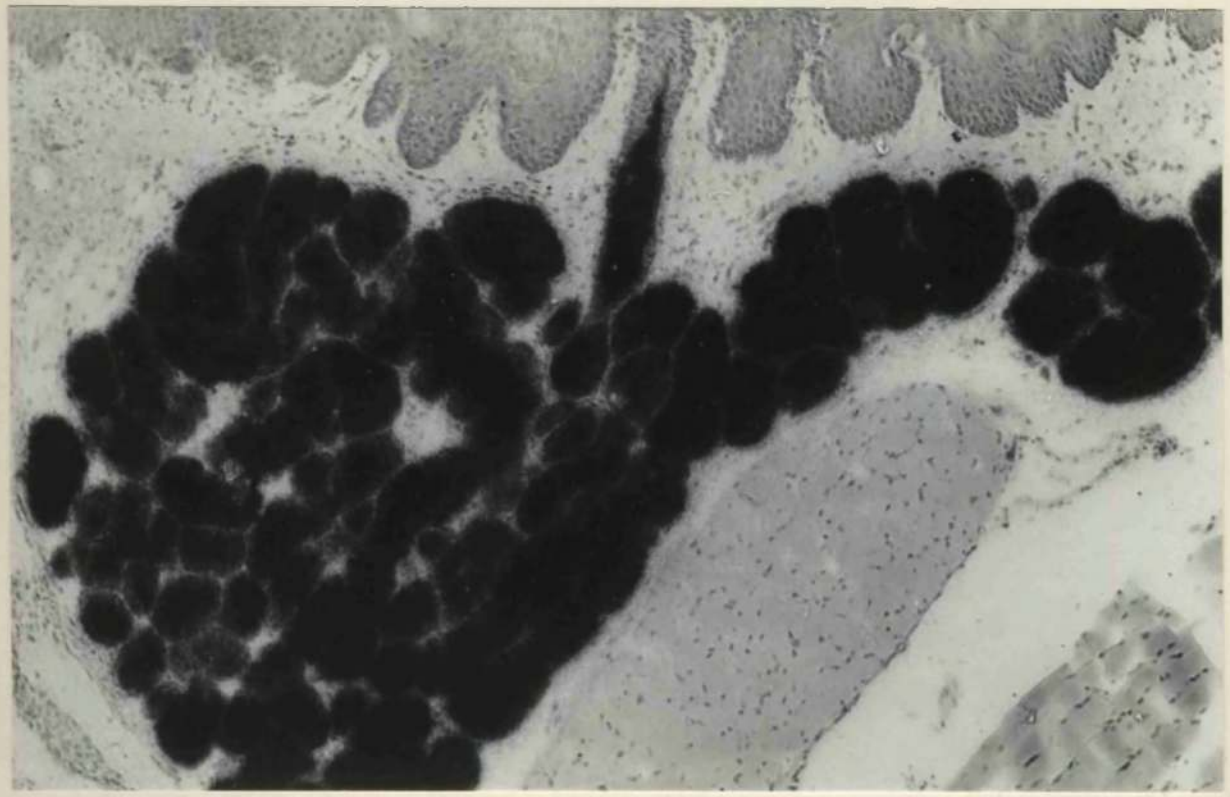

FIG.1,9

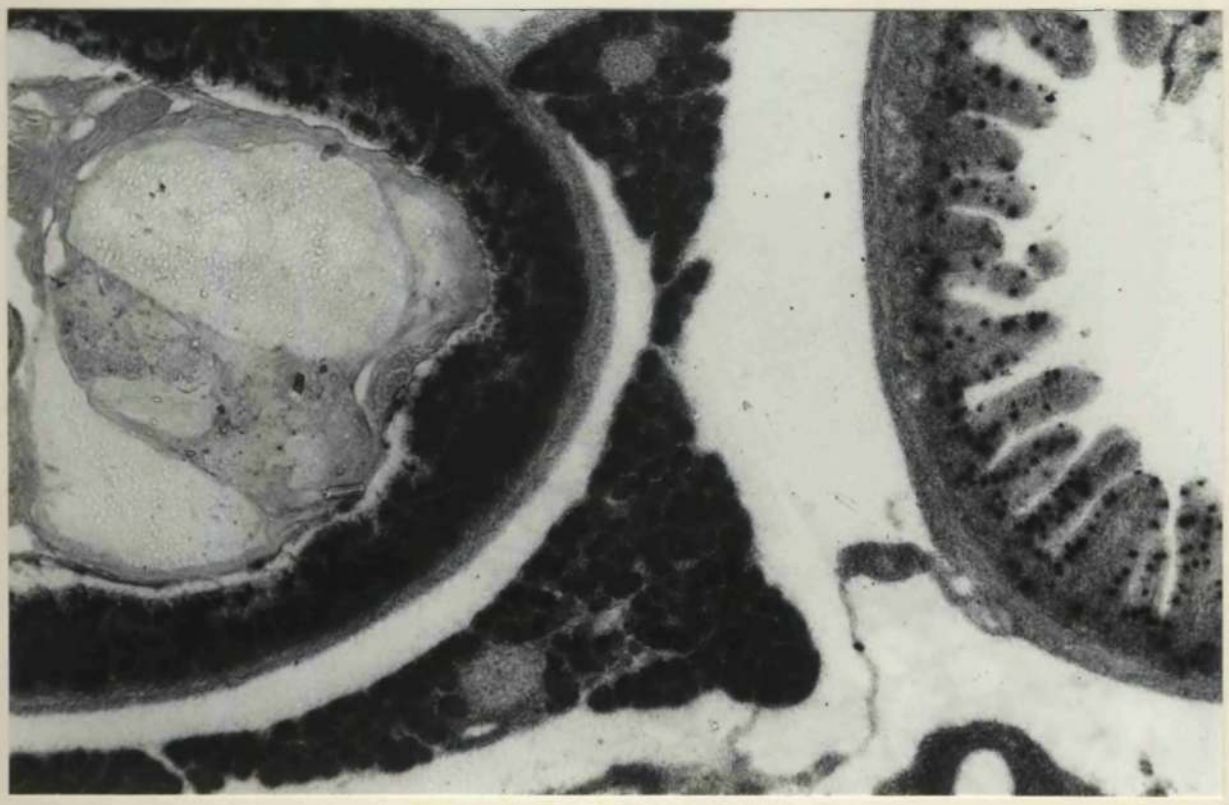

FIG.1,10

Fig. 1,11. - Spleen. The giant cells have a high content of sulphur-35. Mouse. Autoradiograph, Kodak AR10. X 100.

Fig. 1,12. - Brain. A diffuse sulphate-image is shown in brain and cerebellum. The dura mater also contains sulphur-35. The most intense image is an skull. Mouse. Autoradiograph, Kodak AR50. X 90.

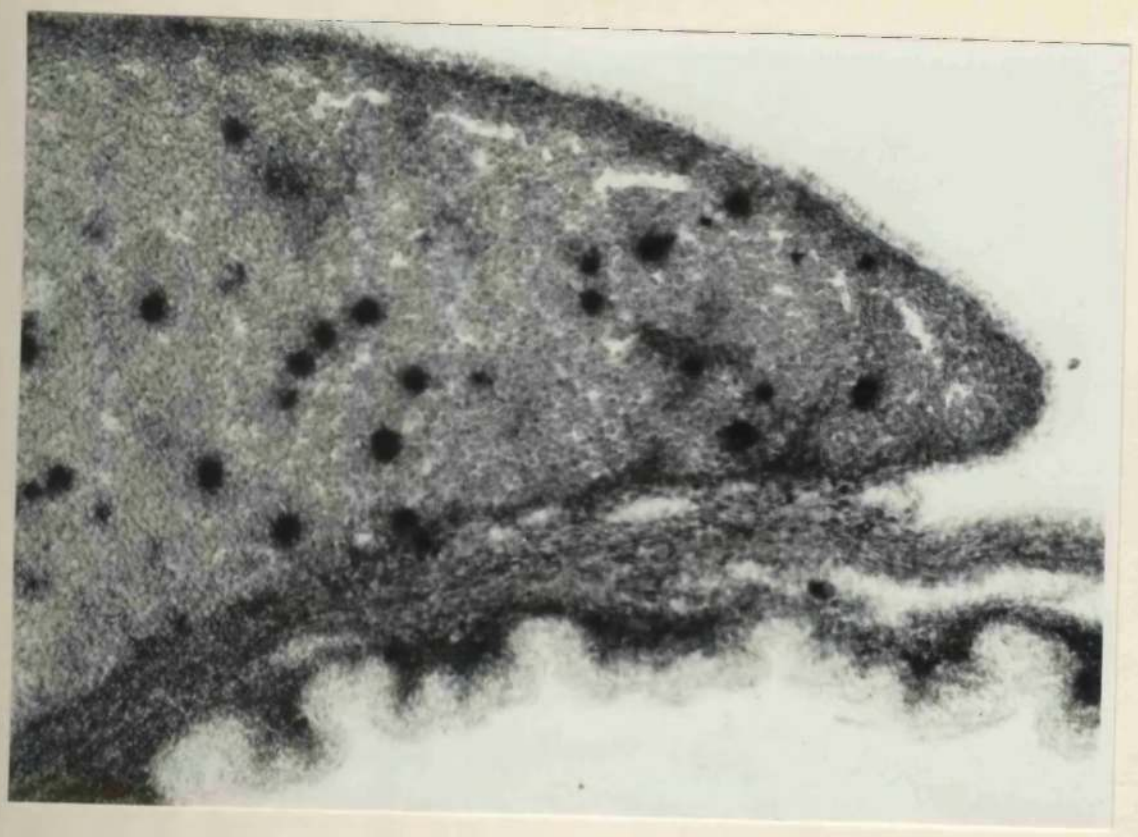

FIG. 1,11

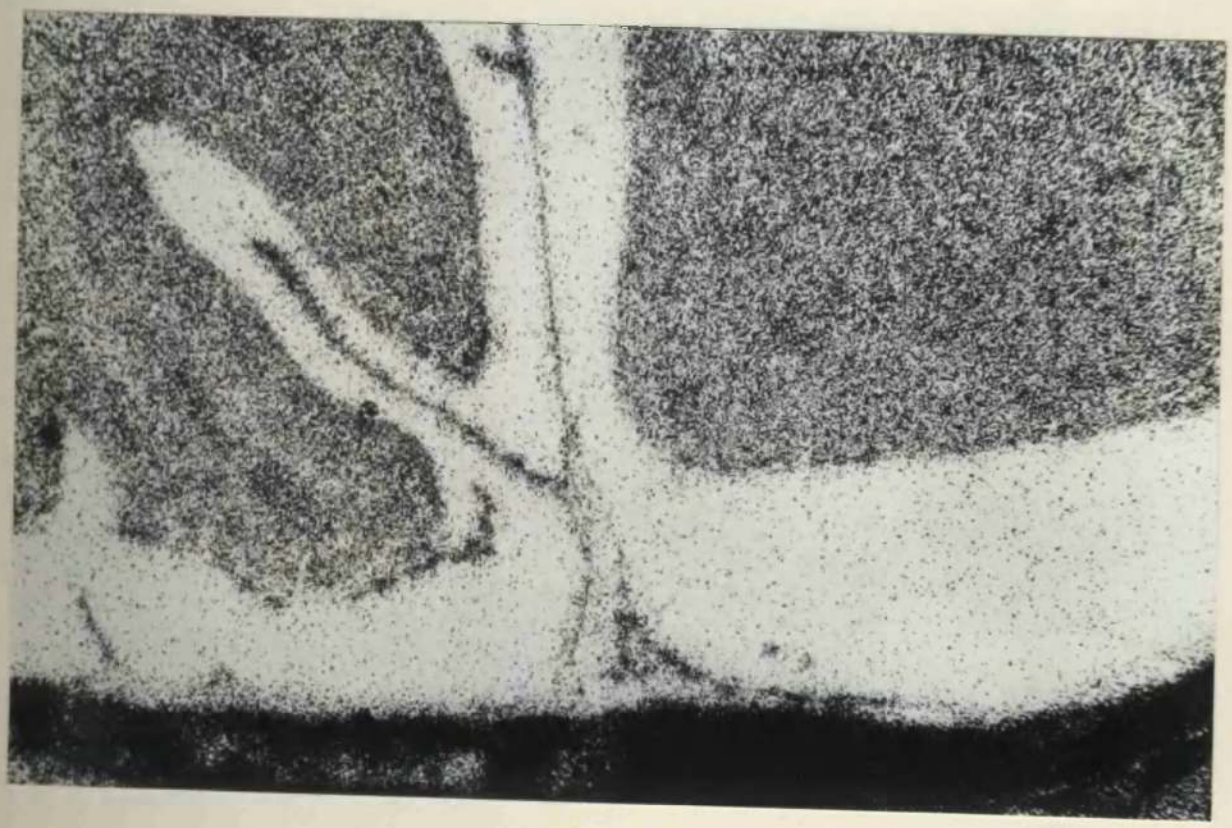

FIG. 1,12

Fig. 1,13. – Peripheral nerve. A diffuse sulphate-image is seen over the nerves. The perineural layer contains more sulphur-35 and the most intense image is over the wall of a blood vessel. Mouse. Autoradiograph, Kodak AR10. X 225.

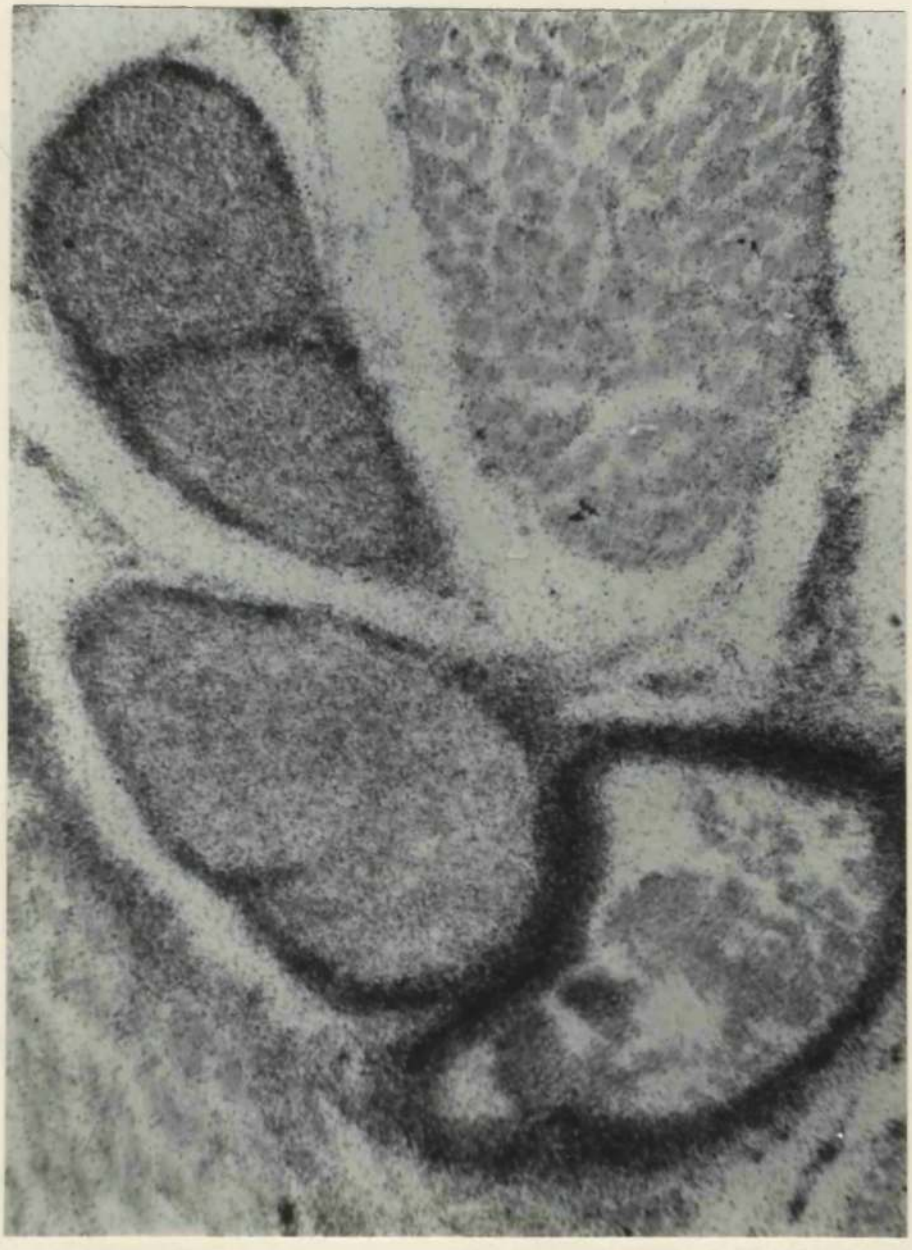

FIG. 1,13

Fig. 1,14. - Silicotic foci of 51 days duration. The largest on the right consists mostly of dense collagen with fibroblasts at the periphery. The other two are still largely fibroblastic with only early formation of collagen fibres. Mouse. Iron haematoxylin and Van Gieson. X 150.

Fig. 1,15. - Corresponding autoradiograph shows highest concentration of radio-sulphate in the areas of fibroblasts and young fibres. The mature collagen of the larger focus is inert. Mouse. Kodak AR10. X 150.

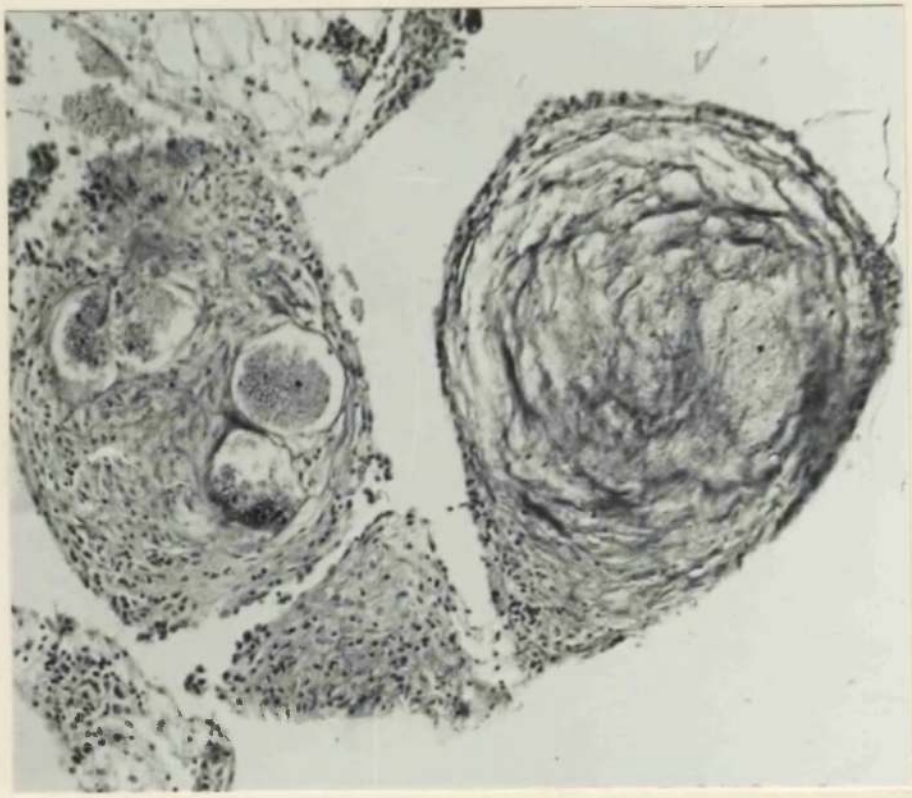

FIG.1,14

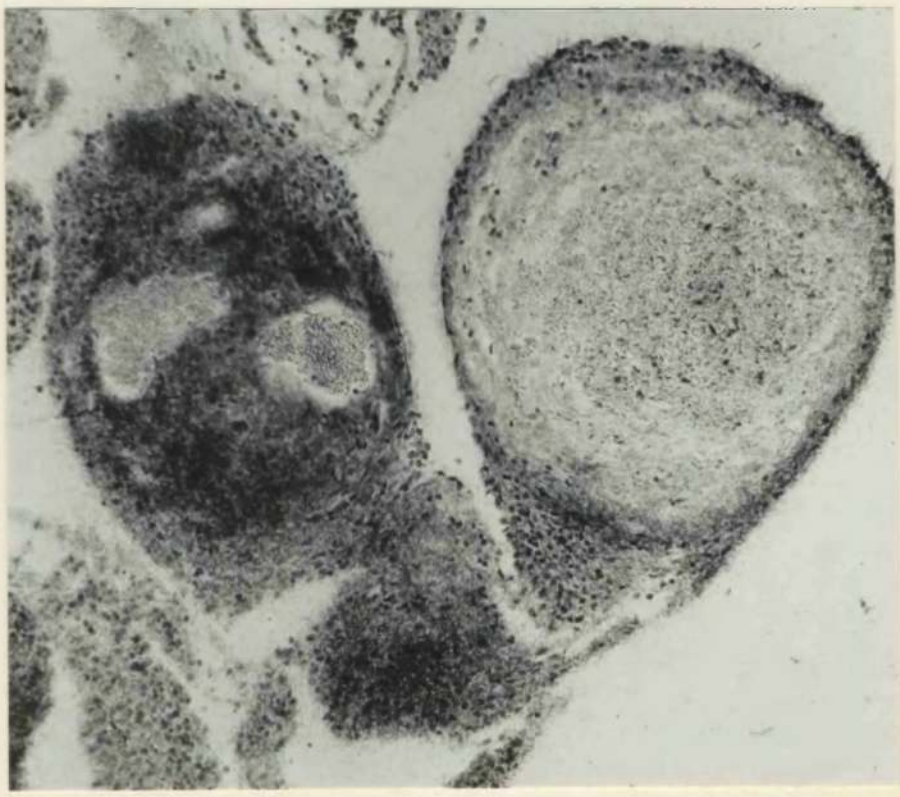

FIG.1,15

Fig. 1,16. - Mast cells in dermis showing characteristic punctate images. Mouse. Autoradiograph, Kodak AR10. X 250.

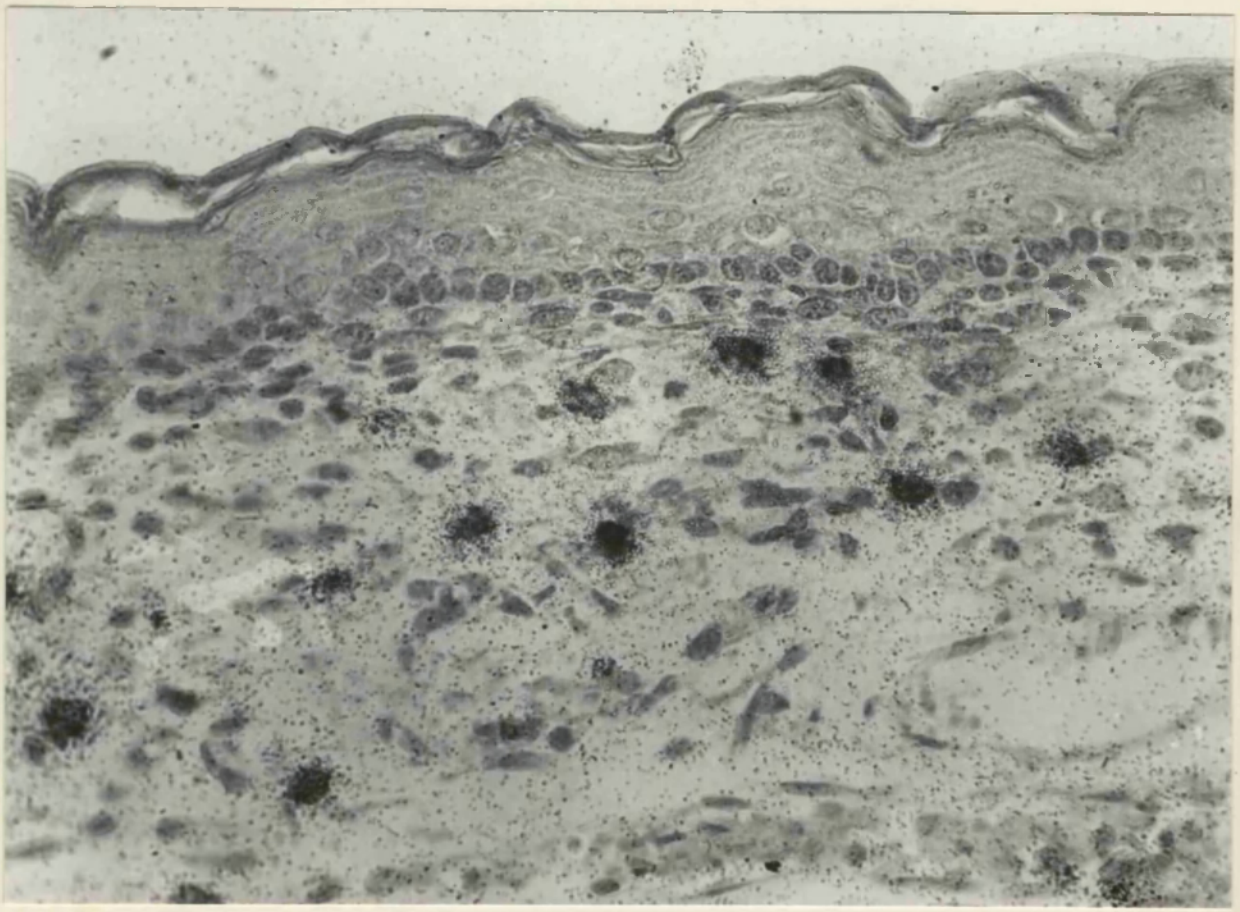

FIG.1,16

Fig. 1,17. - Fibroblasts and argyrophilic fibre network in chick gizzard. Gordon and Sweets' method. X 230.

Fig. 1,18. - Autoradiograph of a neighbouring section. The sulphur-35 image duplicates the silver pattern. The image is diffuse over cells and fibres. Kodak AR10. X 150.

FIG.1,17

FIG.1,18

Fig. 1,19. - Sulphur-35 in developing lumbar fascia. The intense central image is of cartilage in a vertebral body. Mouse. Autoradiograph, Kodak AR50. X 75.

Fig. 1,20. - Cross section of tail. Intense central image over cartilage; lighter diffuse images over tendon bundles, and numerous mast-cell images in dermis. Mouse. Autoradiograph, Kodak AR10. X 80.

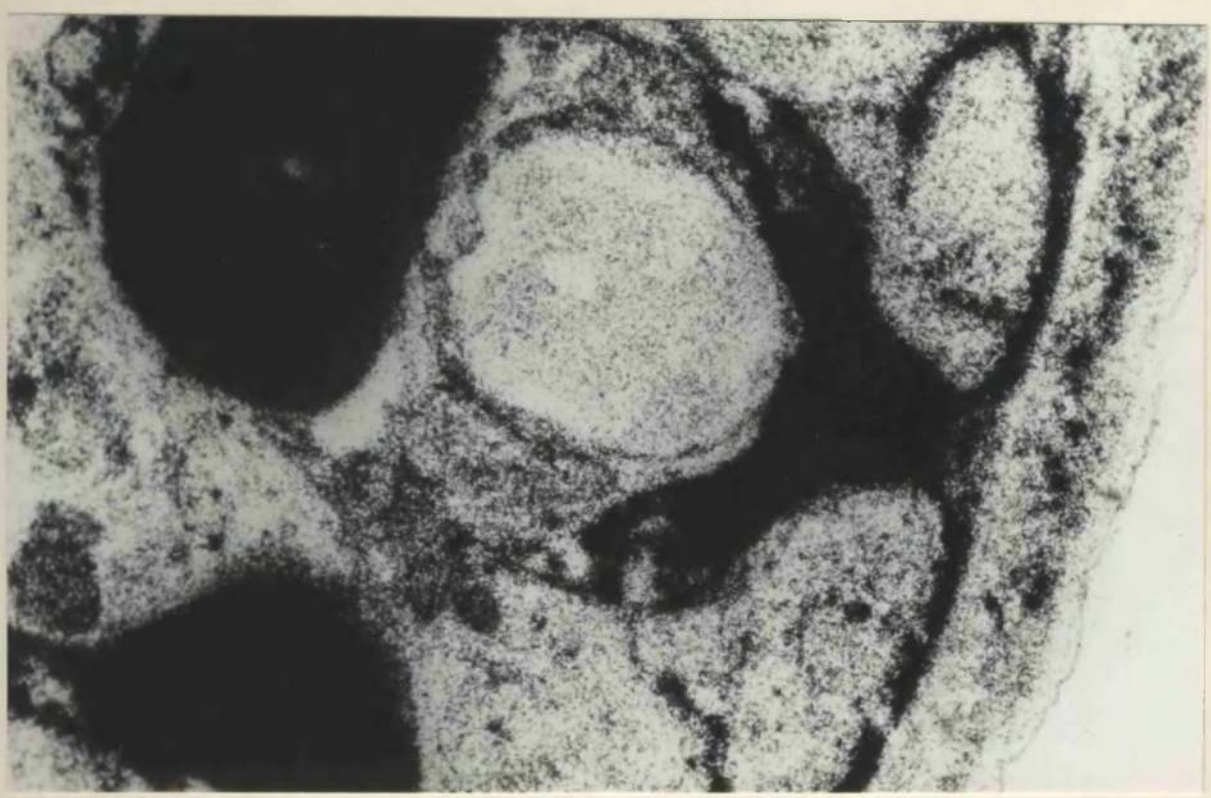

FIG.1,19

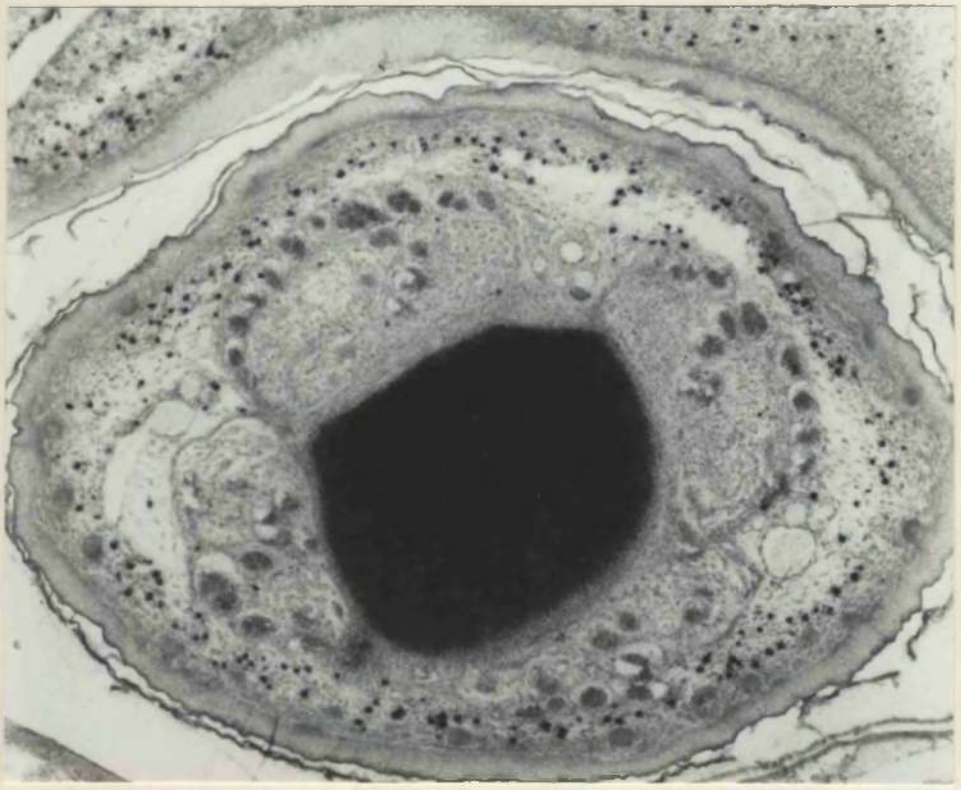

FIG.1,20

Fig. 1,21. - Tendon at insertion in cartilage. Sulphur-35 image is diffuse over cells and fibres, and decreases in intensity with distance from the insertion. No mast cells in the tendon. Rat. Autoradiograph, Kodak AR10. X 70.

Fig. 1,22. - Mast cells, fibroblasts and fibres in rat snout; the cellular image of the mast cell contrasts with the diffuse image of the fibroblasts and related fibres. Autoradiograph, Kodak AR10. X 750.

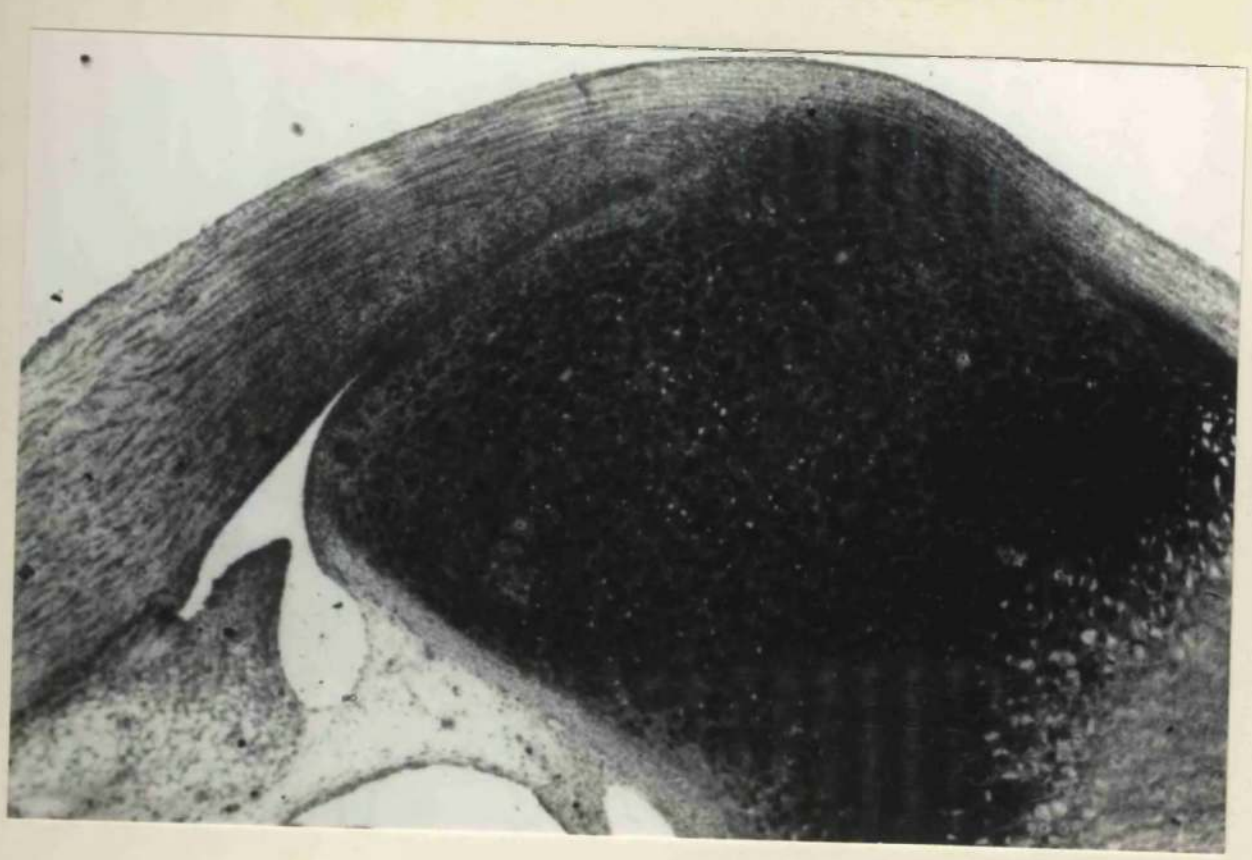

FIG.1,21

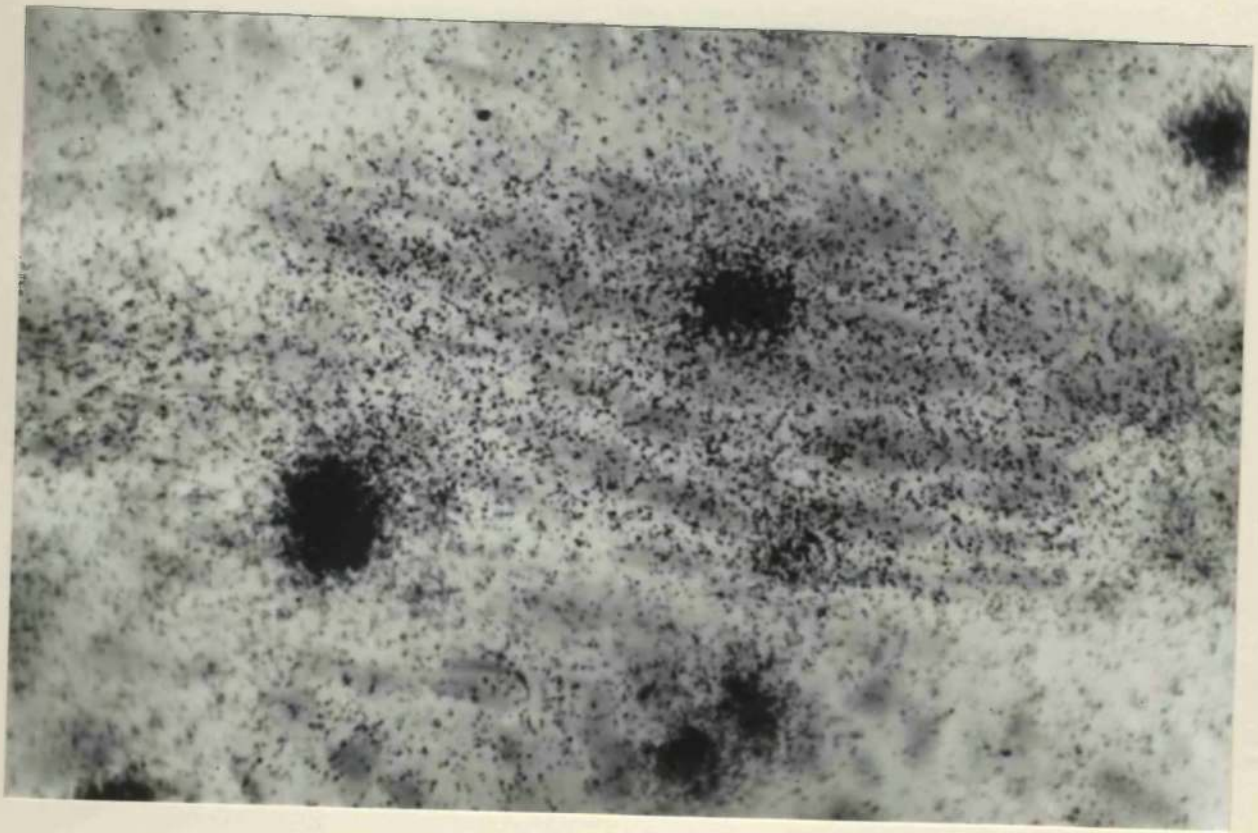

FIG.1,22

Fig. 1,23. - Sarcoma showing numerous mast cells in area of well differentiated spindle-cells (left), and no mast cells in pleomorphic area with local invasion of muscle (right). Rat. Autoradiograph, Kodak AR10. X 60.

Fig. 1,24. - Sarcoma showing marked sulphate-utilisation in regularly orientated bands and whorls of tumour cells and related fibres. Rat. Autoradiograph, Kodak AR10. X 60.

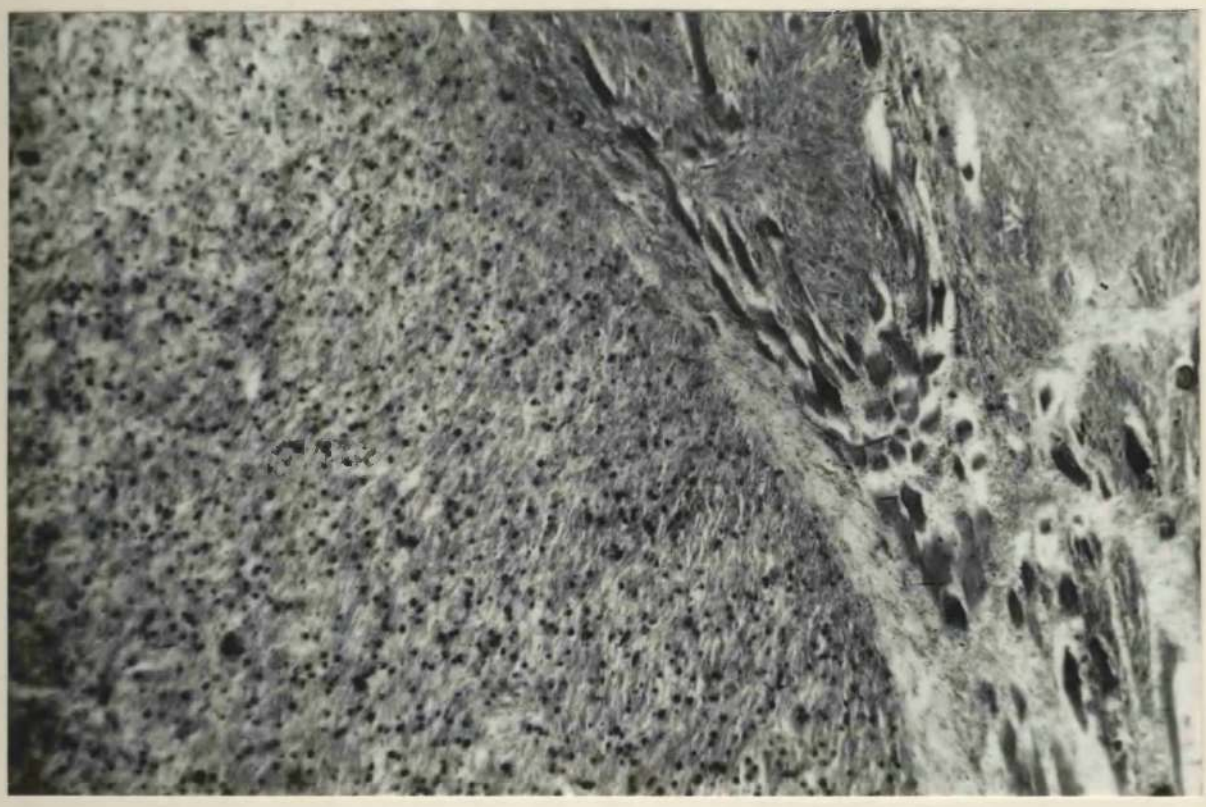

FIG.1,23

FIG.1,24

Fig. 1,25. - Band of collagen in sarcoma. Rat. Haematoxylin and eosin. X 1000.

Fig. 1,26. - Autoradiograph of neighbouring section showing diffuse sulphate-image over the collagen. Kodak AR10. X 1000.

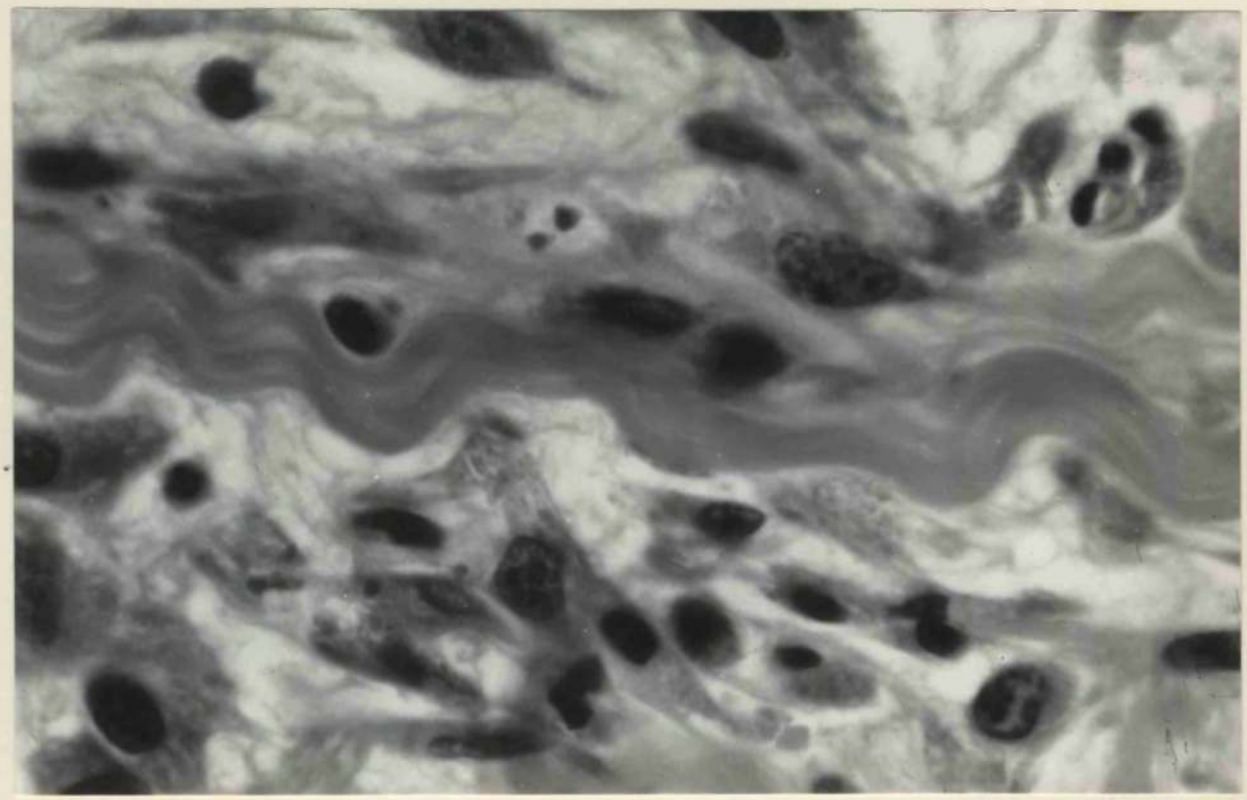

FIG. 1,25

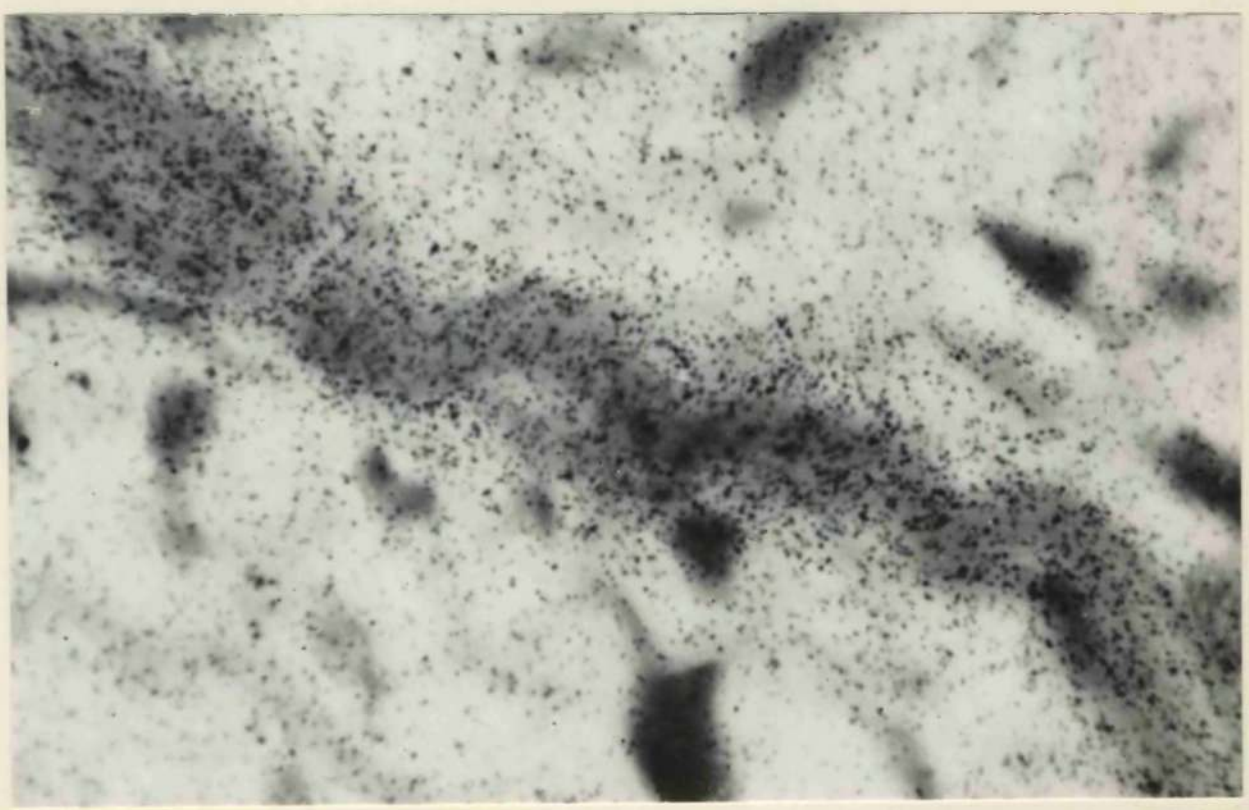

FIG.1,26

Fig. 1,27. - Collagenous band between tumour nodules. Rat. Alcian blue and chlorantine fast red. X 150.

Fig. 1,28. - Autoradiograph of neighbouring section showing diffuse image over cells and fibres. Mast-cell images are present in the nodules. Kodak AR50. X 150.

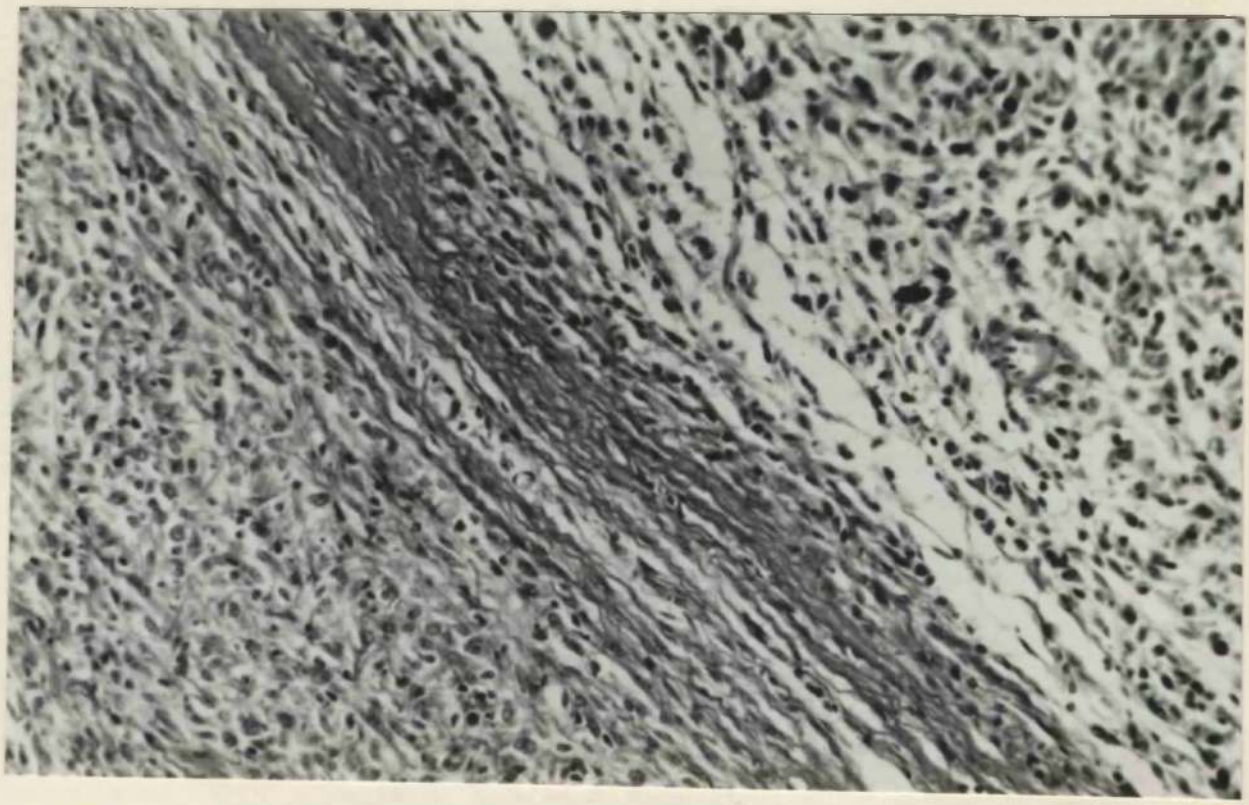

FIG.1,27

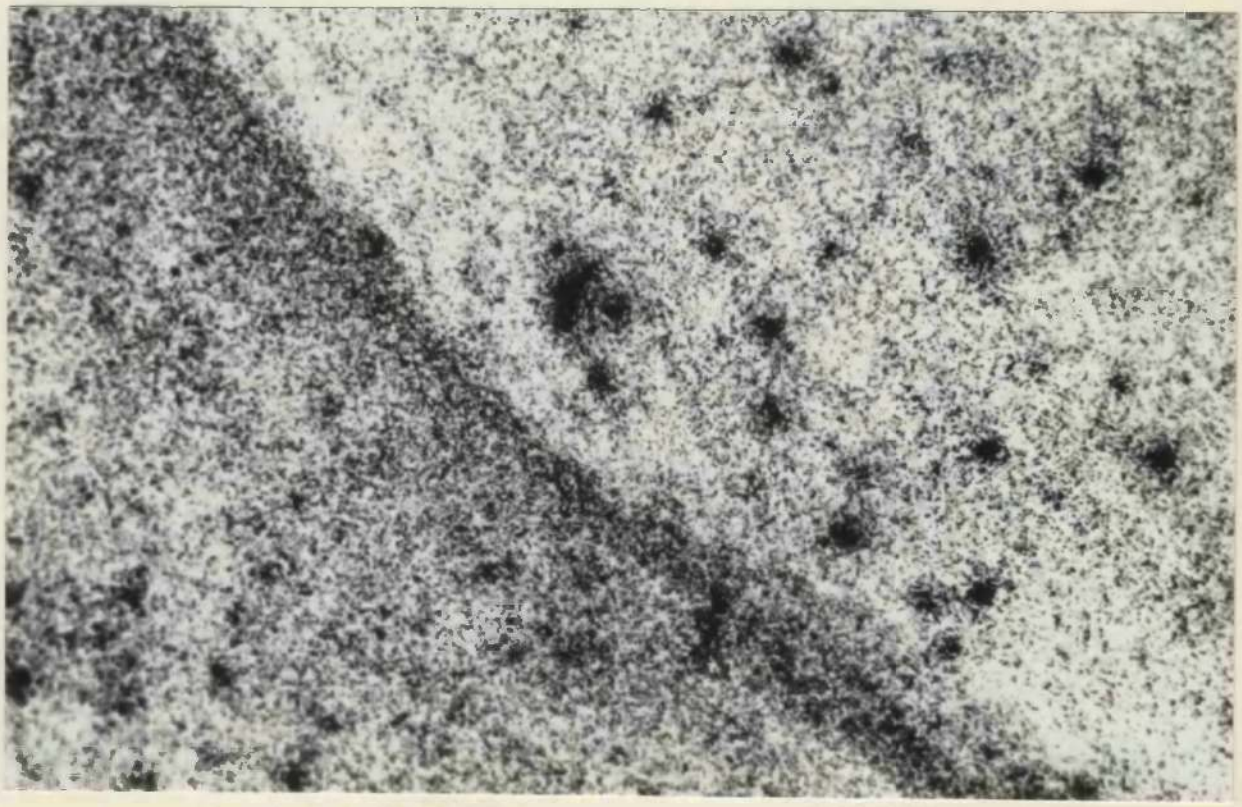

FIG.1,28

Fig. 1,29. - Papilloma showing bound sulphate in stroma. Mouse. Autoradiograph, Kodak AR10. X 19.

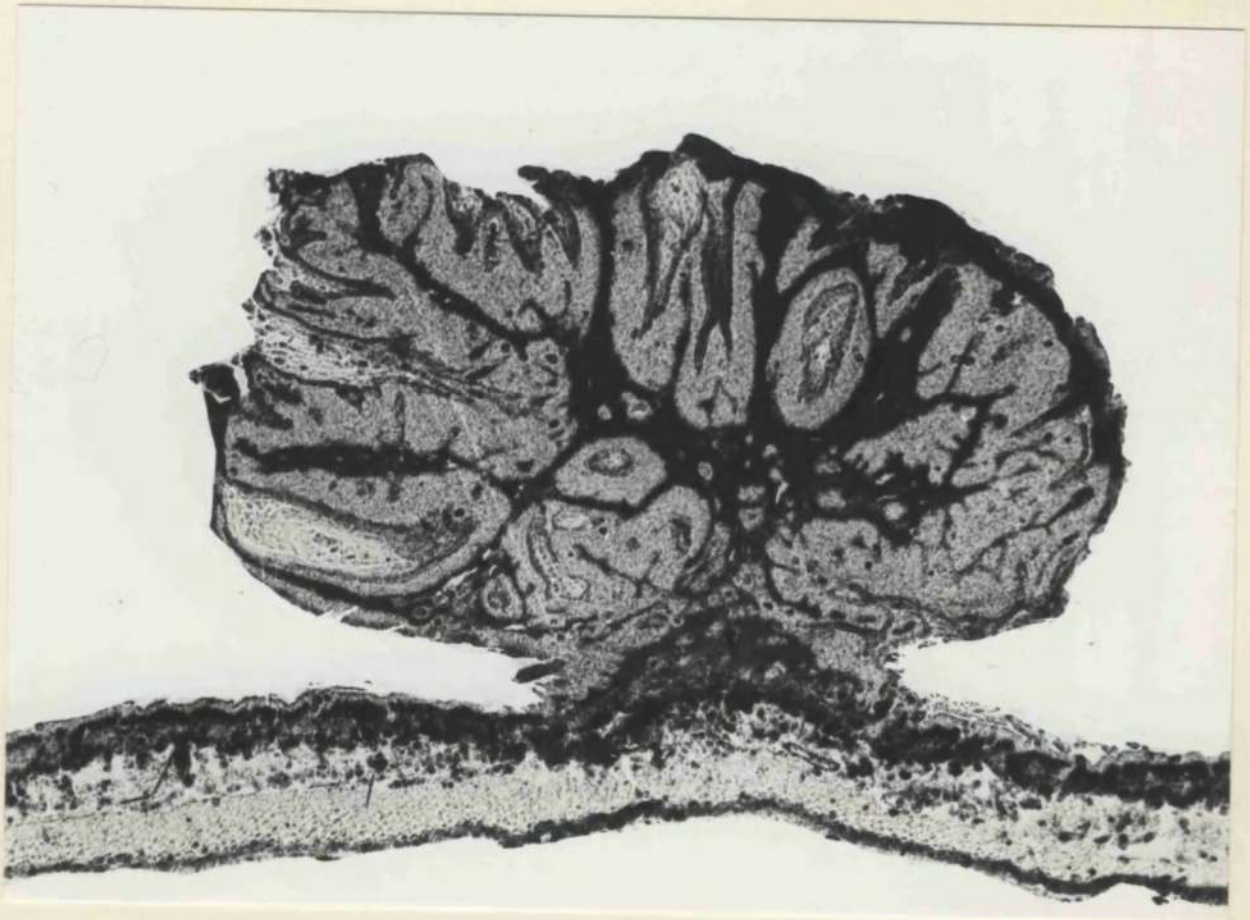

FIG.1, 29

Fig. 1,30. - Carrageenin focus at 7 days after injection of carrageenin. The sulphate-image is light and diffuse over the peripheral zone of fibrogenesis. Mouse. Autoradiograph, Kodak AR10. X 16.

Fig. 1,31. - Carrageenin focus at 9 days. The image is now more intense and is also present over a zone of fibrogenesis in muscle (bottom right). Mast cells are present in the dermis. Mouse. Autoradiograph, Kodak AR10. X 12.

Fig. 1,32. - Carrageenin focus at 11 days. The image has decreased in intensity, but is still present diffusely over cells and fibres on the periphery of the focus. Mouse. Autoradiograph, Kodak AR10. X 15.

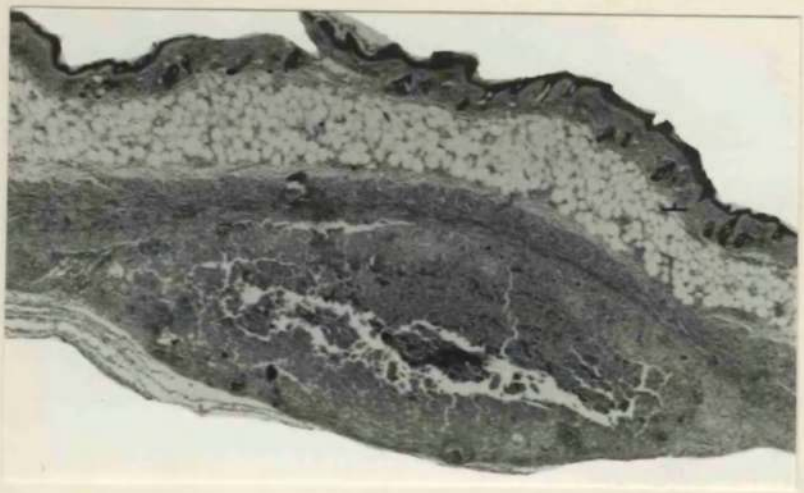

FIG. 1,30

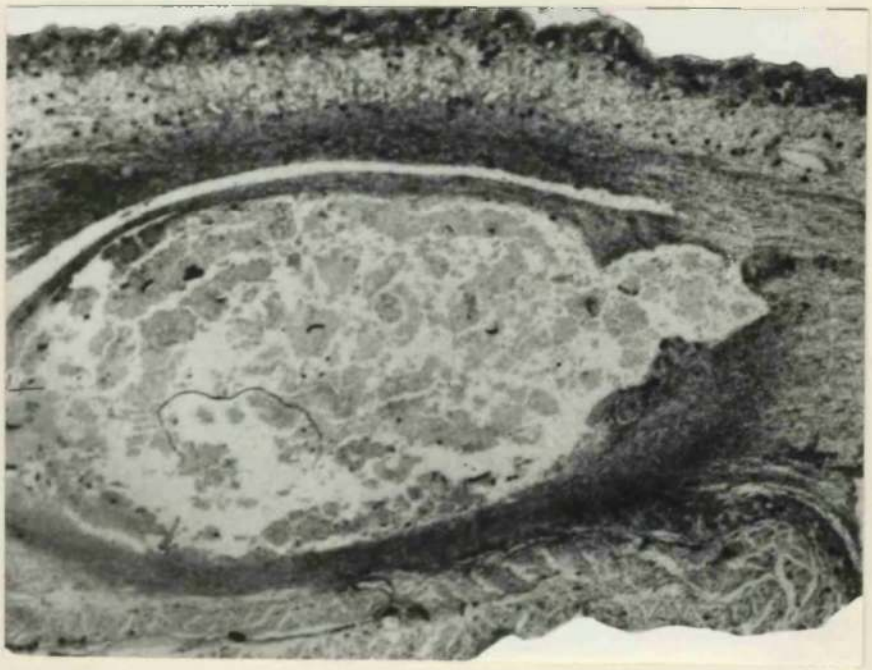

FIG. 1,31

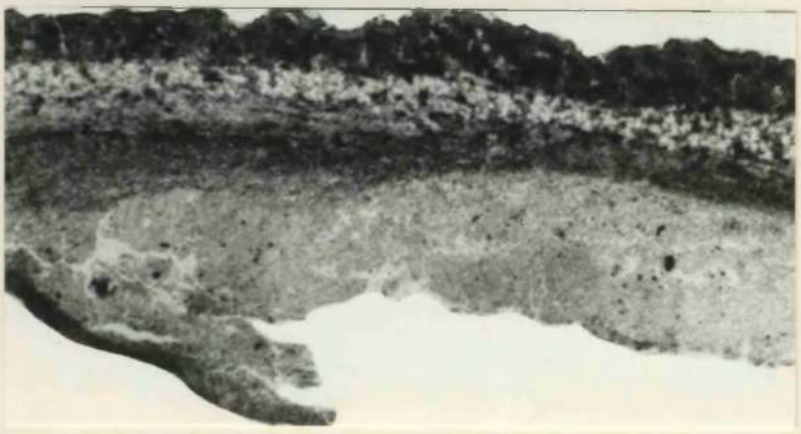

FIG. 1,32

Fig. 1,33. - Sulphur-35 utilisation by granulation tissue on periphery of abscess. The images are present diffusely over fibroblasts and fibres. A few mast cells are seen outside the main zone of fibrogenesis. Mouse. Autoradiograph, Kodak AR10. X 16.

FIG. 1,33

Fig. 1,34. - Bound sulphate in granulation tissue in resolving peritonitis on the hepatic peritoneal layer. No mast cells present. Mouse. Autoradiograph, Kodak AR50. X 140.

Fig. 1,35. - Skin graft (top) on chorio-allantoic membrane of chick. Sulphate-utilisation is shown by the diffuse image over the fibroblasts and fibres of the granulation tissue. No mast cells demonstrable. The cleft between skin and membrane is an artefact. Autoradiograph, Kodak AR10. X 150.

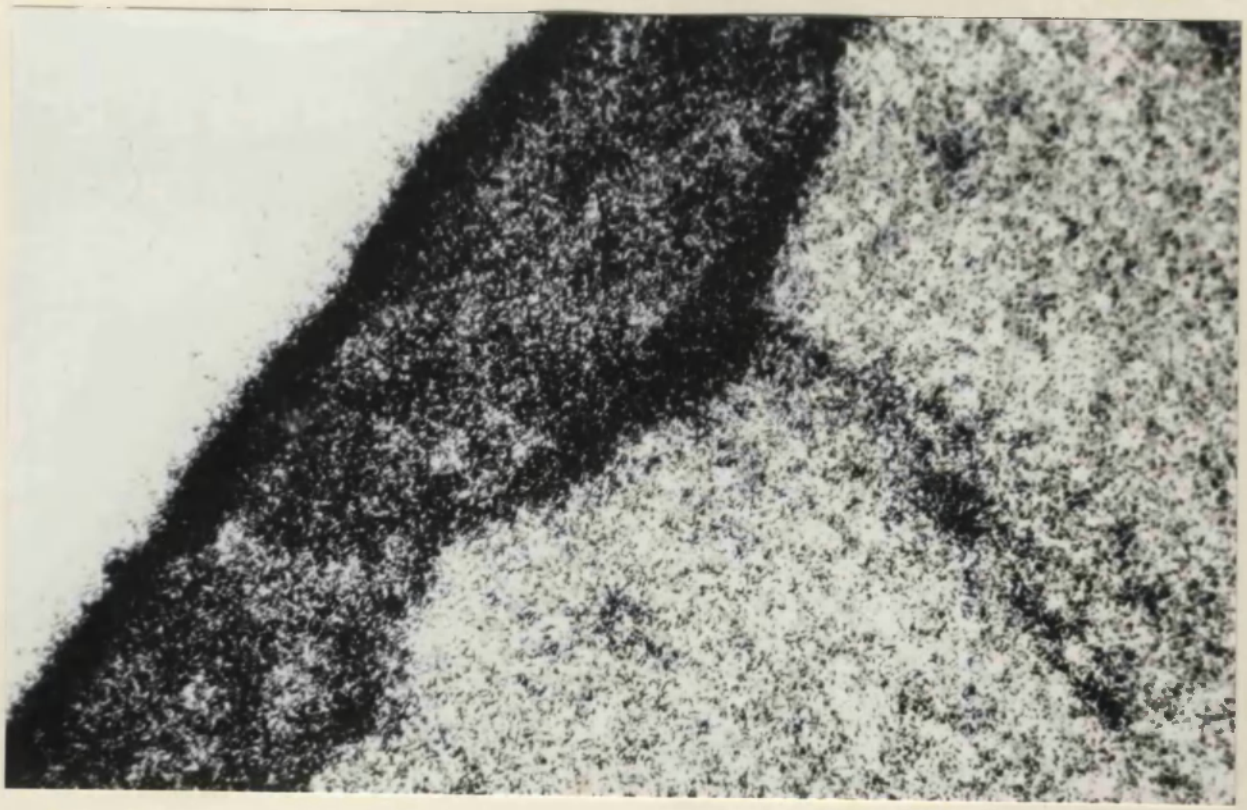

FIG. 1, 34

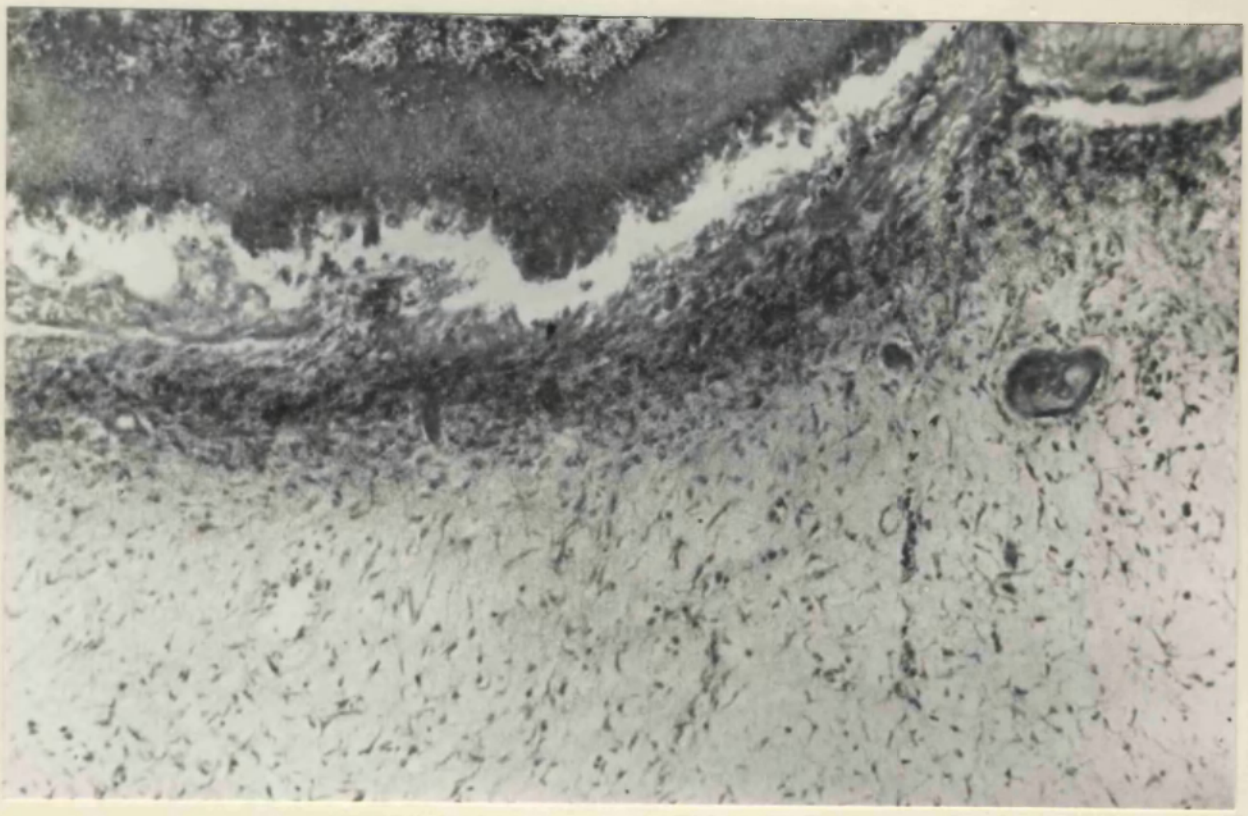

FIG. 1, 35

Fig. 1,36. - Cirrhotic liver. The sulphate-image coincides with the pattern of fibrous tissue. Mouse. Autoradiograph, Kodak AR50. X 90.

Fig. 1,37. - Sulphate-image over wide zone of young connective tissue around implanted catgut in liver. Mouse. Autoradiograph, Kodak AR50. X 90.

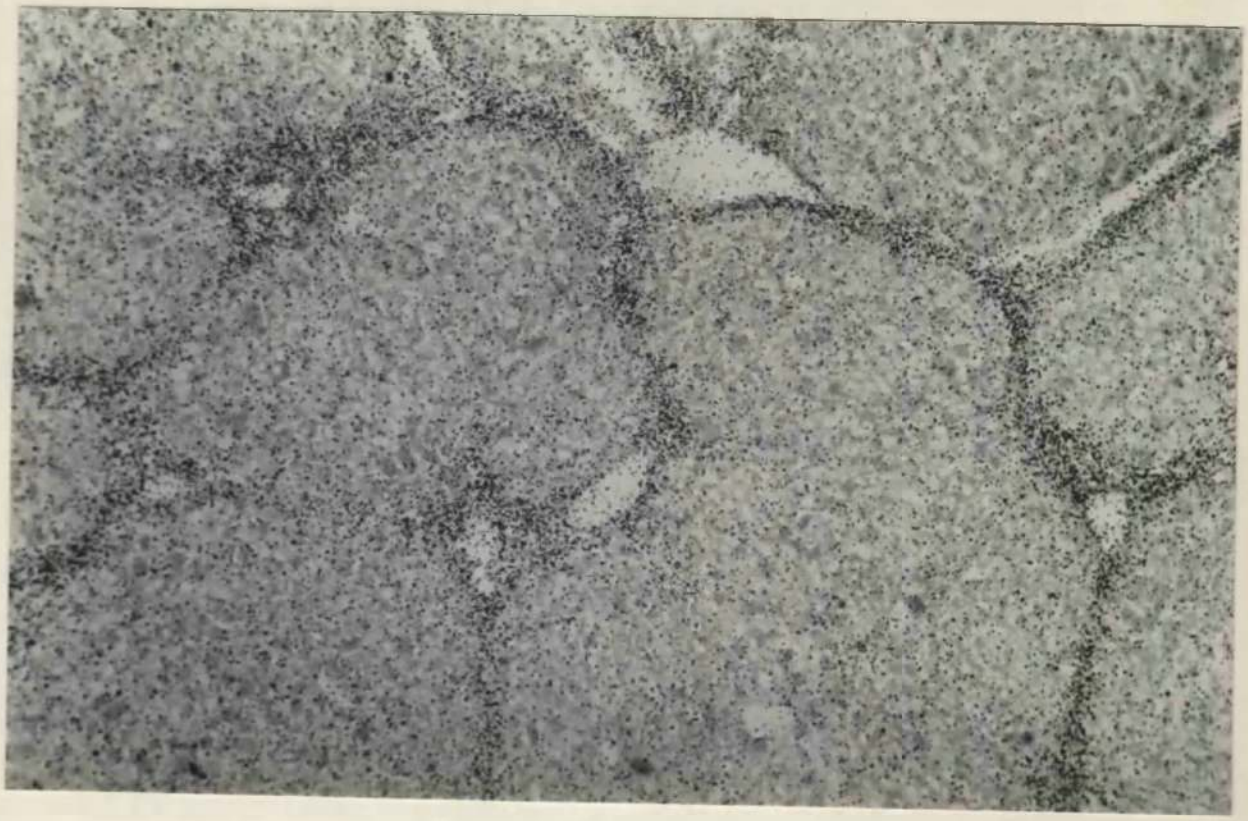

FIG. 1,36

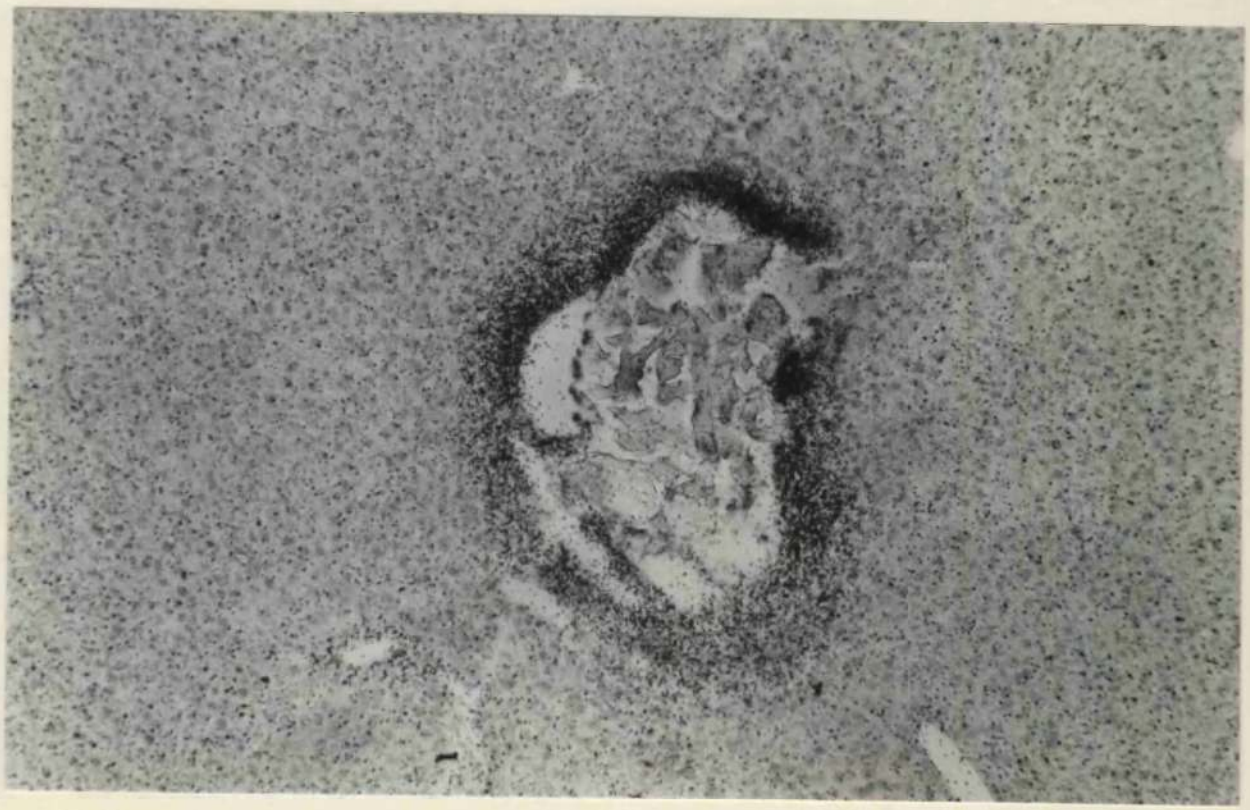

FIG. 1,37

Fig. 1,38. - Catgut lesion in liver at later stage with site of implant still showing utilisation of radio-sulphate in fibrous tissue. Mouse. Autoradiograph, Kodak AR50. X 950.

Fig. 1,39. - Catgut lesion in liver. The extent but not the intensity of the sulphate-image is reduced by administration of cortisone. Mouse. Autoradiograph, Kodak AR50. X 140.

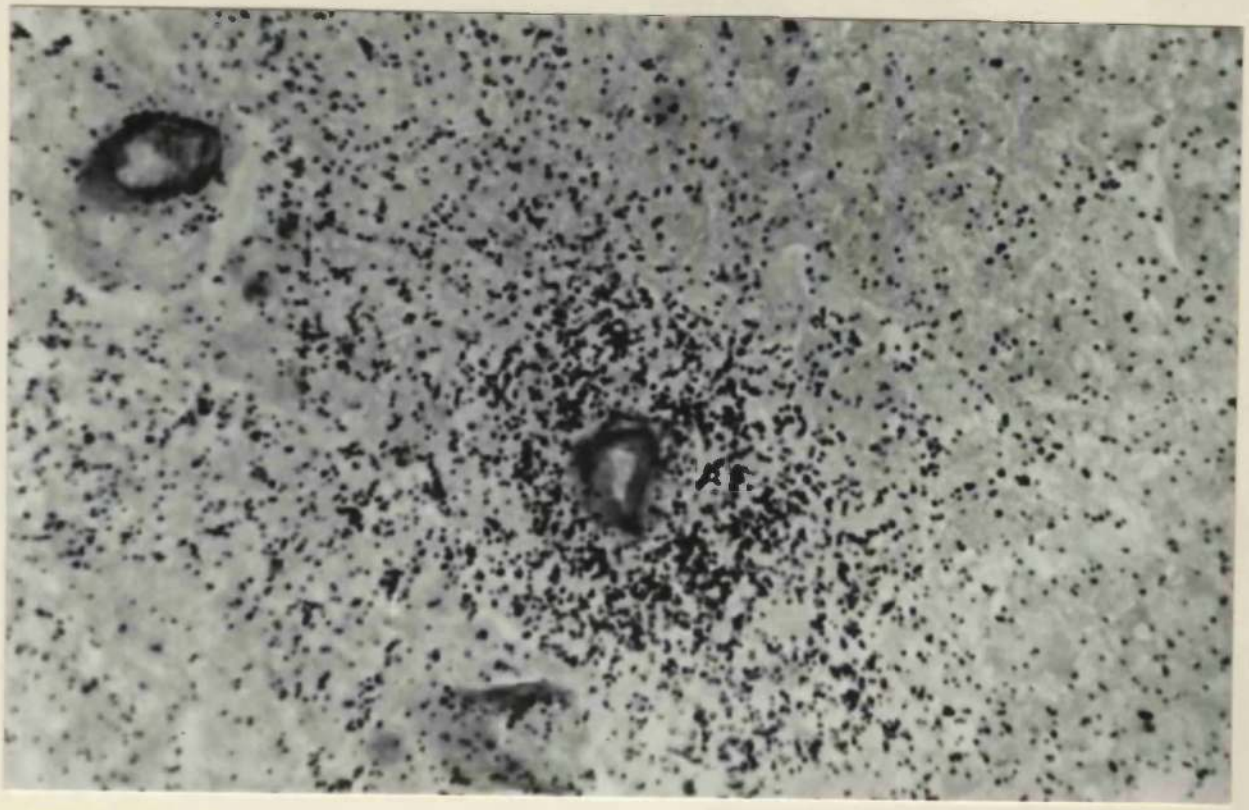

FIG.1,38

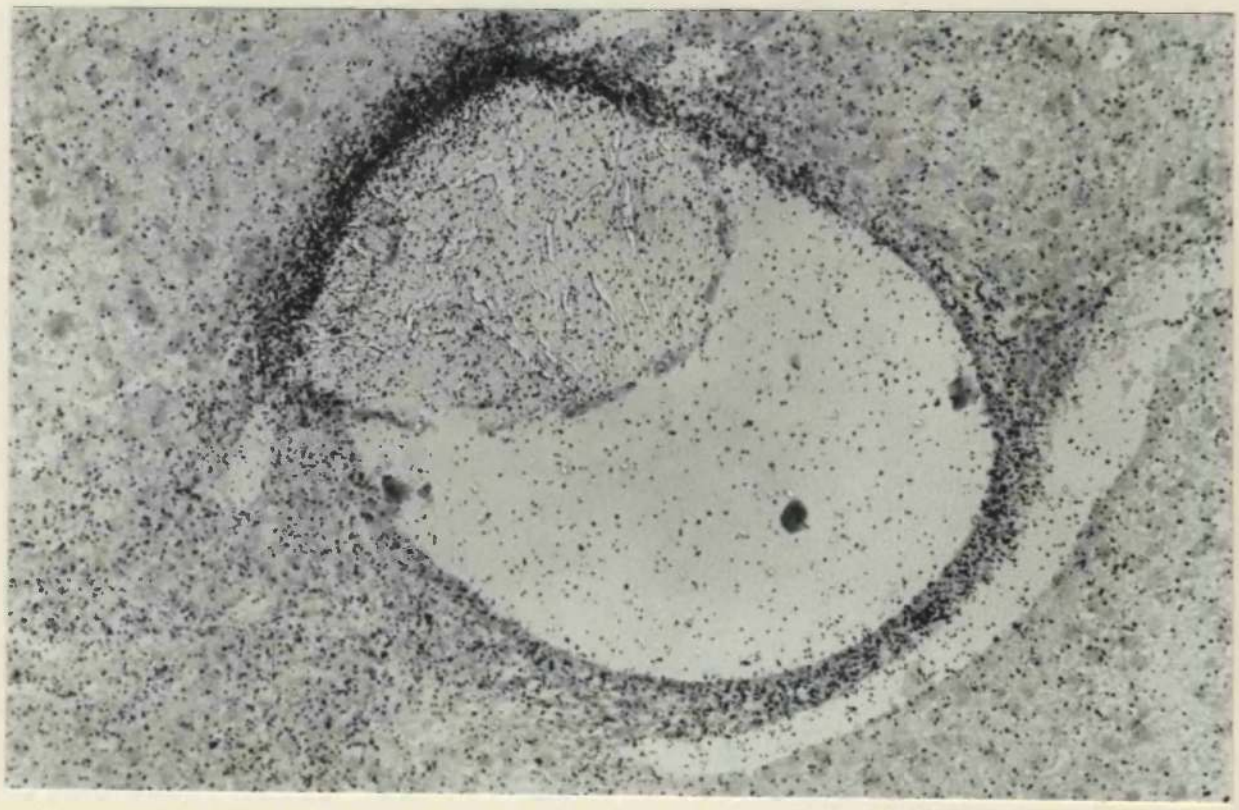

FIG.1,39

Fig. 1,40. - Sulphur-35 utilisation by fibroblasts in stroma of a mixed parotid tumour. The fibres are inert. Man. Autoradiograph, Kodak AR10. X 1700.

Fig. 1,41. - Mast cells showing sulphate-image. Urticaria pigmentosa in man. Autoradiograph, Kodak AR10. X 1300.

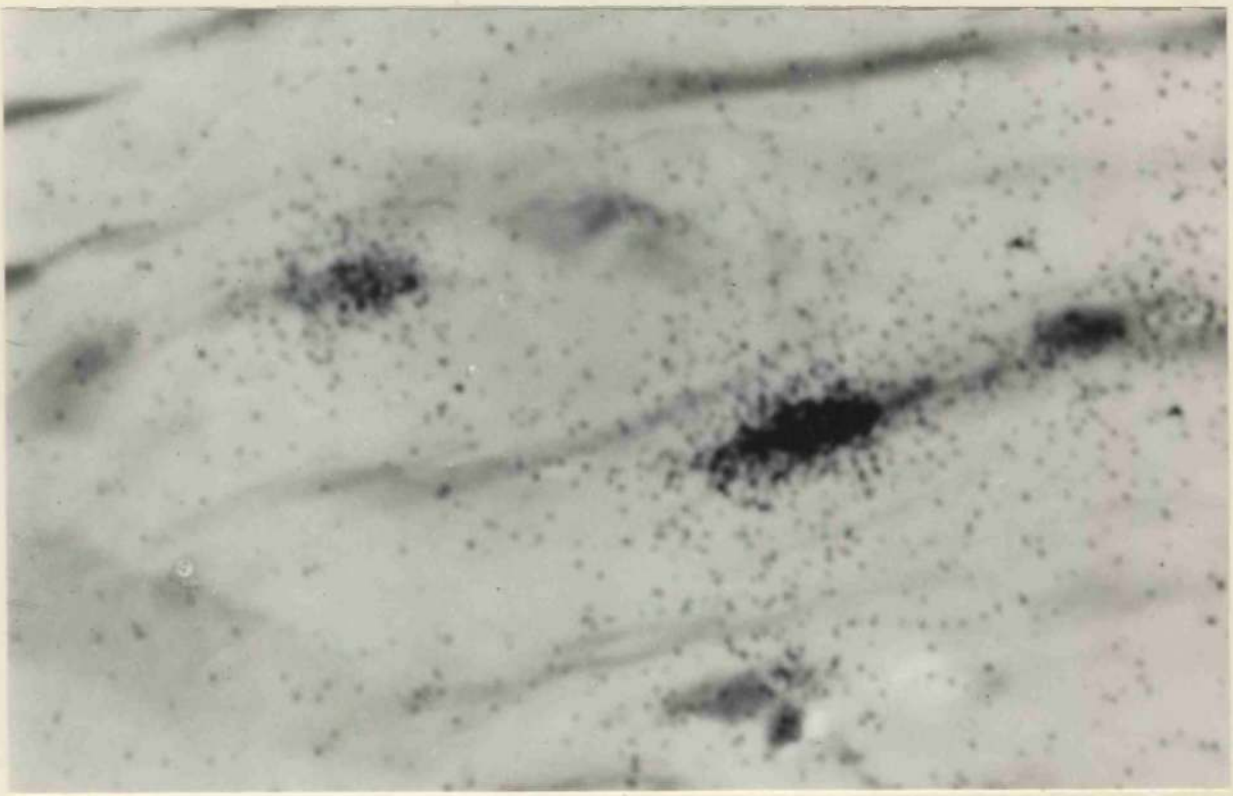

FIG.1,40

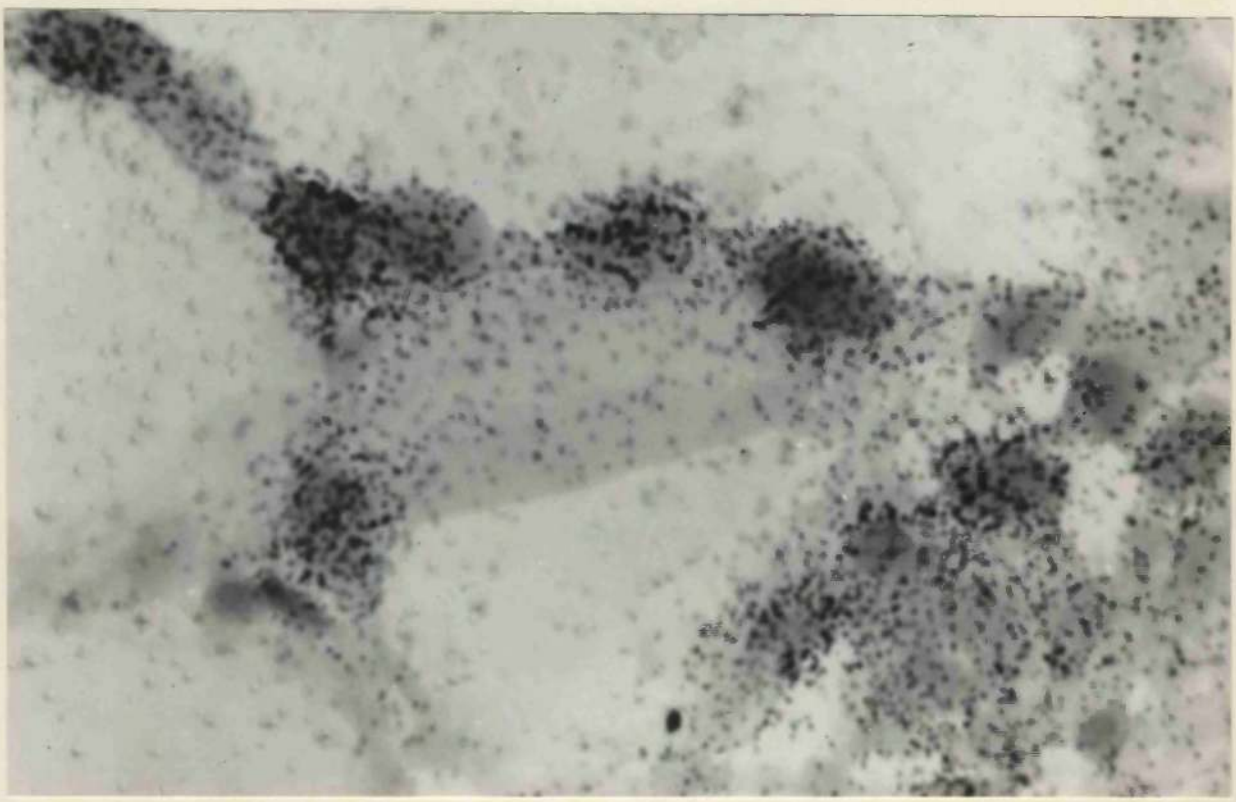

FIG.1,41

Fig. 1,42. - Mast cells in mesenteric spread showing sulphate-utilisation after compound 48/80. The cells are remote from vessels. Rat. Autoradiograph, Kodak AR50. X 950.

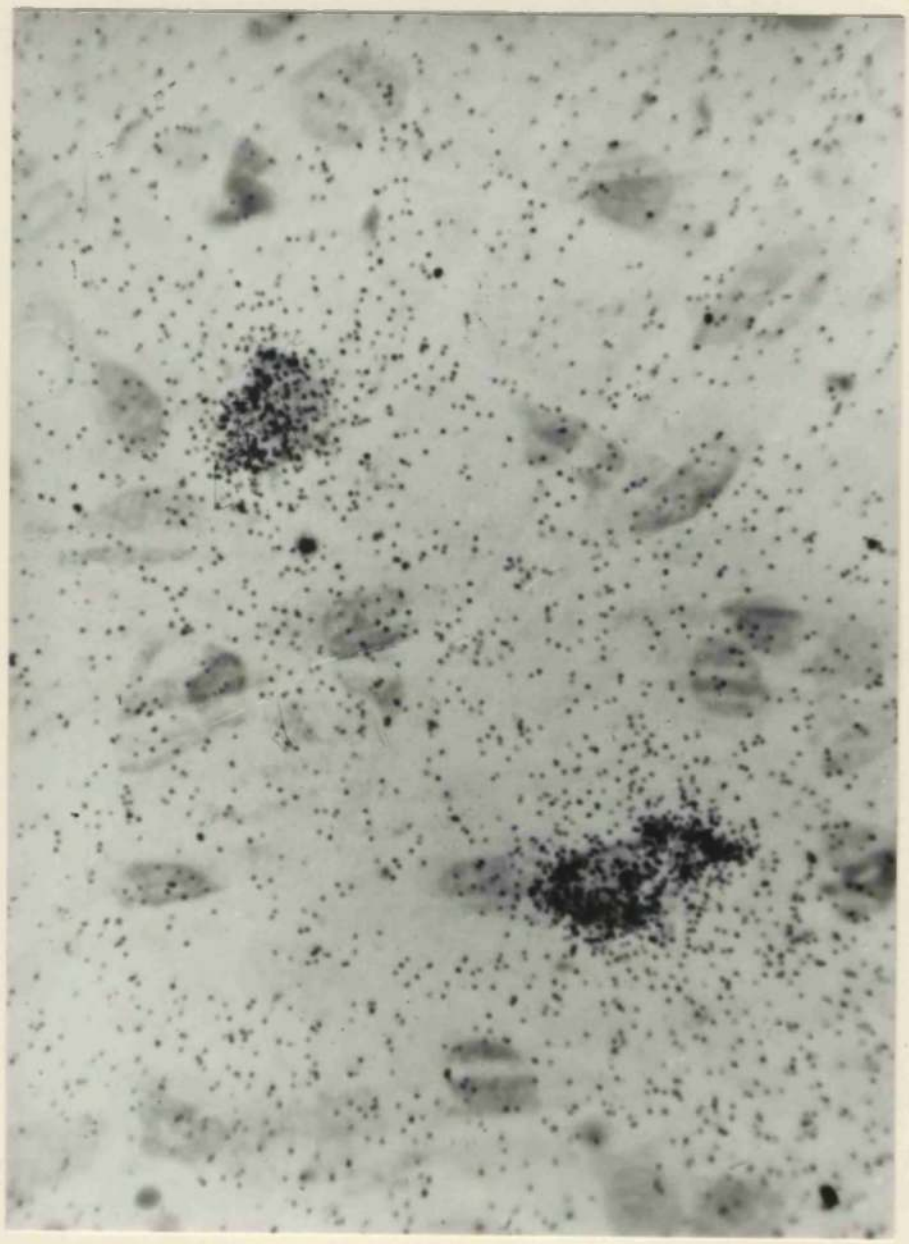

FIG.1,42

P A R T II

Tables 2,1 - 2,4

TABLE 2,1

ANIMALS USED AND RANGE OF TIME INTERVAL BETWEEN INJECTION OF SULPHUR-35 AND SACRIFICE

Animals	No.	Time interval (hr)
Adult albino mice	11	15-69
Suckling albino mice	9	5-169
Foetal albino mice	3	5-19
Adult white rats	1	19
Suckling white rats	12	5-169
Adult guinea-pigs	1	24

TABLE 2,2

UPTAKE OF SULPHUR-35 IN ADULT ANIMALS ESTIMATED BY INTENSITY OF AUTORADIOGRAPHIC IMAGES

Tissue	Interval between injection of sulphur-35 and sacrifice		
	15-19 hr.	20-24 hr.	64-69 hr.
Dental pulp	±	+	+
Odontoblasts	±	0	±
Predentine	+ +	+ +	+ +
Dentinal matrix	0	0	0
Enamel matrix	0	+	±
Ameloblasts	±	±	±

The intensity of the images is graded as follows: 0, no uptake; ±, occasional weakest uptake; +, weakest uptake; + +, intermediate uptake; + + +, greatest uptake.

TABLE 2,3

UPTAKE OF SULPHUR-35 IN FOETAL AND SUCKLING ANIMALS ESTIMATED BY INTENSITY OF AUTORADIOGRAPHIC IMAGES

Tissue	Interval between injection of sulphur-35 and sacrifice							
	5–9 hr.	10–14 hr.	15–19 hr.	64–69 hr.	90–94 hr.	115–119 hr.	135–139 hr.	164–169 hr.
External enamel epithelium	++	+	++					
Internal enamel epithelium	++	+	++					
Stellate reticulum	+to ++	+	+to ++					
Dental papilla	++	+	+					
Dental pulp	++	++	++	++	++to +++	++to +++	++	++to +++
Odontoblasts	++	±to ++	±to ++	+	±to +	0	0	0
Predentine	+++	+++	+++	++	++	+	+	+
Dentinal matrix	+	+	+	+	++	+++	+++	++
Enamel matrix	++	++	++	+	++	++	++	+
Ameloblasts	++	±to ++	±to ++	±to +	+	0	0	0
Stratum intermedium	++	+	+	±to +	+	±	0	0

Where blank spaces occur in the table no examination of the appropriate tissue was made at the time tabulated. The symbols 0, ±, +, ++, and +++ have the same significance as in Table 2,2.

TABLE 2.4

STAINING REACTION OF DENTAL TISSUES COMPARED WITH SULPHUR-35 UPTAKE IN FOETAL, SUCKLING AND ADULT ANIMALS

Tissue	Staining method					
	1	2	3	4	5	Sulphur-35 uptake
External enamel epithelium	Variable	+ve	+ve	+ve	Variable	+ve
Internal enamel epithelium	Variable	+ve	+ve	+ve	Variable	+ve
Stellate reticulum (cells and matrix)	+ve	+ve	Variable	Variable	+ve	+ve
Dental papilla (cells and matrix)	+ve	+ve	+ve	+ve	+ve	+ve
Dental pulp (cells and matrix)	+ve	+ve	+ve	Variable	+ve	+ve
Odontoblasts	Variable	+ve	Variable	Variable	Variable	+ve
Dentinal tubules (peripheral part)	+ve	+ve	Variable	-ve	+ve	Not determined
Predentine	Variable	-ve	Variable	-ve	+ve	+ve
Dentinal matrix	-ve	-ve	Variable	Variable	-ve	+ve
Enamel matrix	Variable	Variable	Variable	Variable	-ve	+ve
Ameloblasts	-ve	+ve	Variable	Variable	Variable	+ve
Stratum intermedium	Variable	+ve	+ve	-ve	-ve	+ve

Staining methods are numbered as follows: 1, alcian blue; 2, alcian blue-chlorantine fast red; 3, periodic acid-Schiff; 4, toluidine blue at pH 2.6; 5, toluidine blue, at pH 4.9. No quantitative significance is attached to the symbols in this table. +ve, positive; -ve, negative.

P A R T II

Figs. 2,1 - 2,39

Figs. 2,1. - Autoradiograph showing sulphur-35 utilisation by human cartilage cells in vitro. Kodak AR50. X 90.

Fig. 2,2. - Sulphate-utilisation in vitro by cartilage homograft 19 years after implanting. Man. Autoradiograph, Kodak AR50. X 90.

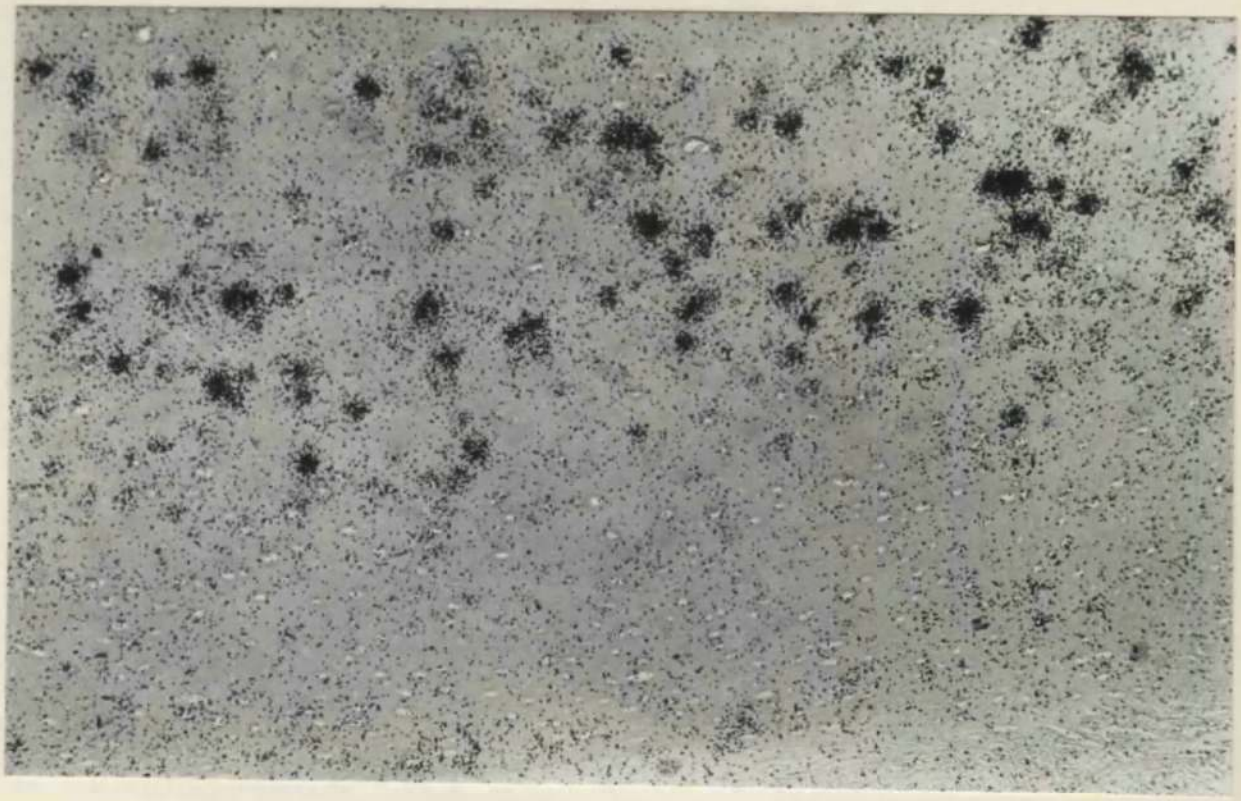

FIG.2,1

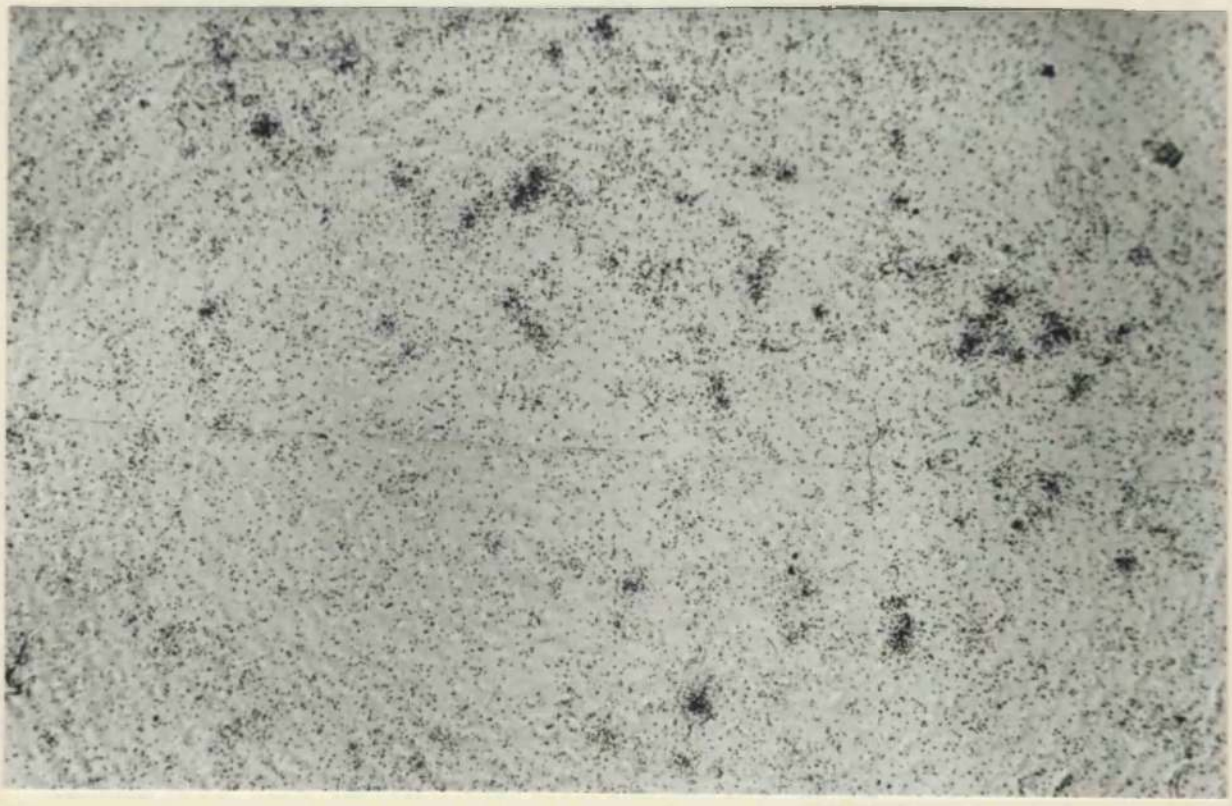

FIG.2,2

Fig. 2,3. - Lathyritic rat (left) and control to show lesions in long bones.

FIG.2,3

Fig. 2,4. - Epiphyseal cartilage showing early fibrillar change in matrix. Rat. Haematoxylin and eosin. X 350.

Fig. 2,5. - Autoradiograph of neighbouring section showing absence of radio-sulphate in the altered matrix. Rat. Kodak AR10. X 270.

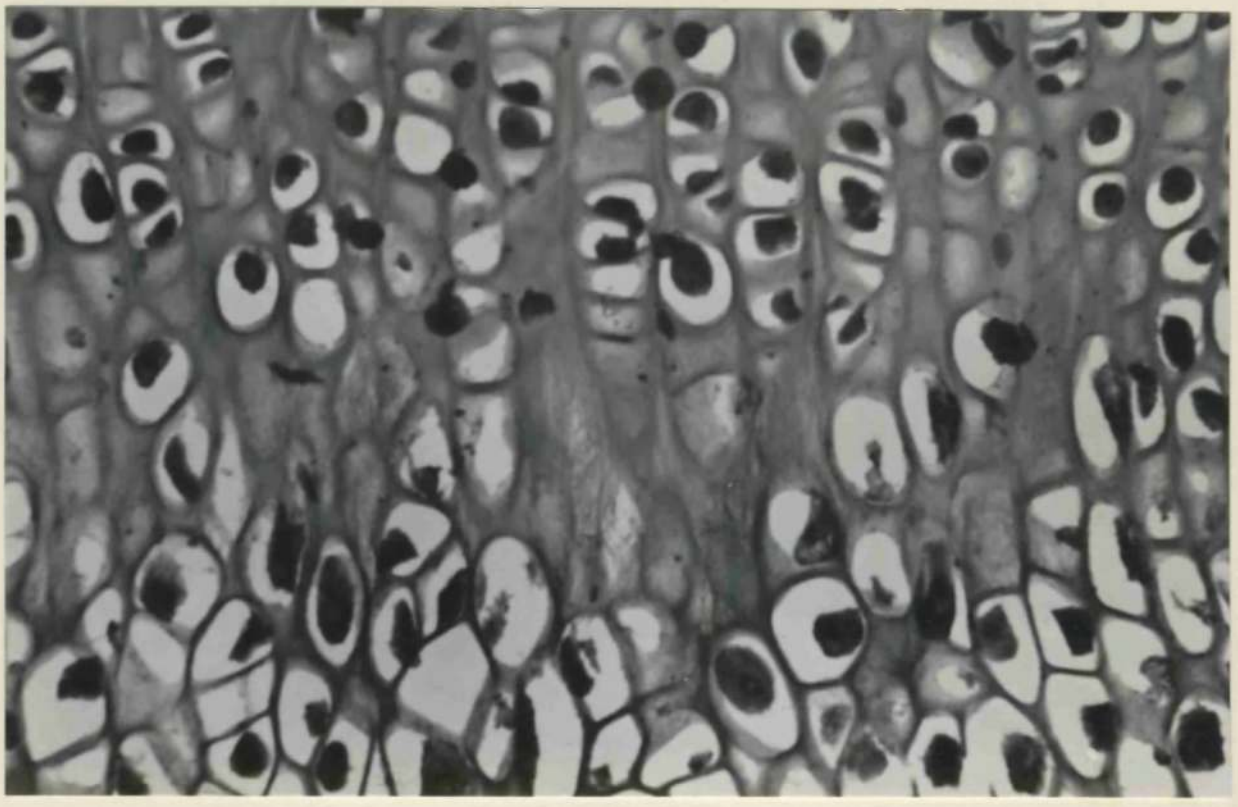

FIG. 2,4

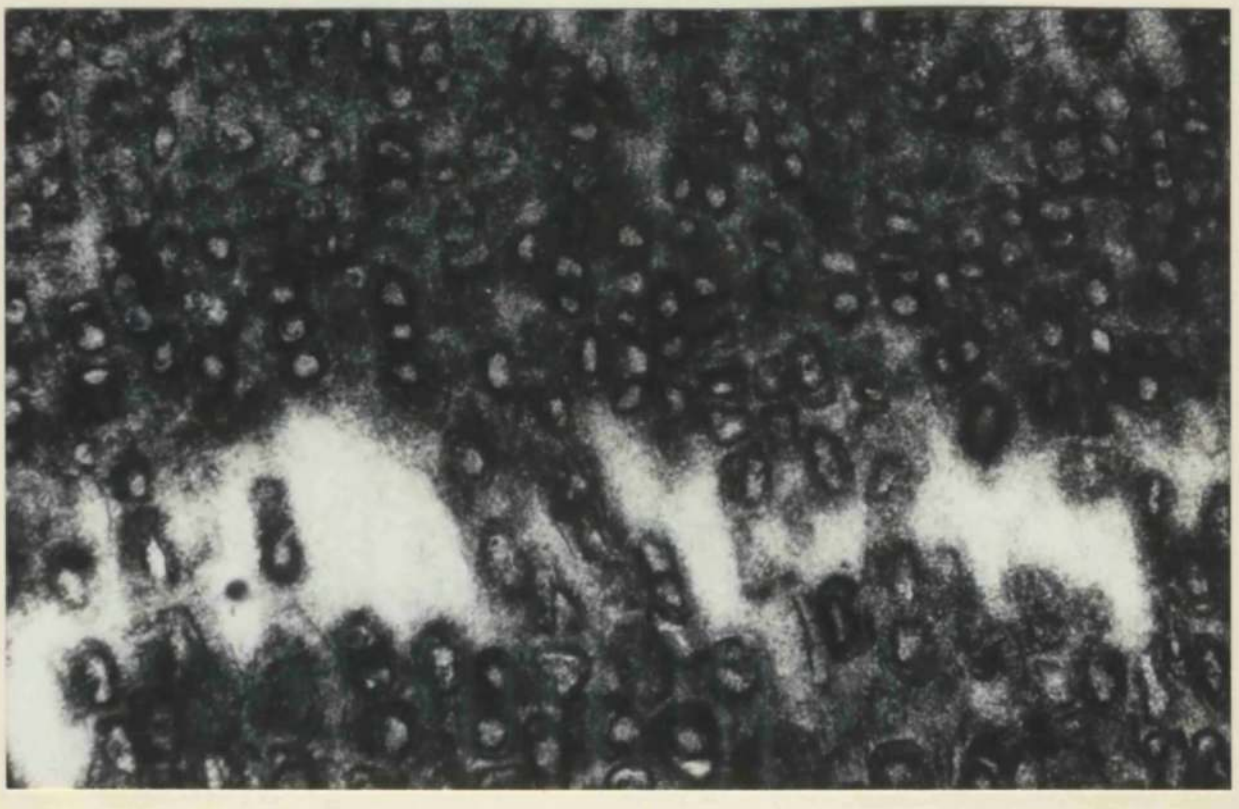

FIG. 2,5

Fig. 2,6. - Delayed union of fracture in lathyritic rat.

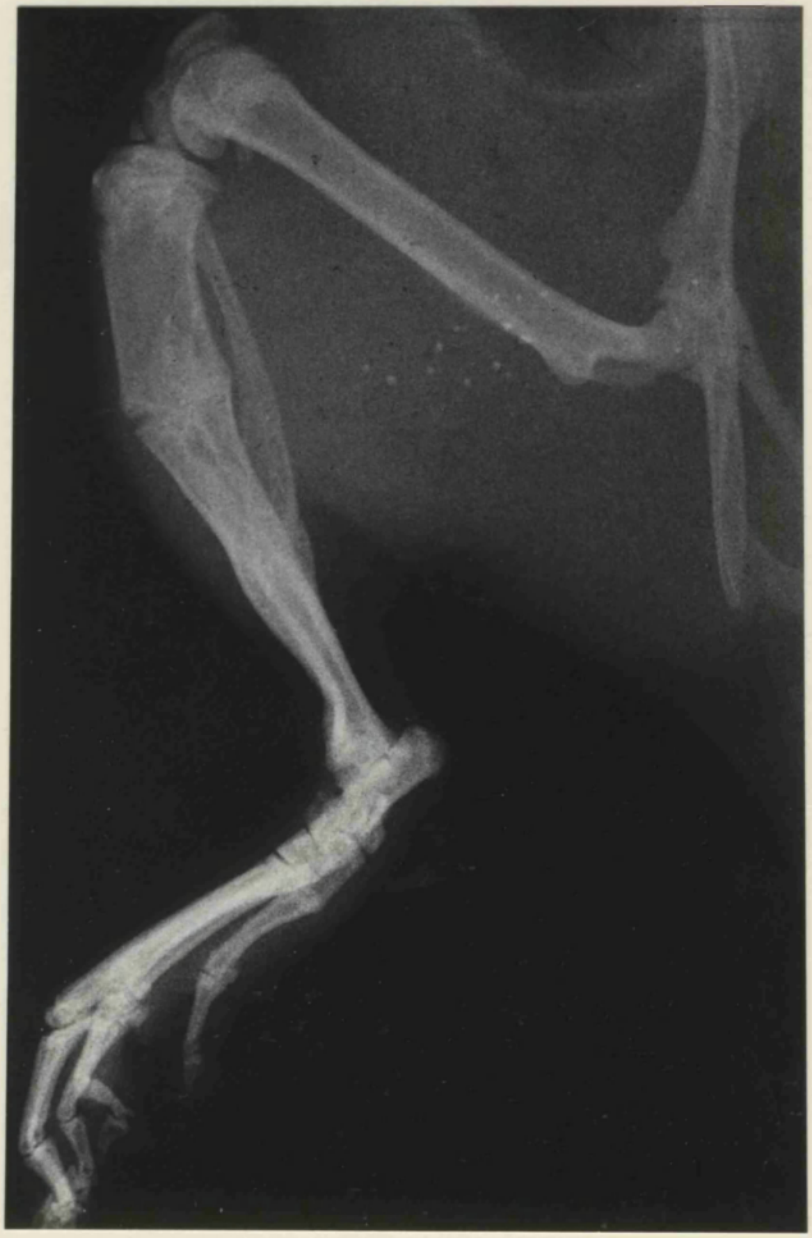

FIG. 2, 6

Fig. 2,7. - Epiphyseal cartilage showing altered matrix and irregular proliferation of chondrocytes. Rat. H. and E. X 110.

Fig. 2,8. - Autoradiograph of neighbouring section. No radiosulphate is present in the altered matrix. Rat. Kodak AR10. X 100.

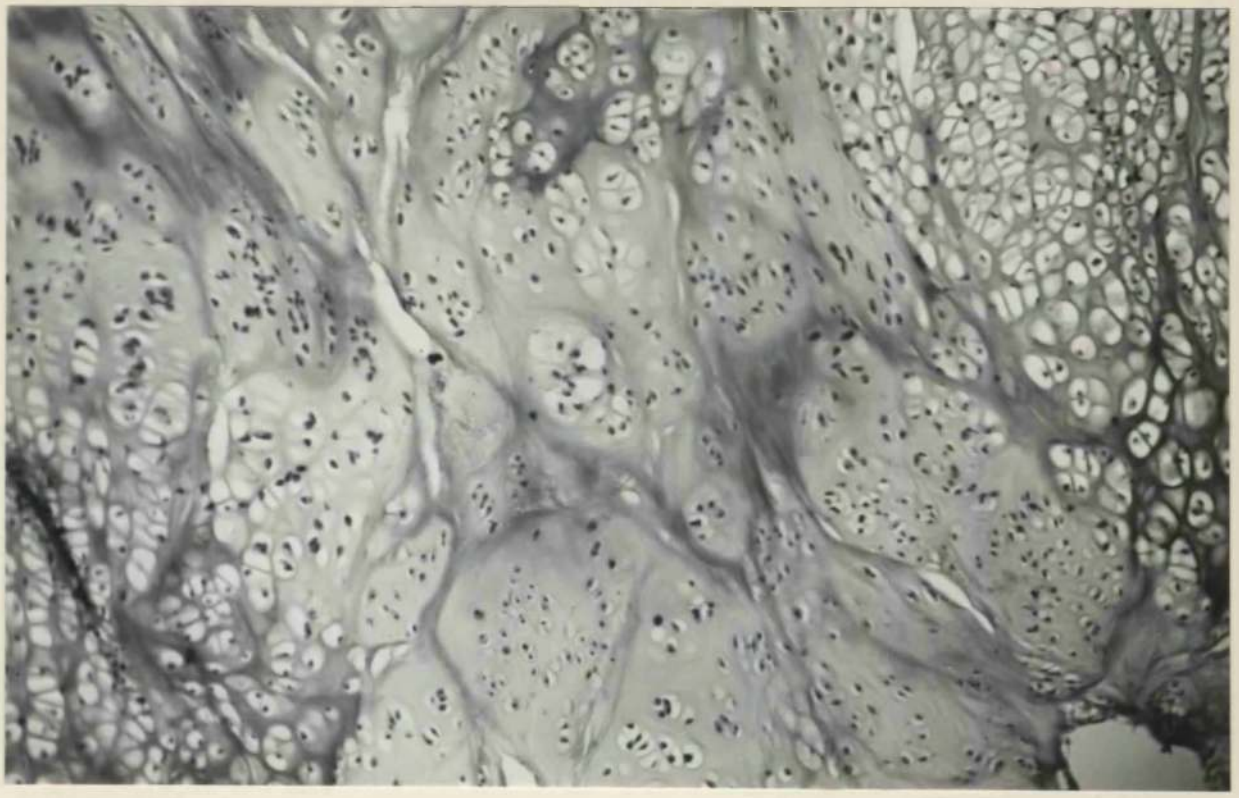

FIG. 2, 7

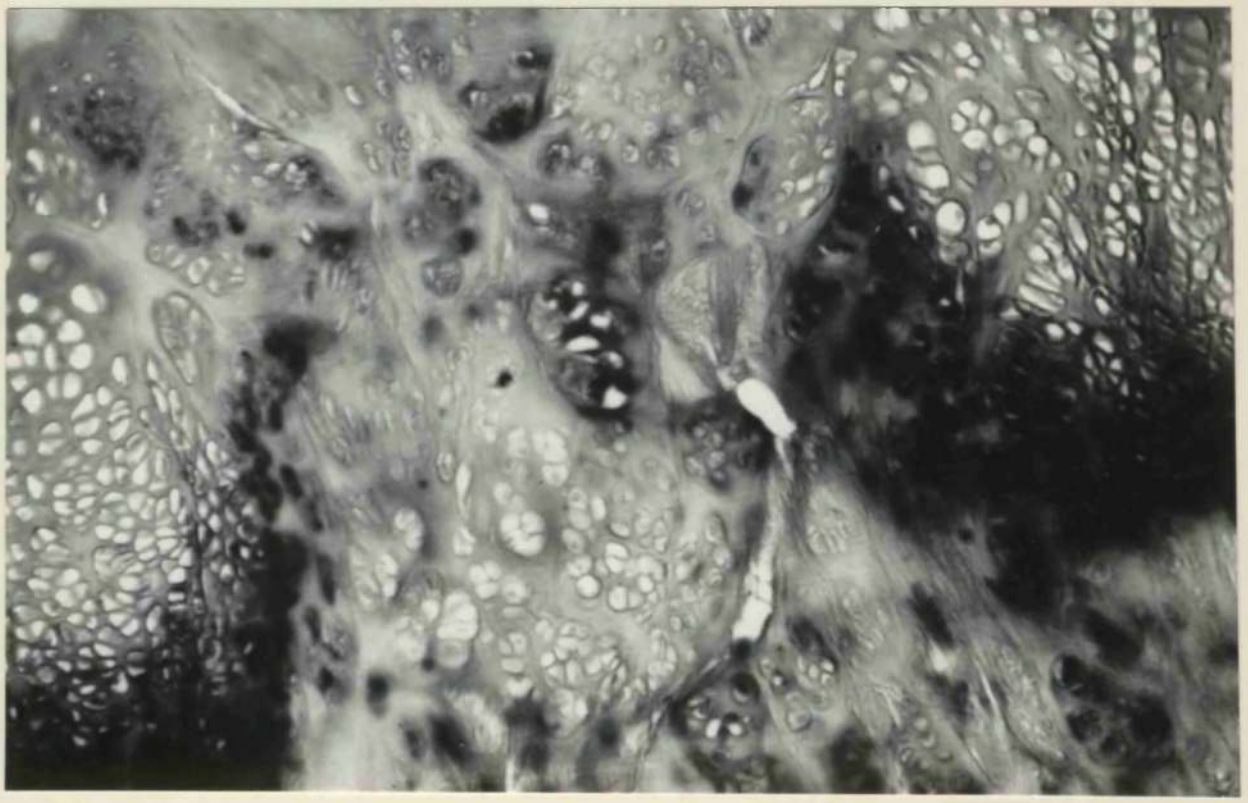

FIG. 2, 8

Fig. 2,9. - Autoradiograph showing radio-sulphate distribution in normal endochondral ossification in rat. Kodak AR10. X 100.

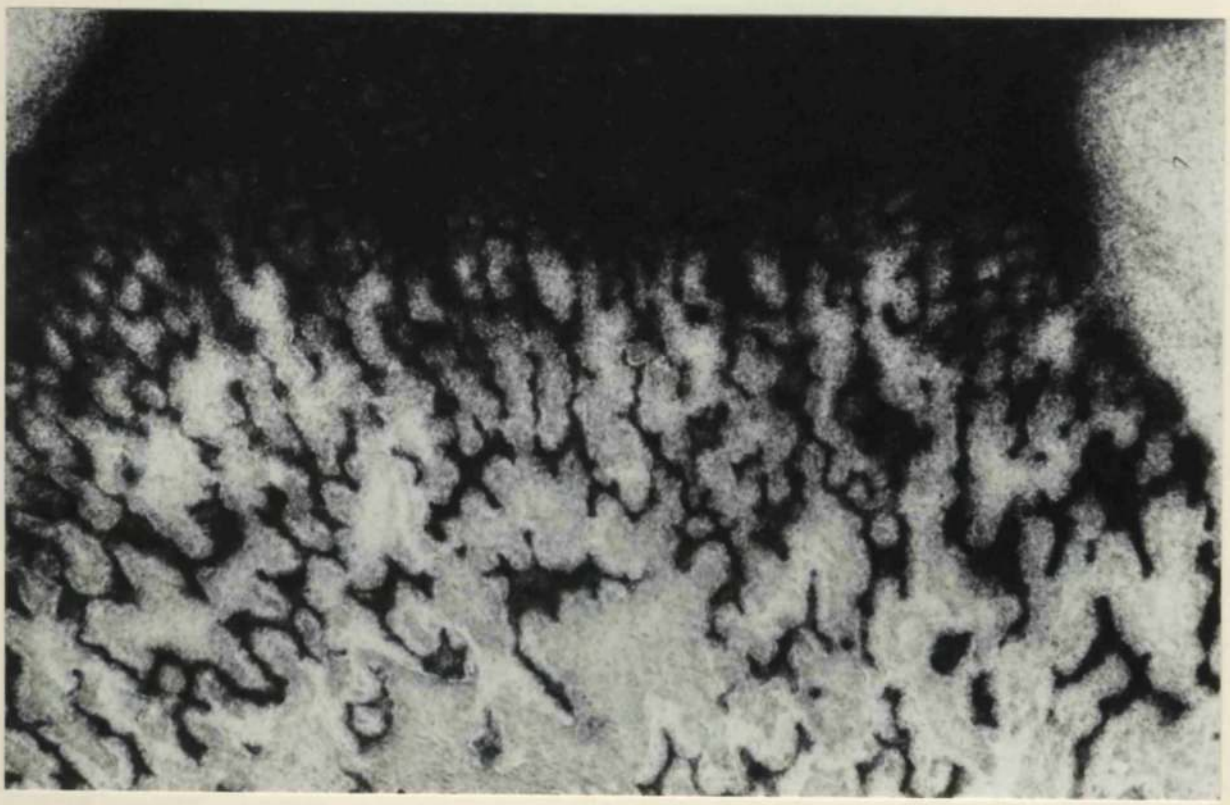

FIG. 2,9

Fig. 2,10. - Periosteal proliferation in lathyrism. An early osteogenic field. Rat. Toluidine blue. X 100.

Fig. 2,11. - Autoradiograph of neighbouring section. The sulph image is diffuse over cells and fibres. Rat. Kodak AR50. X 100.

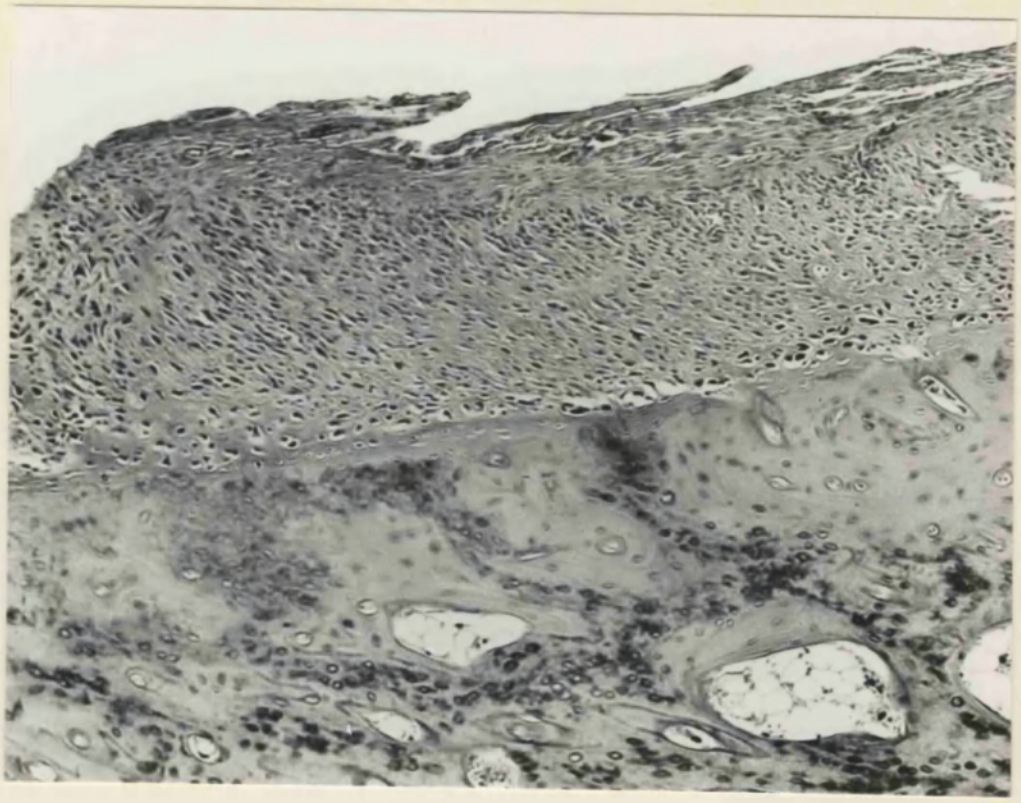

FIG. 2,10

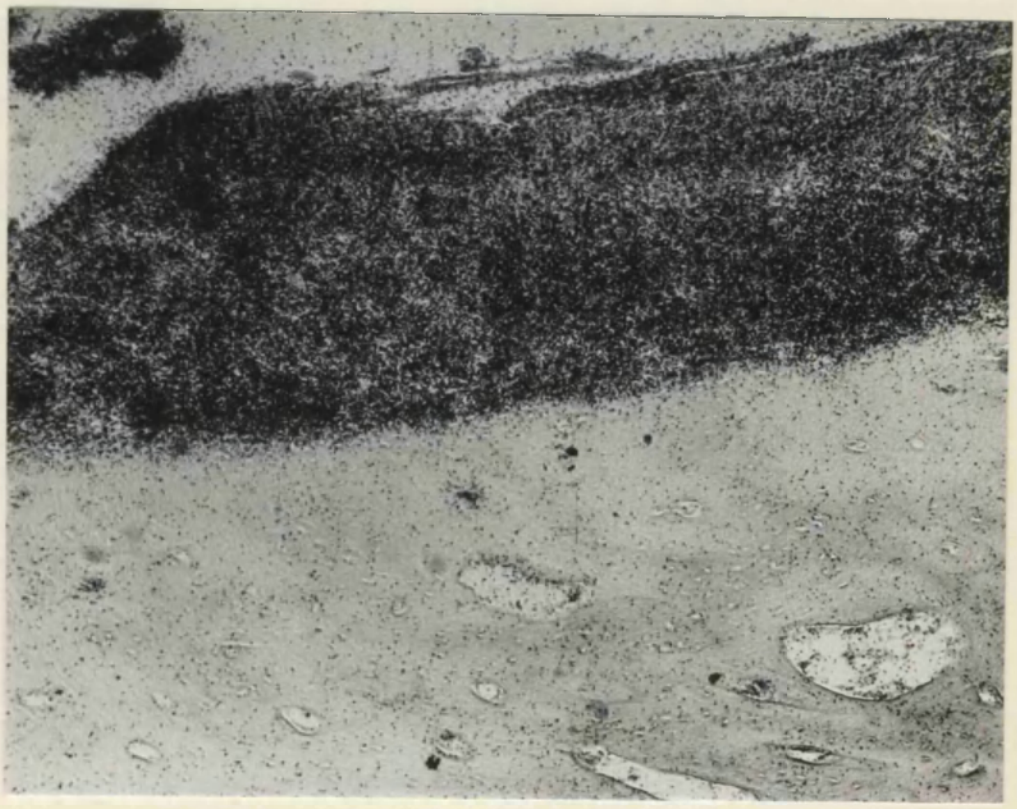

FIG. 2,11

Fig. 2,12. - Osteogenic field in lathyrism at stage of fibro-cellular condensation. The sulphate-image is over areas of imminent osteogenesis. Rat. Autoradiograph, Kodak AR10. X 150.

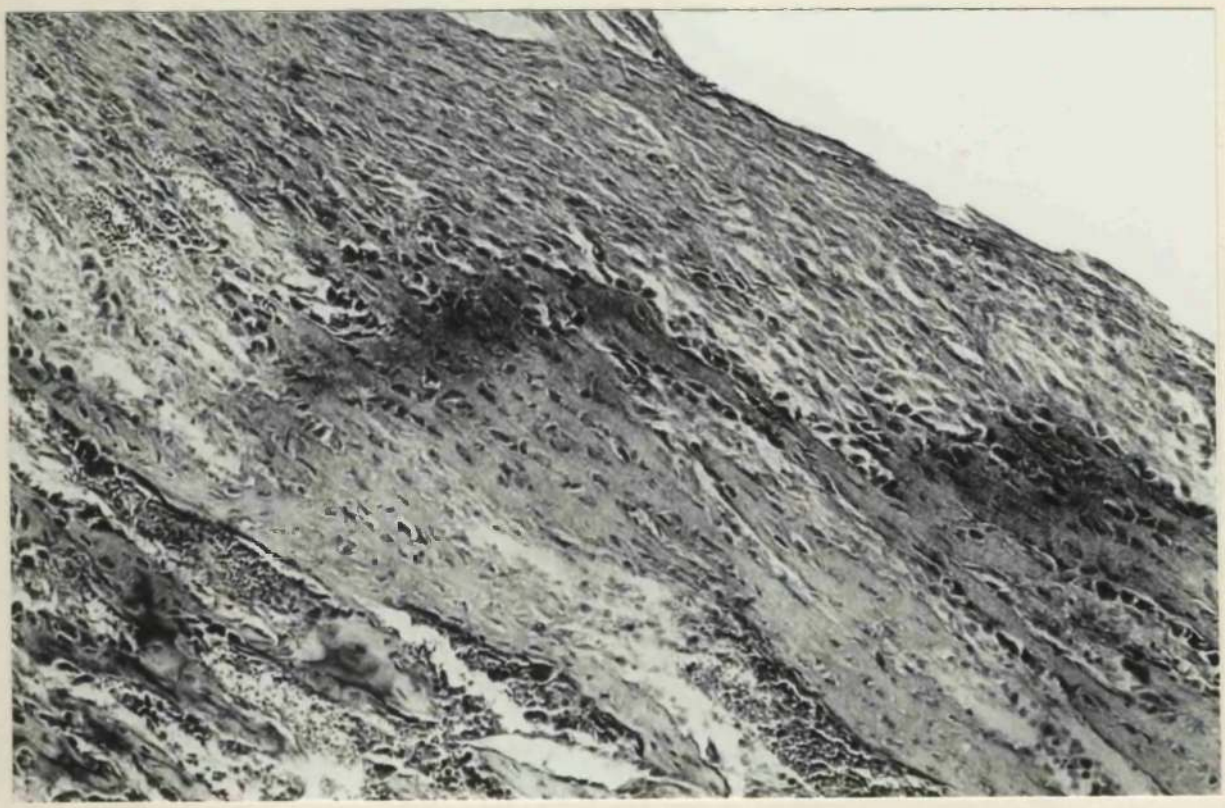

FIG.2,12

Fig. 2,13. - Later stage of intramembranous ossification in lathyritic rat. Toluidine blue. X 90.

Fig. 2,14. - Autoradiograph of neighbouring section. The new woven bone contains abundant labelled sulphated mucopolysaccharide. Rat. Kodak AR50. X 90.

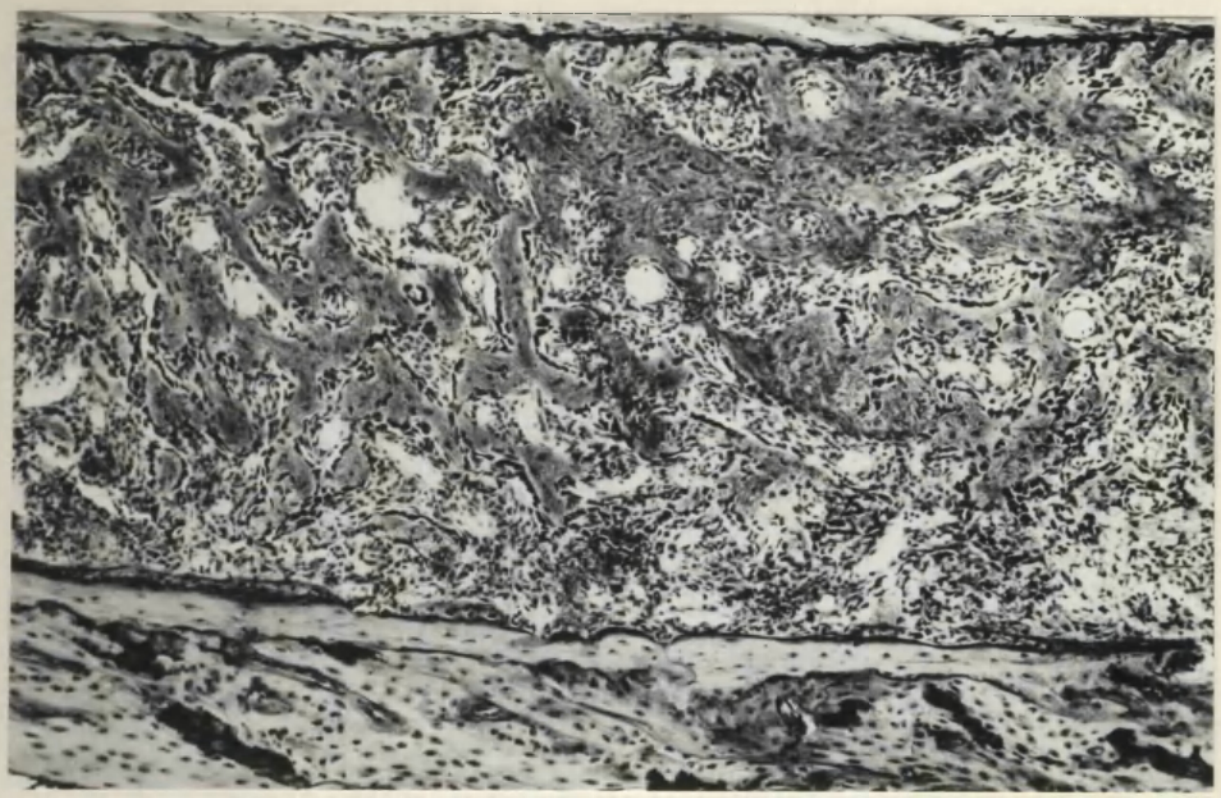

FIG. 2,13

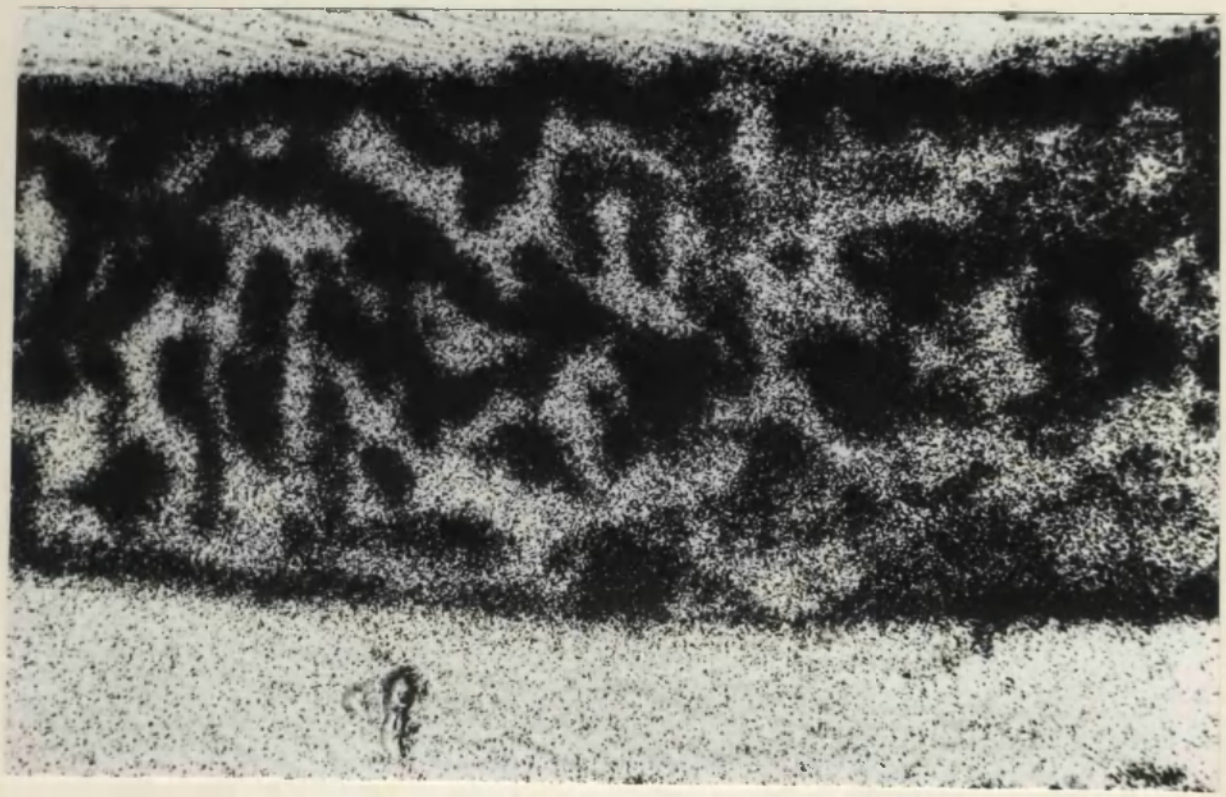

FIG. 2,14

Fig. 2,15. - Autoradiograph of new woven bone. The sulphate-image is diffuse over cells, fibres, and matrix; it is limited by the peripheral osteoblasts. Rat. Kodak AR10. X 250.

Fig. 2,16. - Autoradiograph showing radio-sulphate incorporation in appositional bone laid down on woven bone. Rat. Kodak AR50. X 225.

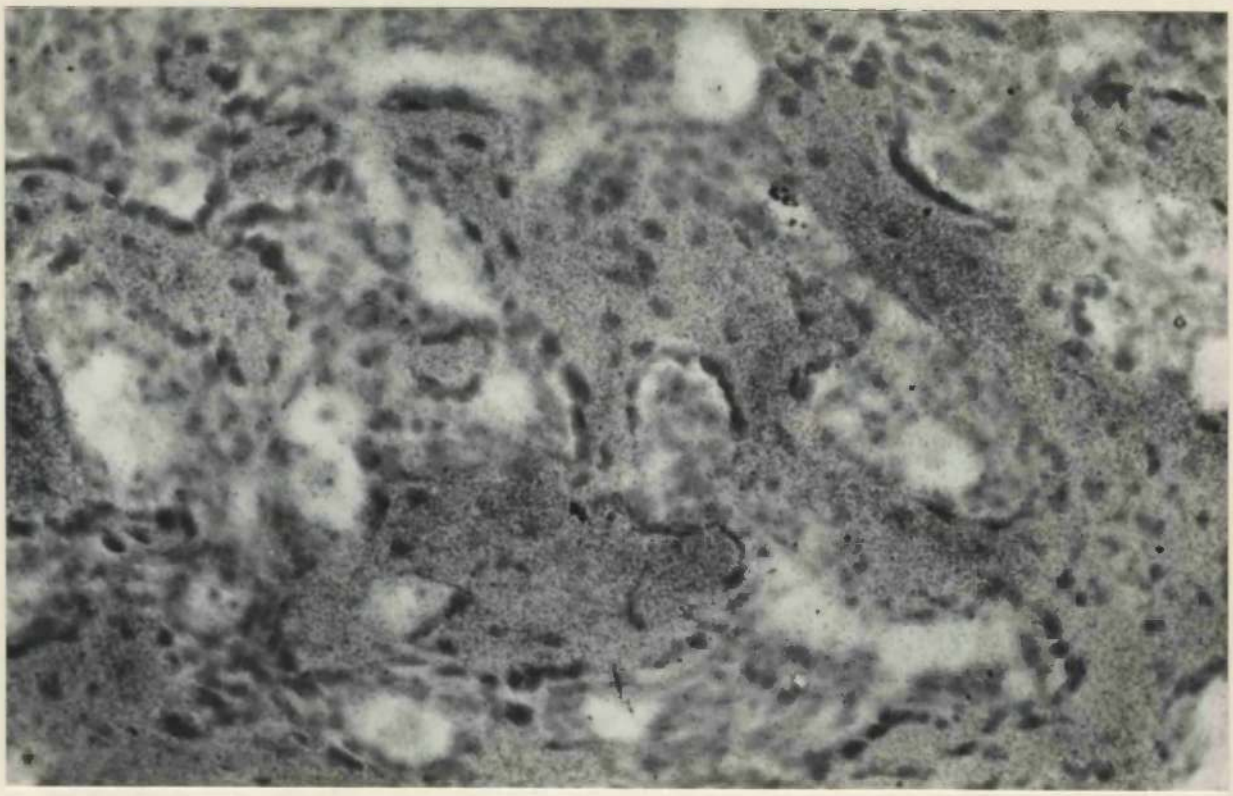

FIG.2,15

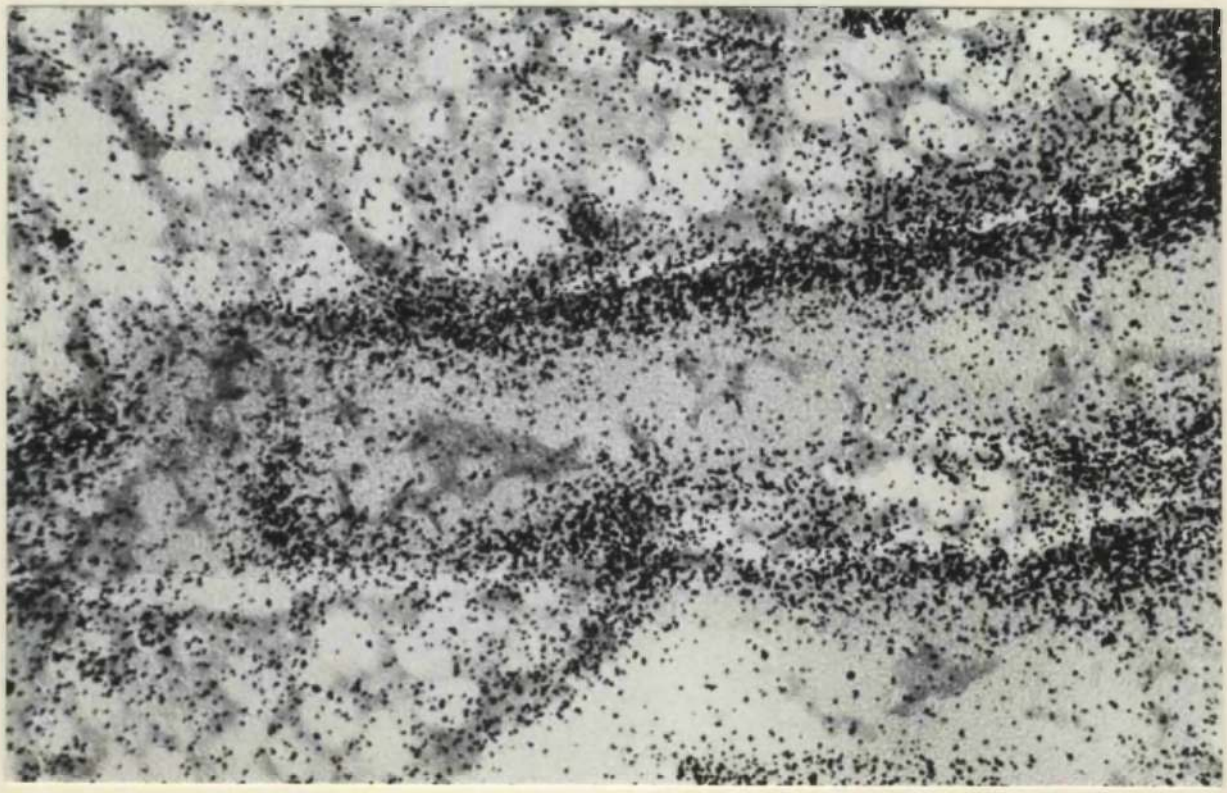

FIG.2,16

Fig. 2,17. - Transverse section of normal rat long bone showing displacement of sulphate-image by further appositional growth from periosteum. Autoradiograph, Kodak AR10. X 90.

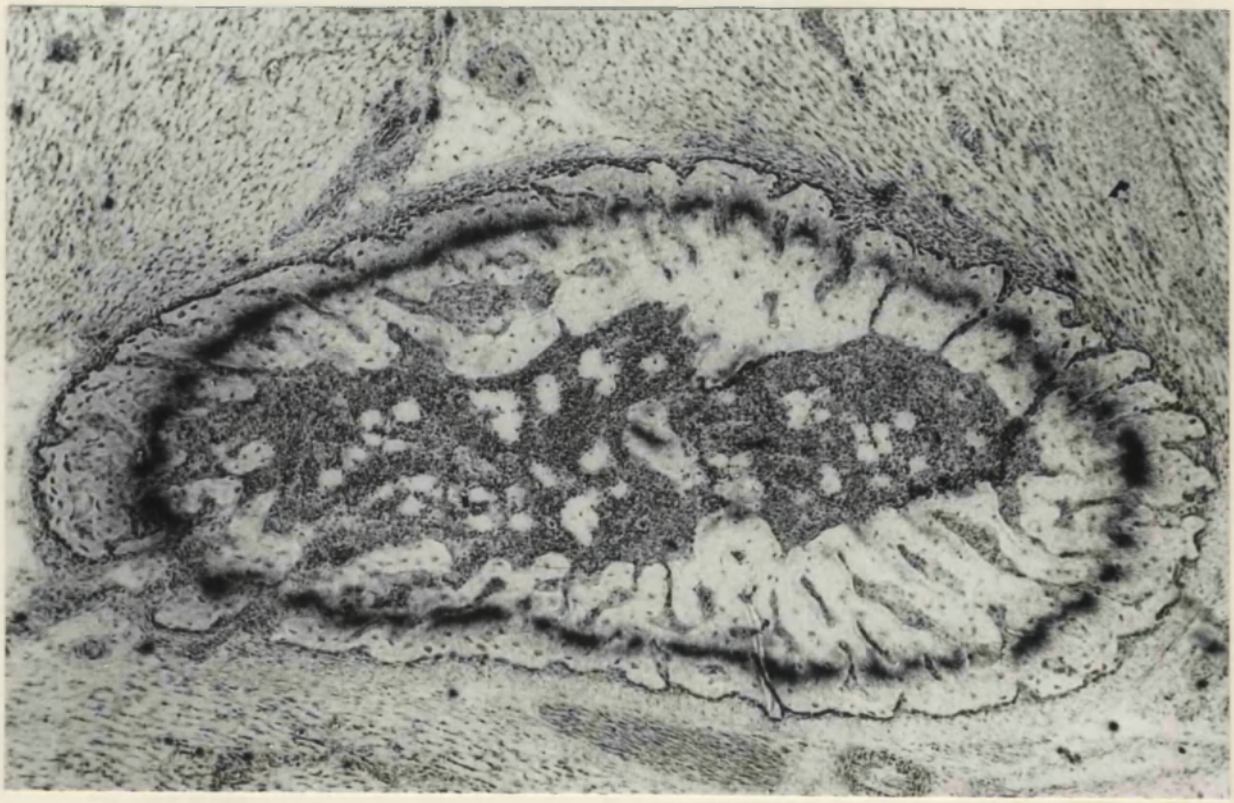

FIG.2,17

Fig. 2,18. - Epiphyseal cartilage in lathyritic rat showing altered matrix and irregular cellular proliferation. H. and E. X 400.

Fig. 2,19. - Autoradiograph of neighbouring section showing presence of radio-glycine in the altered matrix. Rat. Kodak AR50. X 90.

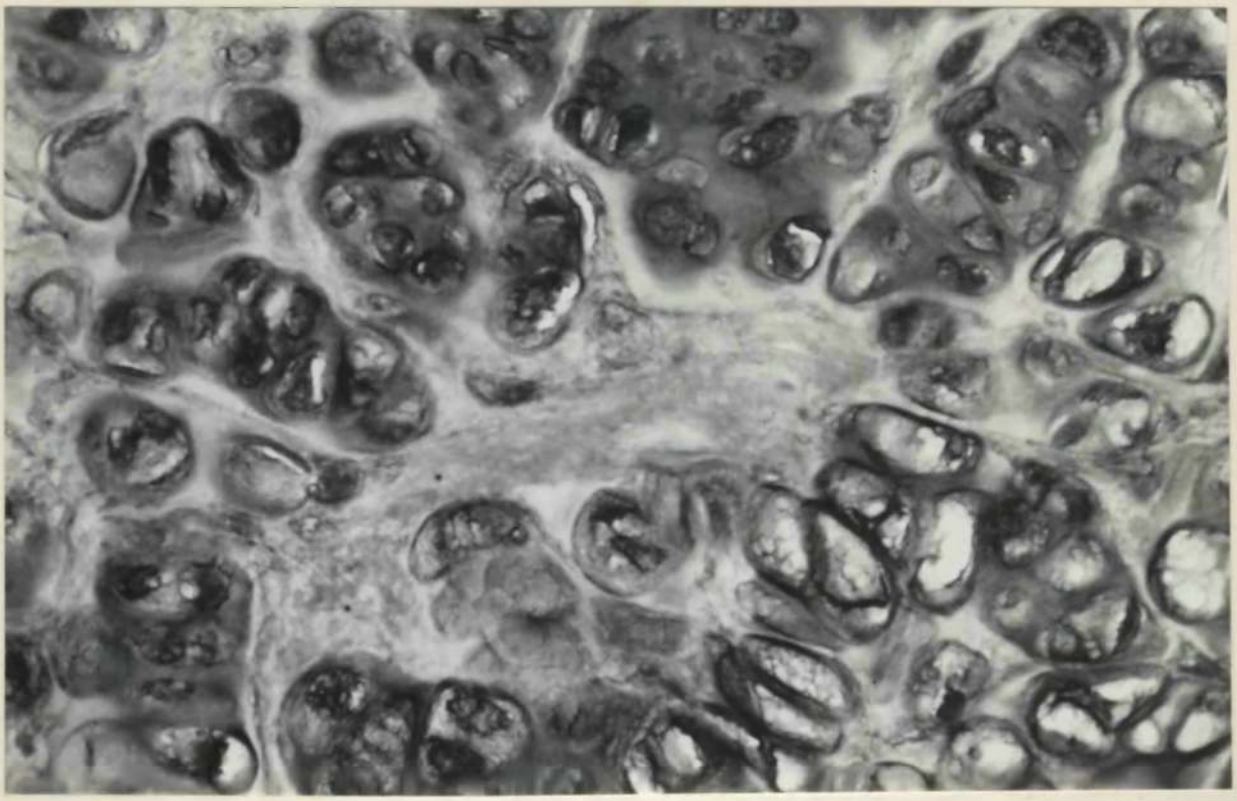

FIG. 2,18

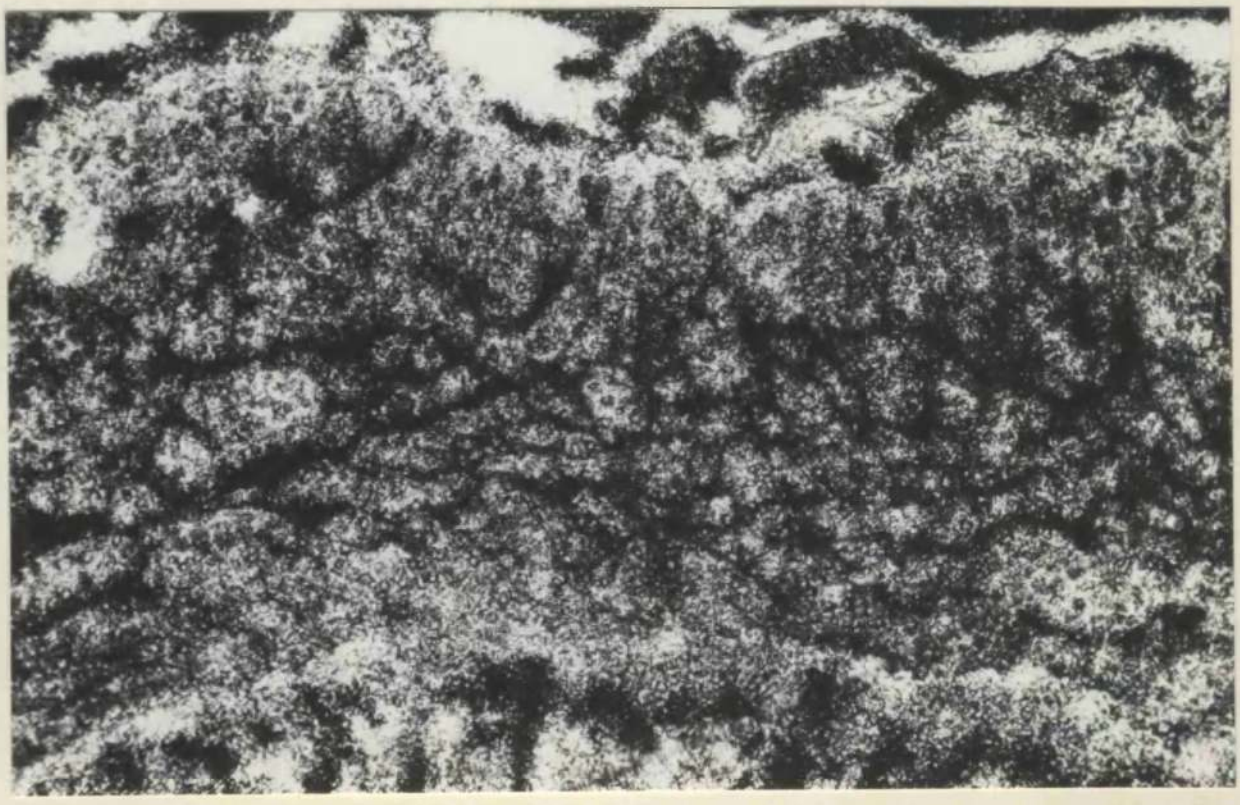

FIG. 2,19

Fig. 2,20. - The clumps of cartilage cells in the lathyritic rat epiphyseal cartilage are clearly separated by radio-glycine labelled matrix. Autoradiograph, Kodak AR10. X 330.

FIG.2,20

Fig. 2,21. - Altered matrix and tear in lathyritic epiphyseal cartilage. Rat. H. and E. X 400.

Fig. 2,22. - Corresponding autoradiograph showing presence of radio-methionine in the altered matrix. Rat. Kodak AR50. X 130.

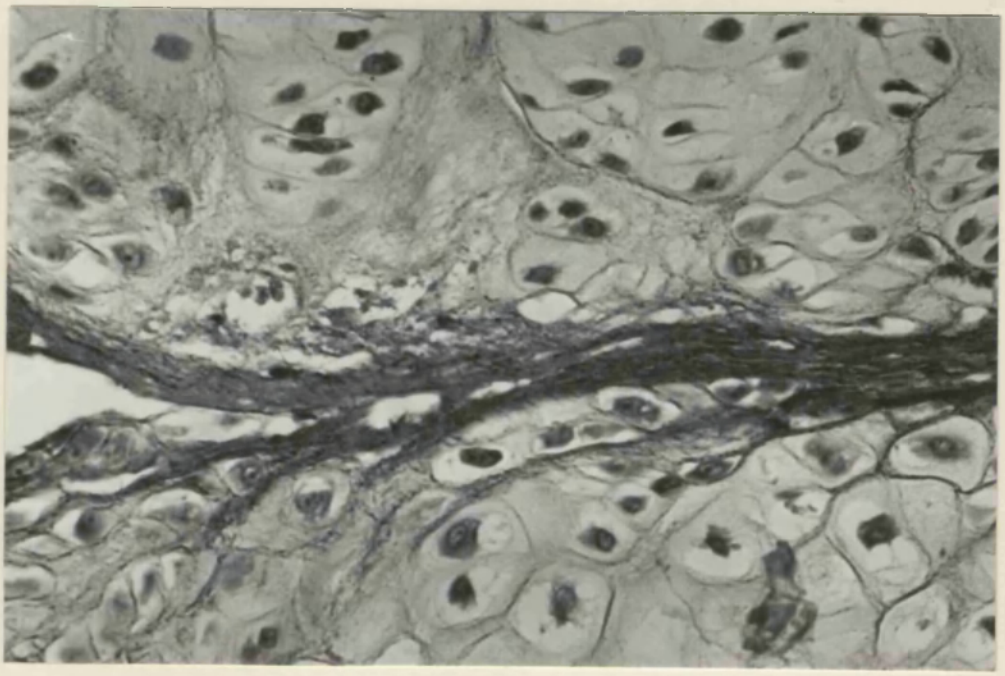

FIG.2,21

FIG.2,22

Fig. 2,23. - Autoradiograph showing radio-glycine in appositional bone laid down on woven bone. Rat. Kodak AR10. X 150.

Fig. 2,24. - Incisor in oblique coronal section: adult rat 19 hours. after injection. Sulphur-35 is present in the predentine as a uniform layer, and is present mainly focally in cells of the dental pulp. Autoradiograph, Kodak AR50. X 220.

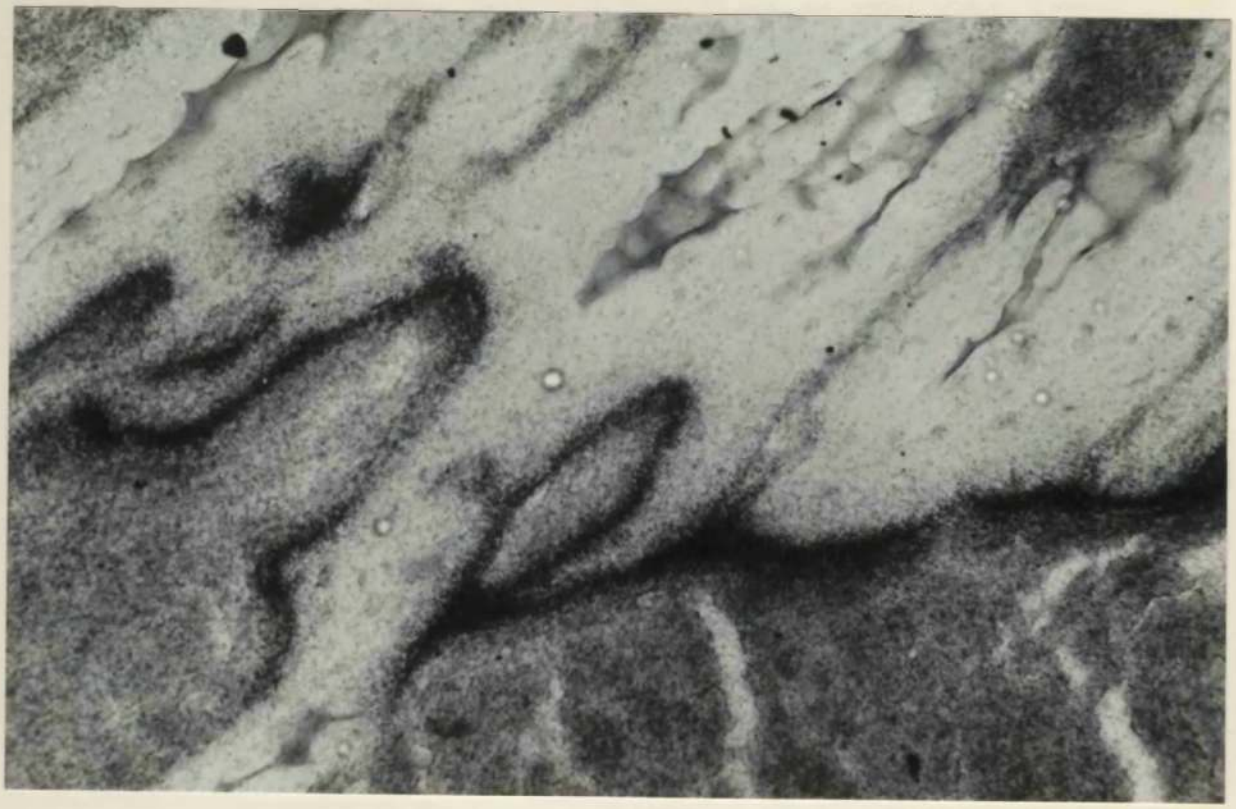

FIG. 2,23

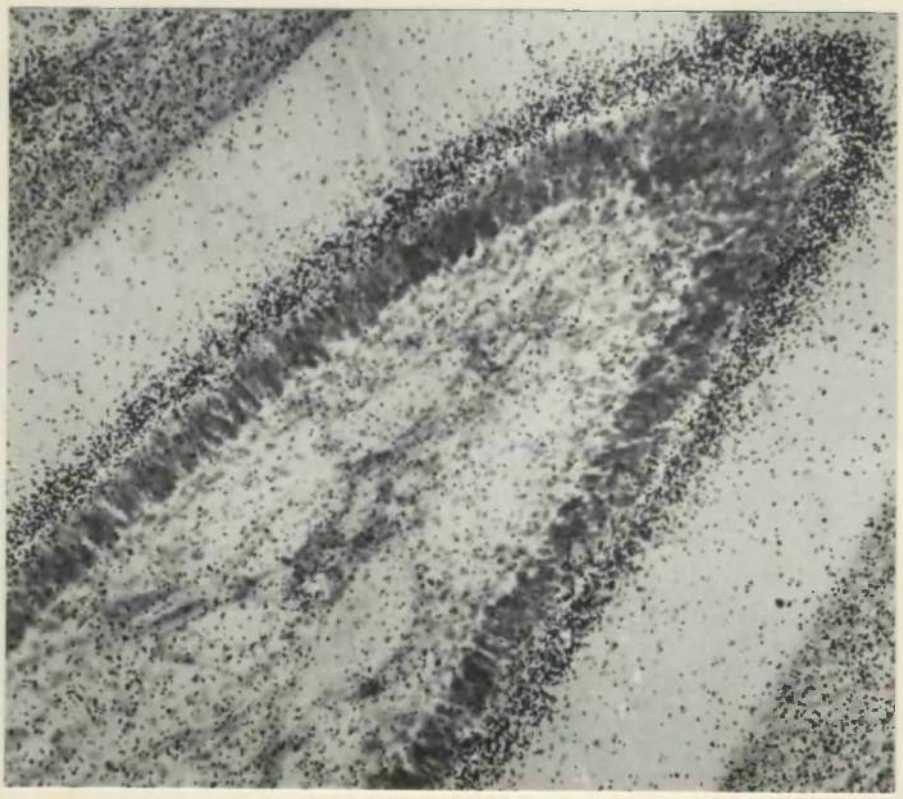

FIG. 2, 24

Fig. 2,25. - Incisor in coronal section: foetal mouse 18 hours after injection. Sulphur-35 is present as a uniform band in the predentine: the enamel matrix separated in processing from the ameloblasts shows a lighter uptake. Autoradiograph, Kodak AR10. X 390.

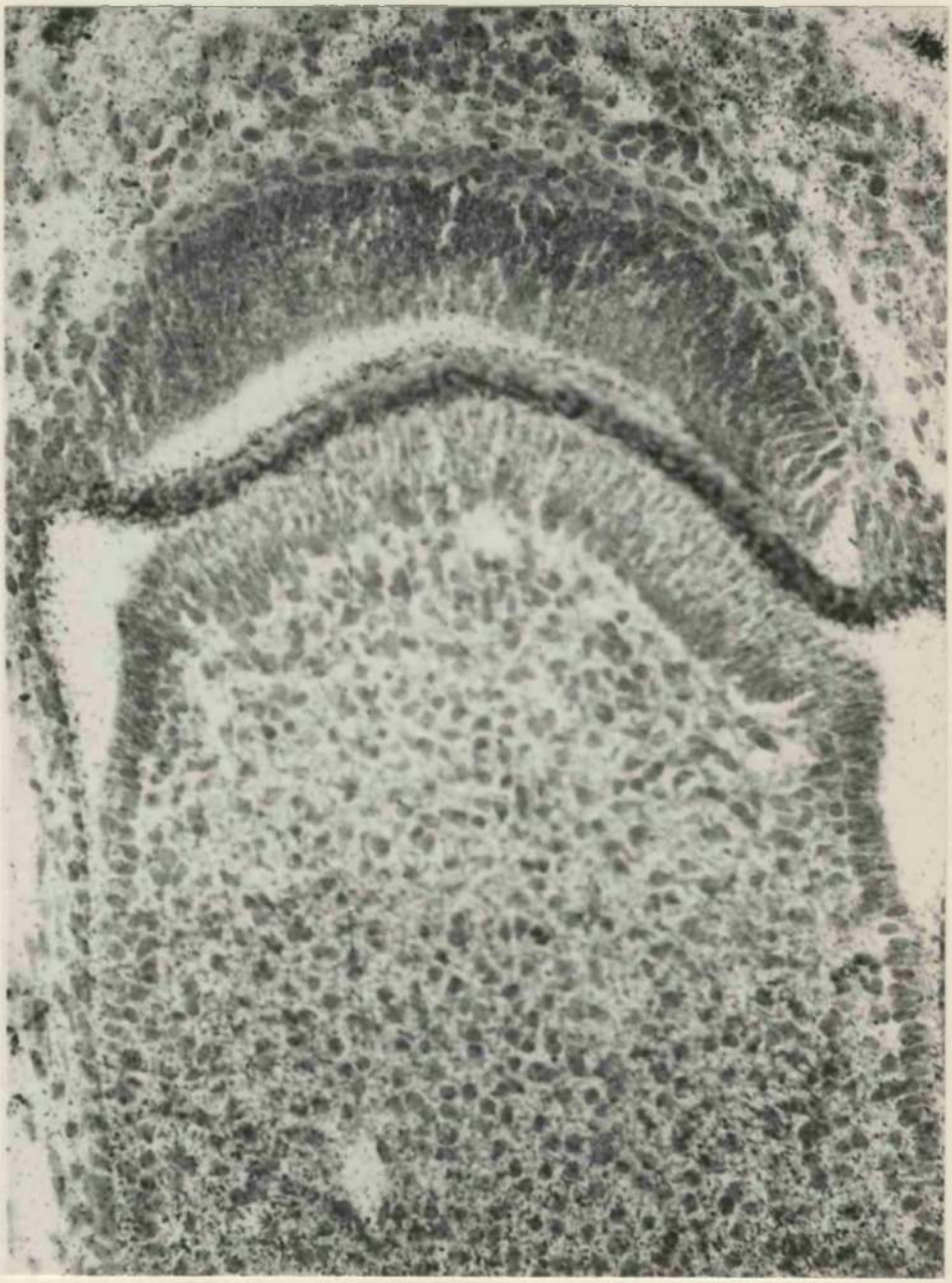

FIG. 2.25

Fig. 2,26. - Molar in sagittal section: suckling rat 94 hours after injection. The radioactive zone in the dentinal matrix is about equidistant from the predentine and the amelo-dentinal junction at the cusp. Autoradiograph, Kodak AR10. X 280.

Fig. 2,27. - Molar in sagittal section: suckling rat 164 hours after injection. The edge of the radioactive zone in the dentinal matrix coincides with the amelo-dentinal junction. Autoradiograph, Kodak AR10. X 200.

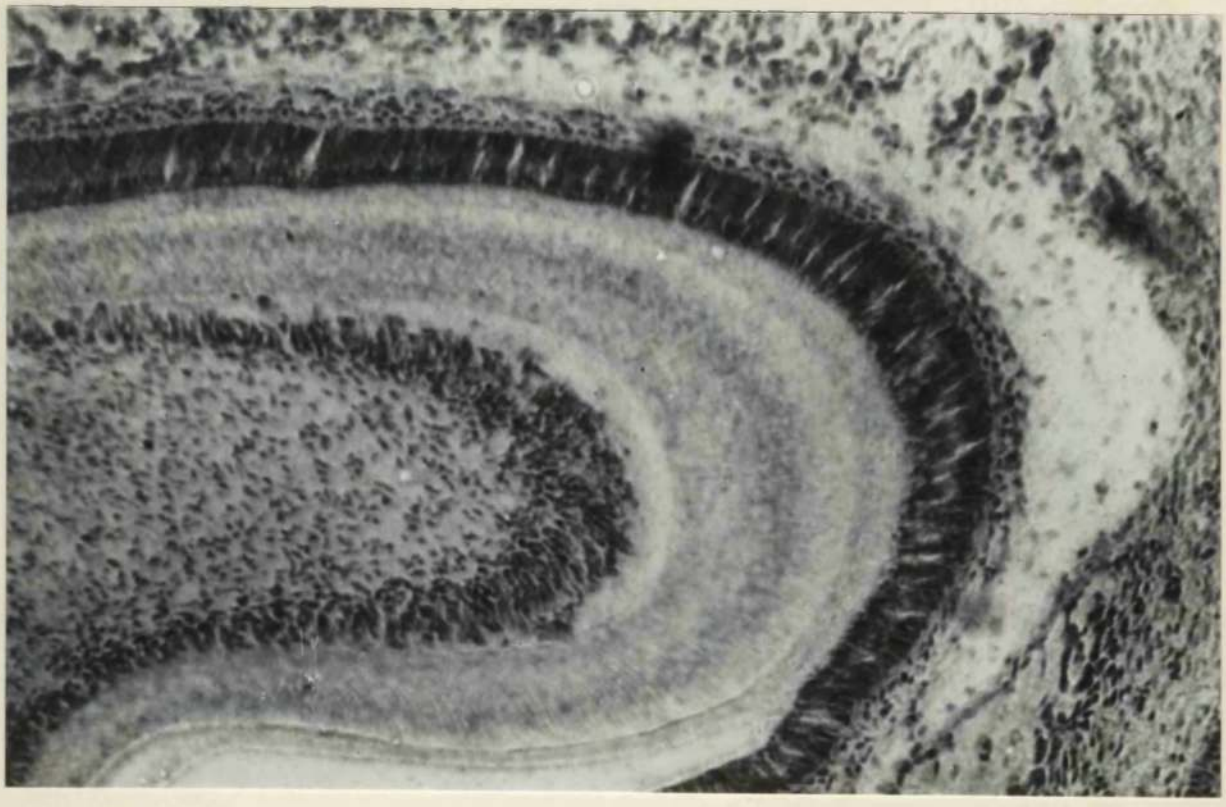

FIG. 2,26

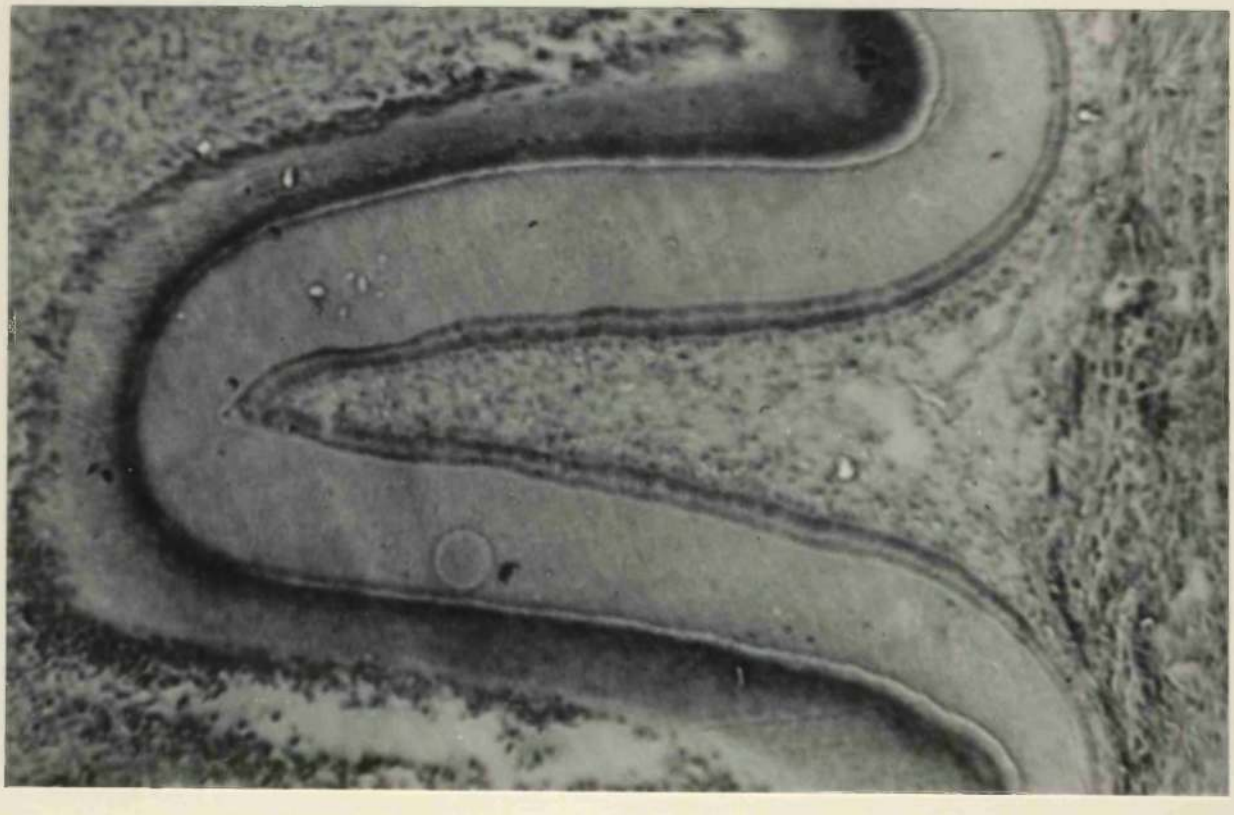

FIG. 2,27

Fig. 2,28. - Incisor in sagittal section: suckling rat 118 hours after injection. Two closely apposed zones of activity are present in the dentinal matrix on the convex side of the tooth (bottom) about one-third of the width of the matrix from the amelo-dentinal junction. A single zone is present in the dentinal matrix on the concave side (top). The enamel matrix (bottom) shows a uniform uptakethroughout its width. Autoradiograph, Kodak AR10. X 300.

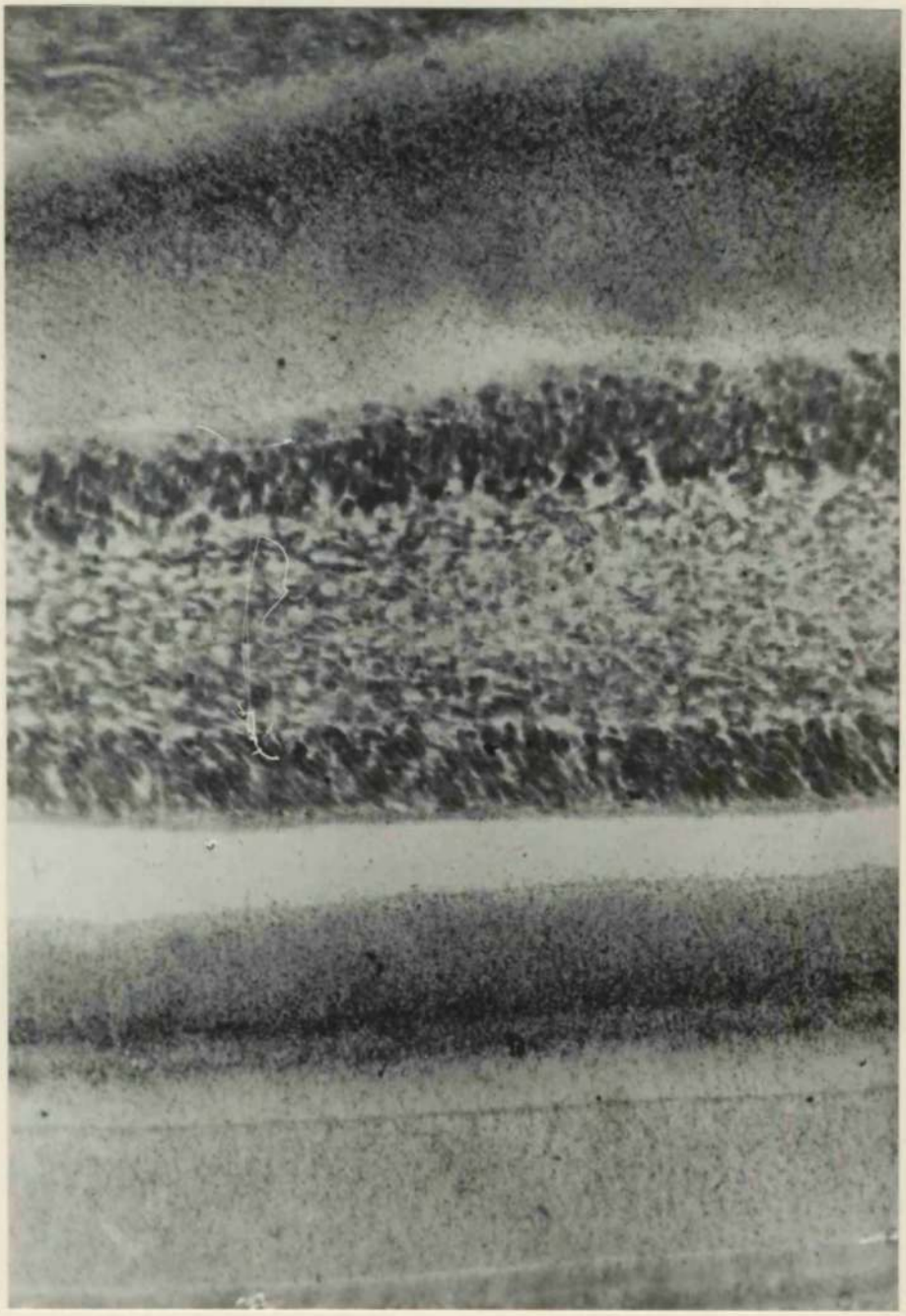

FIG. 2, 28

Fig. 2,29. - Incisor in sagittal section: suckling rat 118 hours after injection. The enamel matrix shows a uniform uptake throughout its width. Cells of the dental pulp (bottom right) show activity. Two zones of activity are present in the dentinal matrix as in fig. 2,28. Autoradiograph, Kodak AR10. X 500.

Fig. 2,30. - Molar in coronal section: suckling rat 8 hours after injection. Sulphur-35 is present in the odontoblasts (bottom left), the ameloblasts, the stratum intermedium, the whole width of the enamel matrix, the predentine, and in some cells of the dental pulp. Autoradiograph, Kodak AR10. X 670.

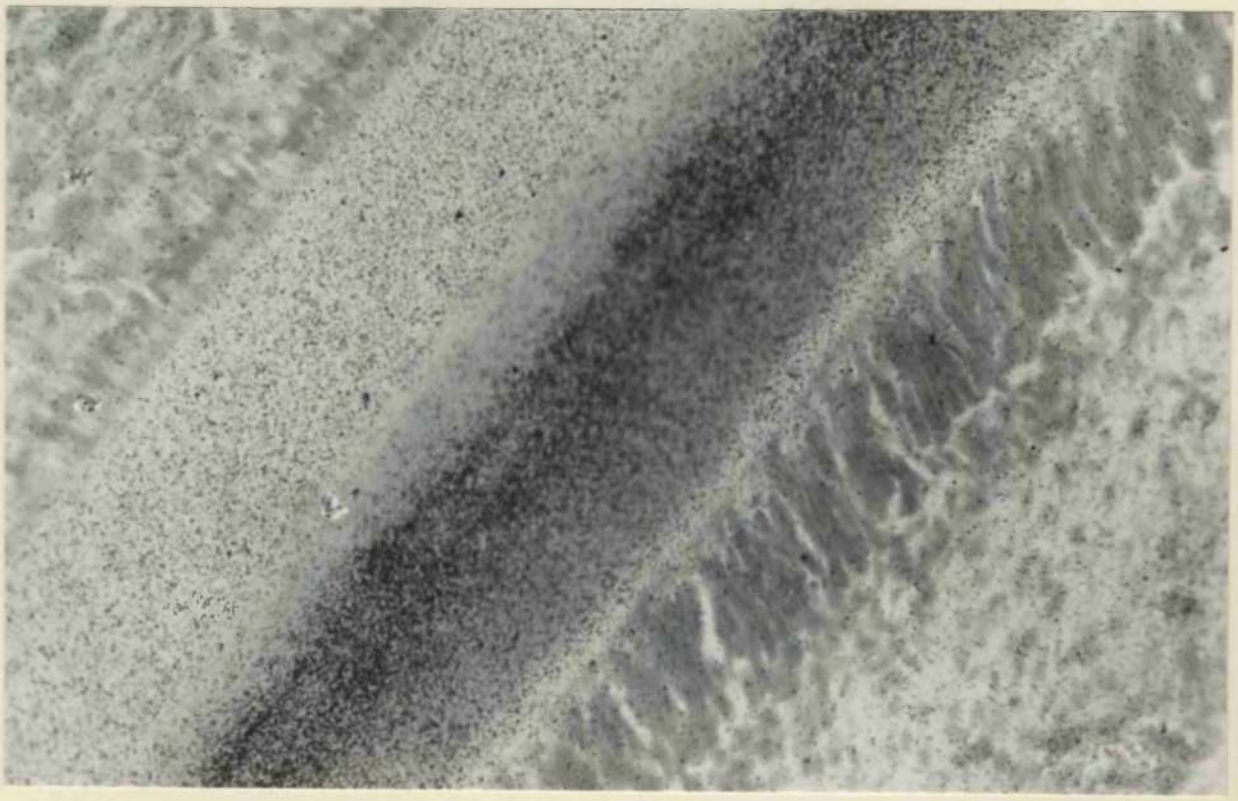

FIG. 2, 29

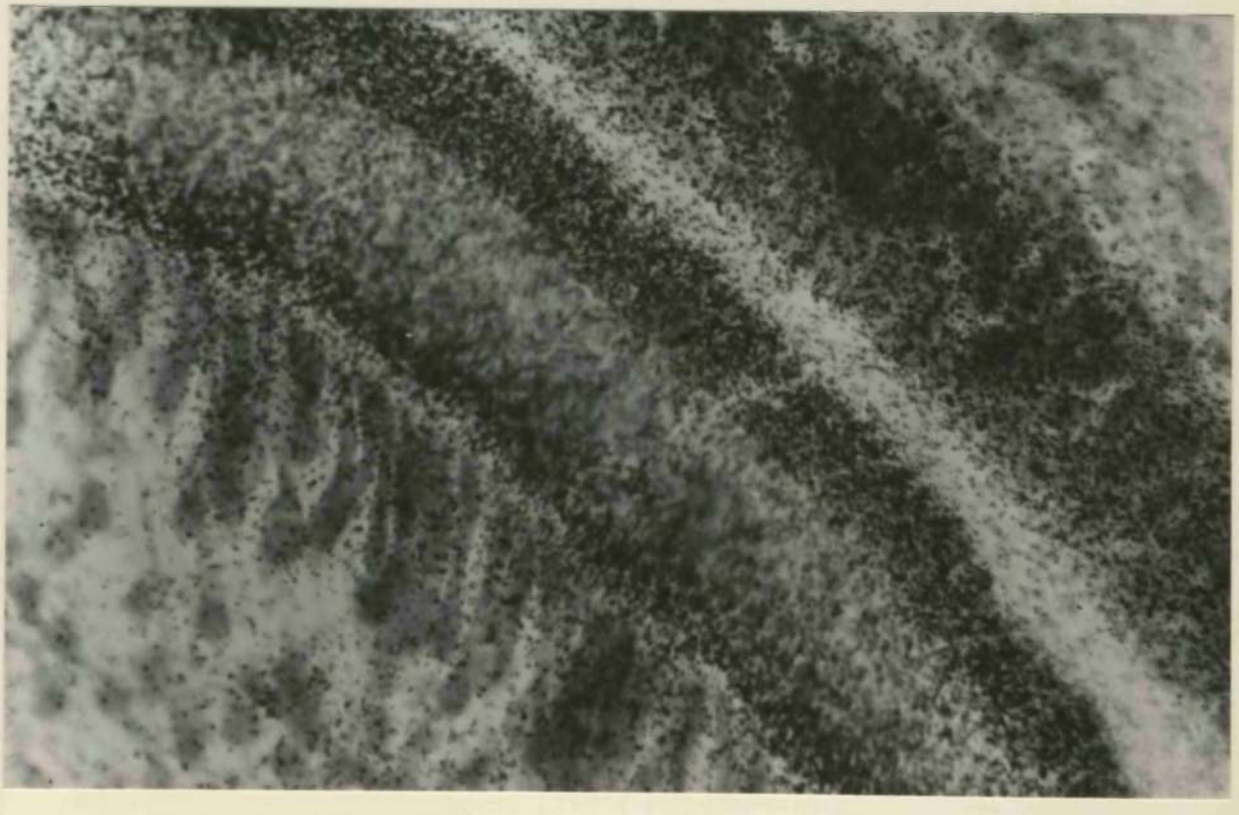

FIG. 2, 30

Fig. 2,31. - Incisor in coronal section: suckling mouse 16 hours after injection. Sulphur-35 is present in the ameloblasts and in the stratum intermedium (top). Autoradiograph, Kodak AR10. X 1350.

Fig. 2,32. - Molar in sagittal section: suckling mouse 12 hours after injection. Sulphur-35 is present most intensely in the supranuclear parts of the ameloblasts. Autoradiograph, Kodak AR10. X 1350.

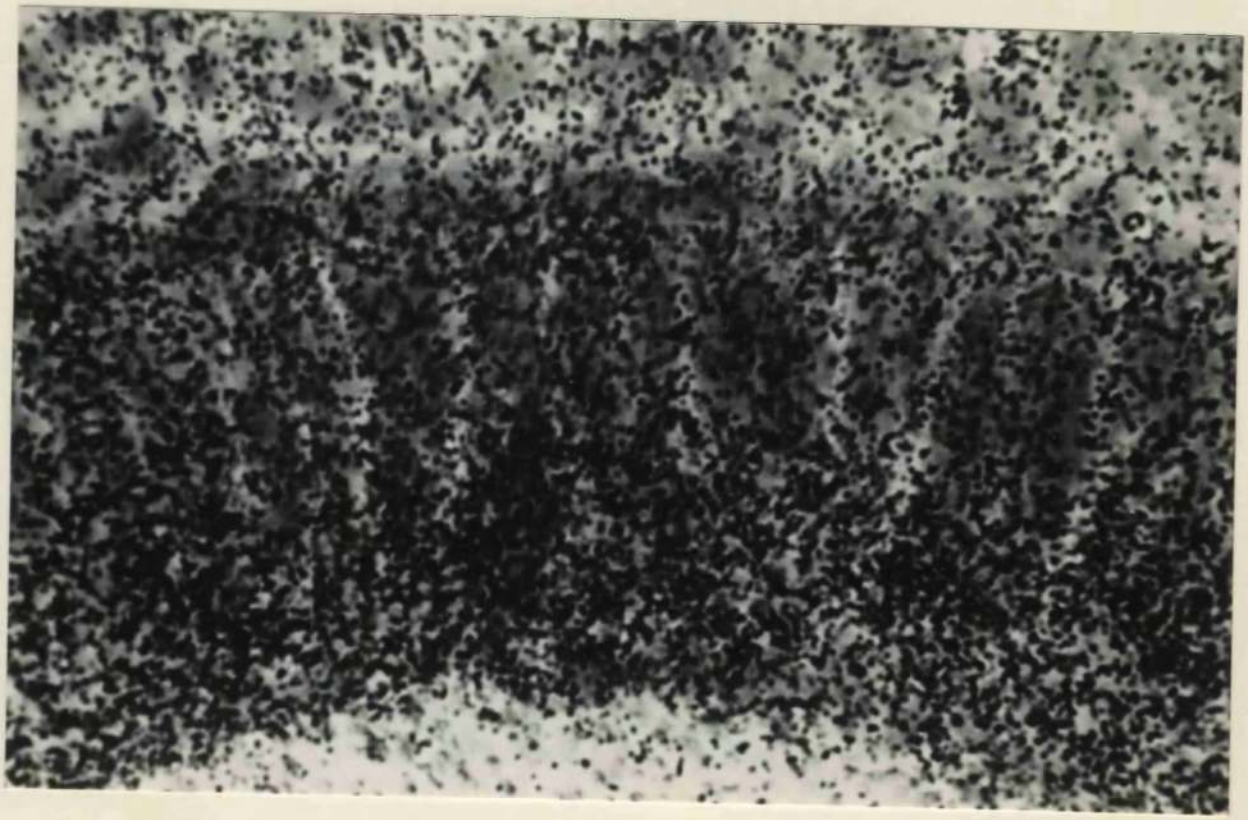

FIG. 2, 31

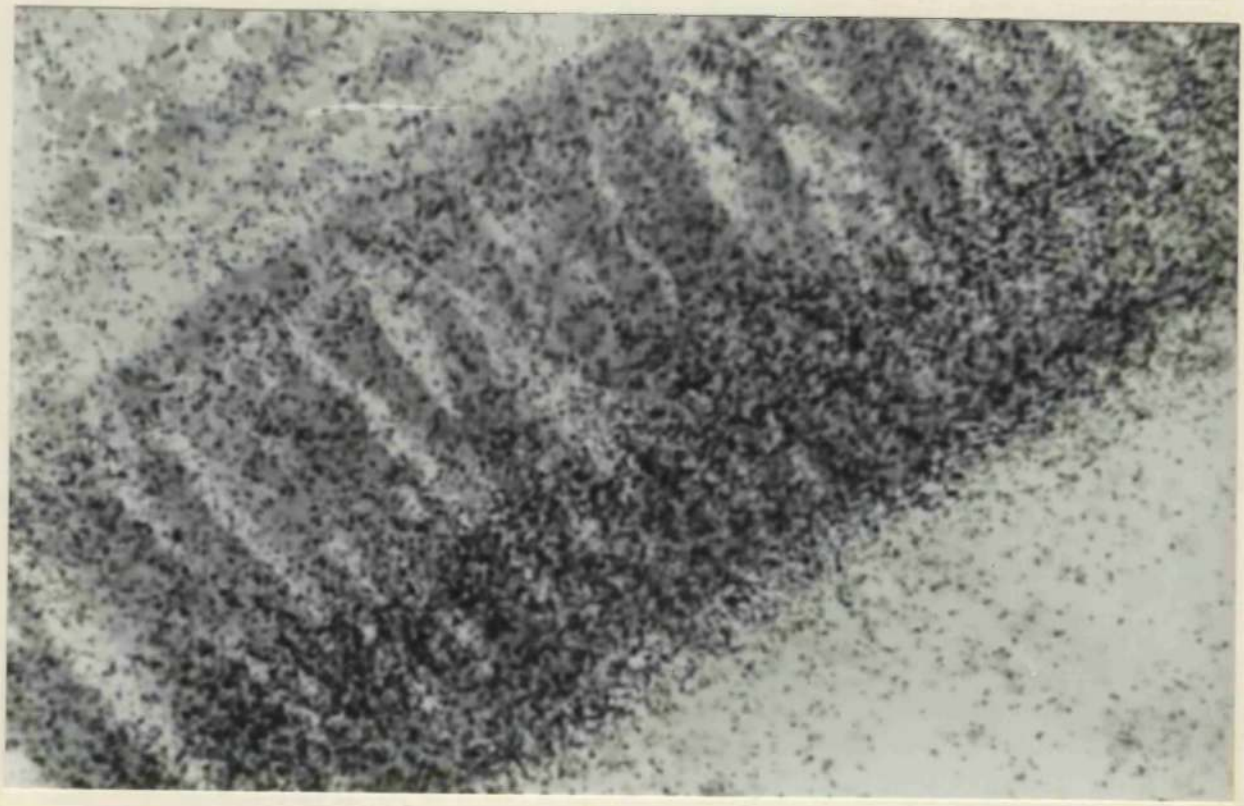

FIG. 2, 32

Fig. 2,33. - Coronal section of developing tooth in foetal mouse 18 hours after injection. The sulphate-image is most intense over the dental papilla and is also seen over the mesodermal tissue on either side of the stalk. Autoradiograph, Kodak AR10. X 225.

Fig. 2,34. - Molar in coronal section: suckling rat 6 hours after injection. Sulphur-35 is present focally in cells of the stellate reticulum and diffusely in the matrix. The stratum intermedium and ameloblasts (bottom, right) also show an uptake. Autoradiograph, Kodak AR10. X 780.

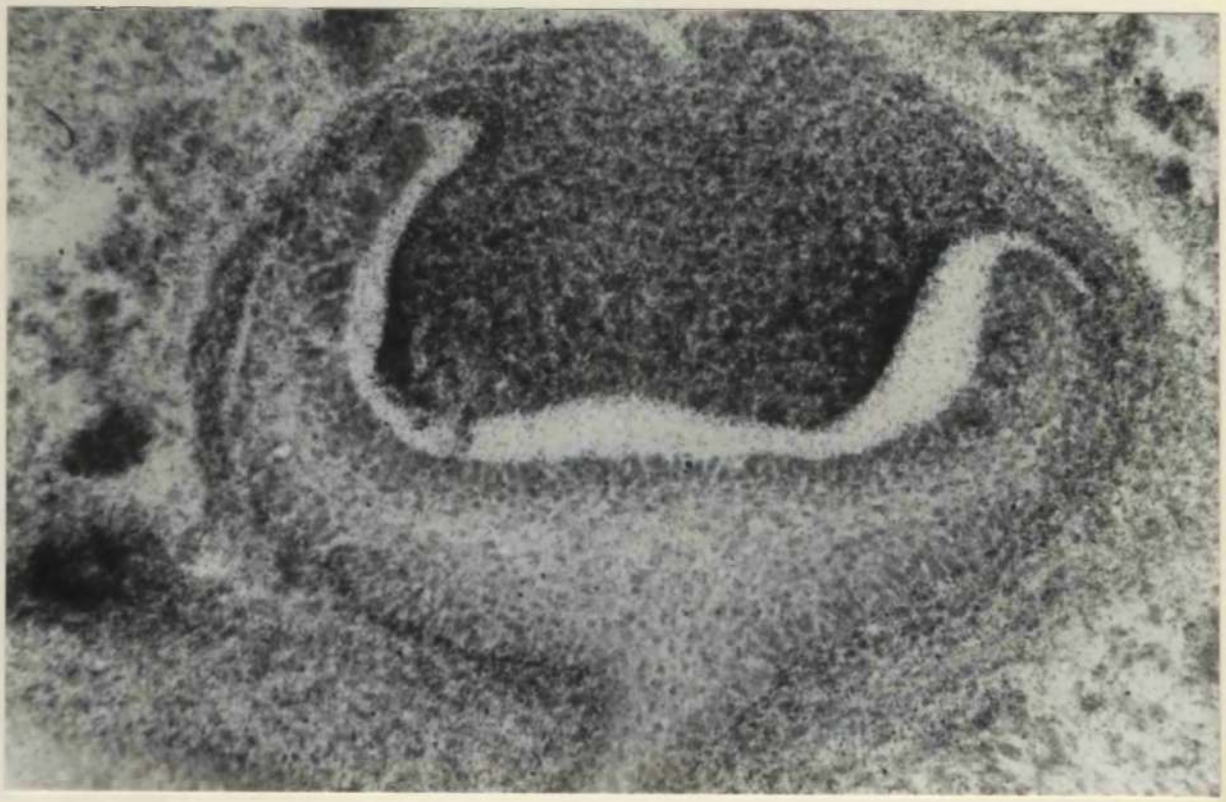

FIG. 2,33

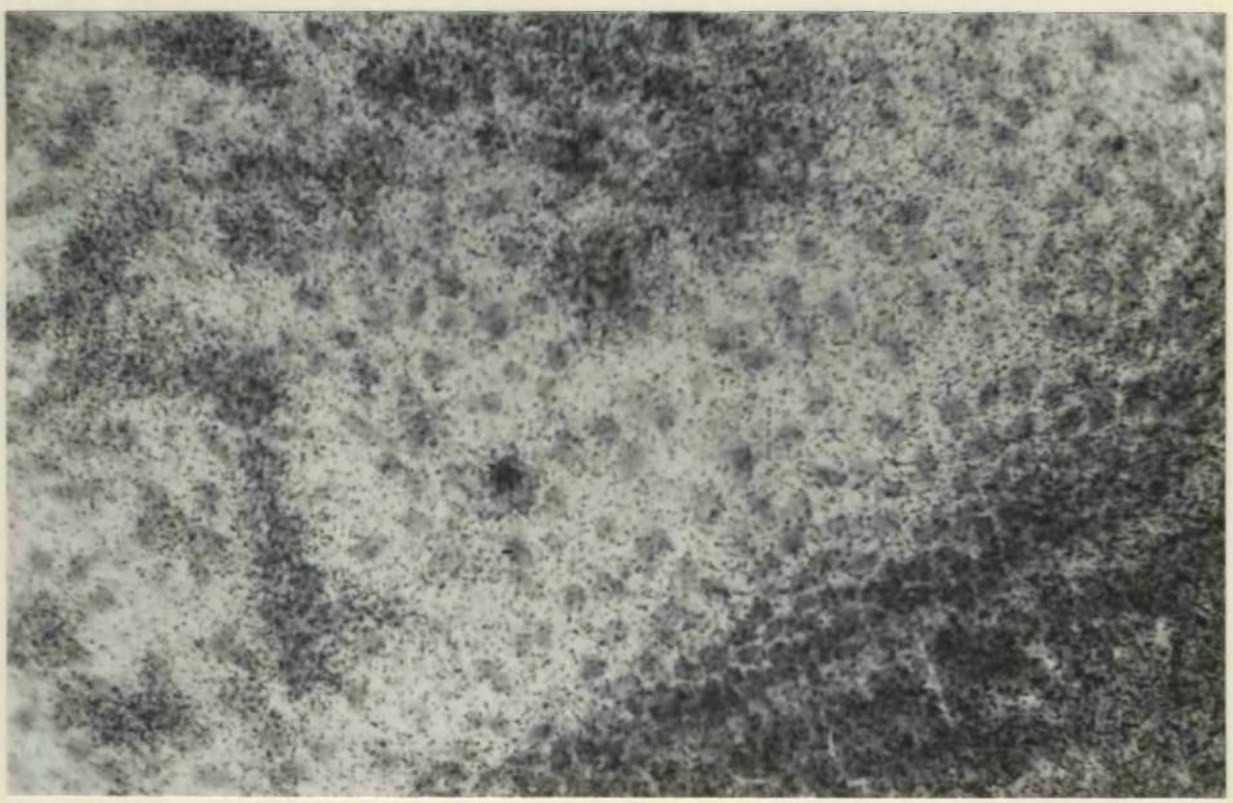

FIG. 2,34

Fig. 2,35. - Diagram of the calciotraumatic response (Irving et al, 1949).

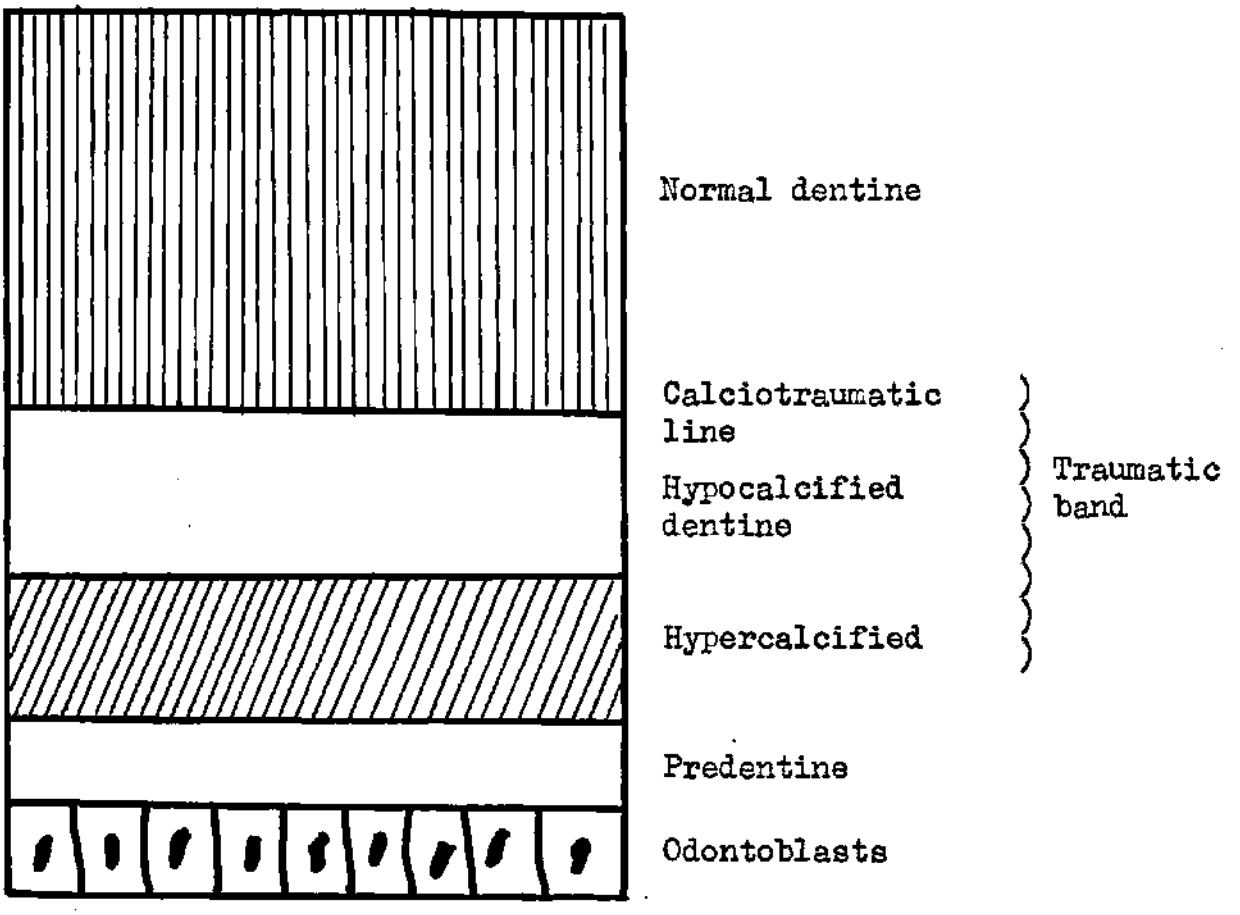
Normal dentine
Calciotraumatic line
Hypocalcified dentine
Traumatic band
Hypercalcified
Predentine
Odontoblasts

FIG. 2,35

Fig. 2,36. - Single calciotraumatic response seen in cross section of rat incisor. H. and E. X 90.

Fig. 2,37. - Corresponding autoradiograph showing sulphate-image in the proximal zone of the calciotraumatic response. A light image is also present in a segment of the calciotraumatic line. Rat. Kodak AR10. X 90.

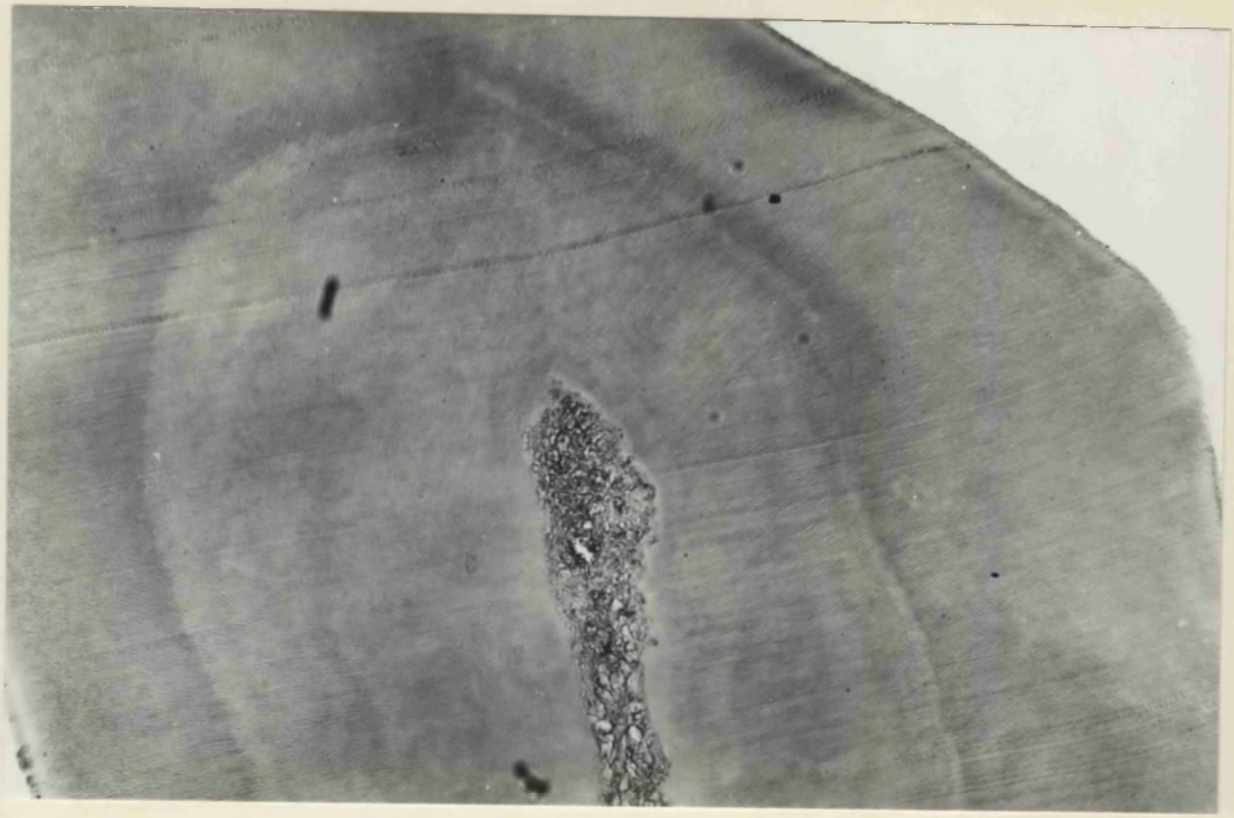

FIG. 2,36

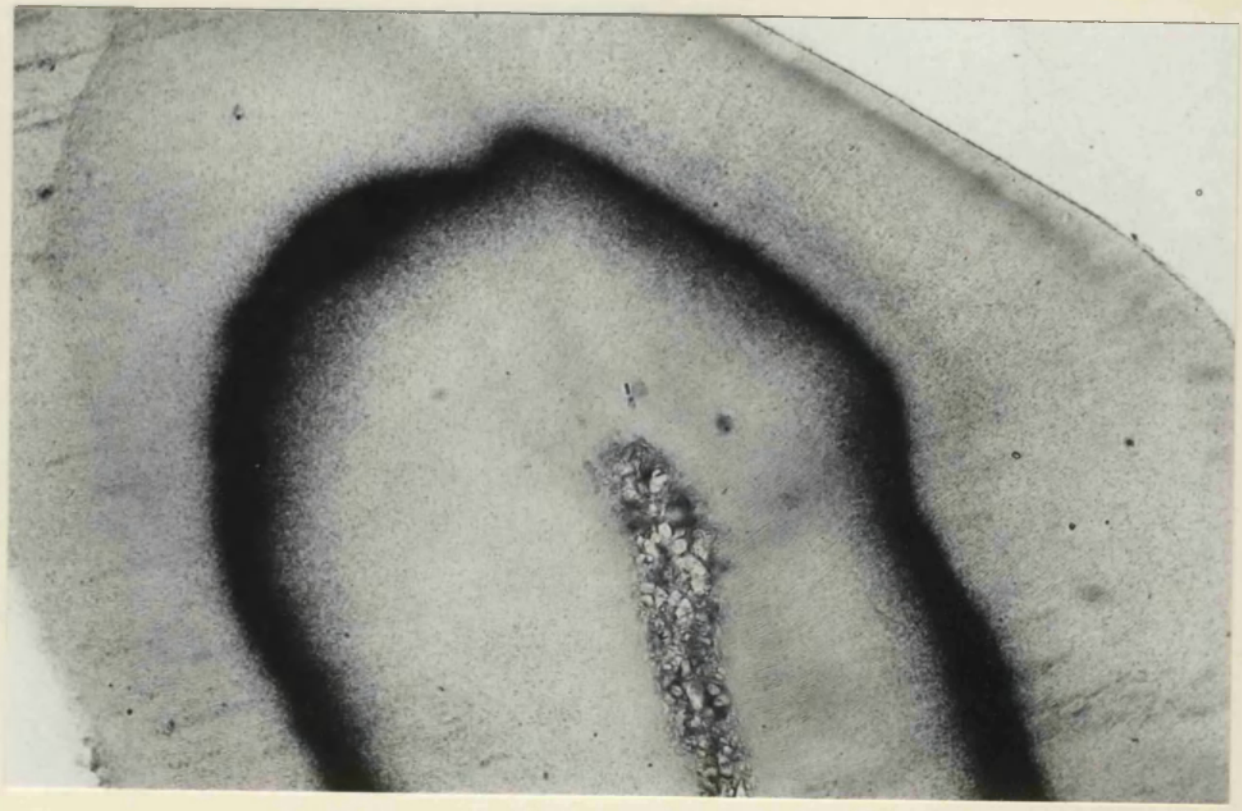

FIG. 2,37

Fig. 2, 38. - Multiple calciotraumatic responses in longitudinal section of rat incisor. Toluidine blue. X 225.

Fig. 2,39. - Autoradiograph showing sulphate-images in proximal zones of 2 calciotraumatic responses in longitudinal section of rat incisor. Kodak AR50. X 225.

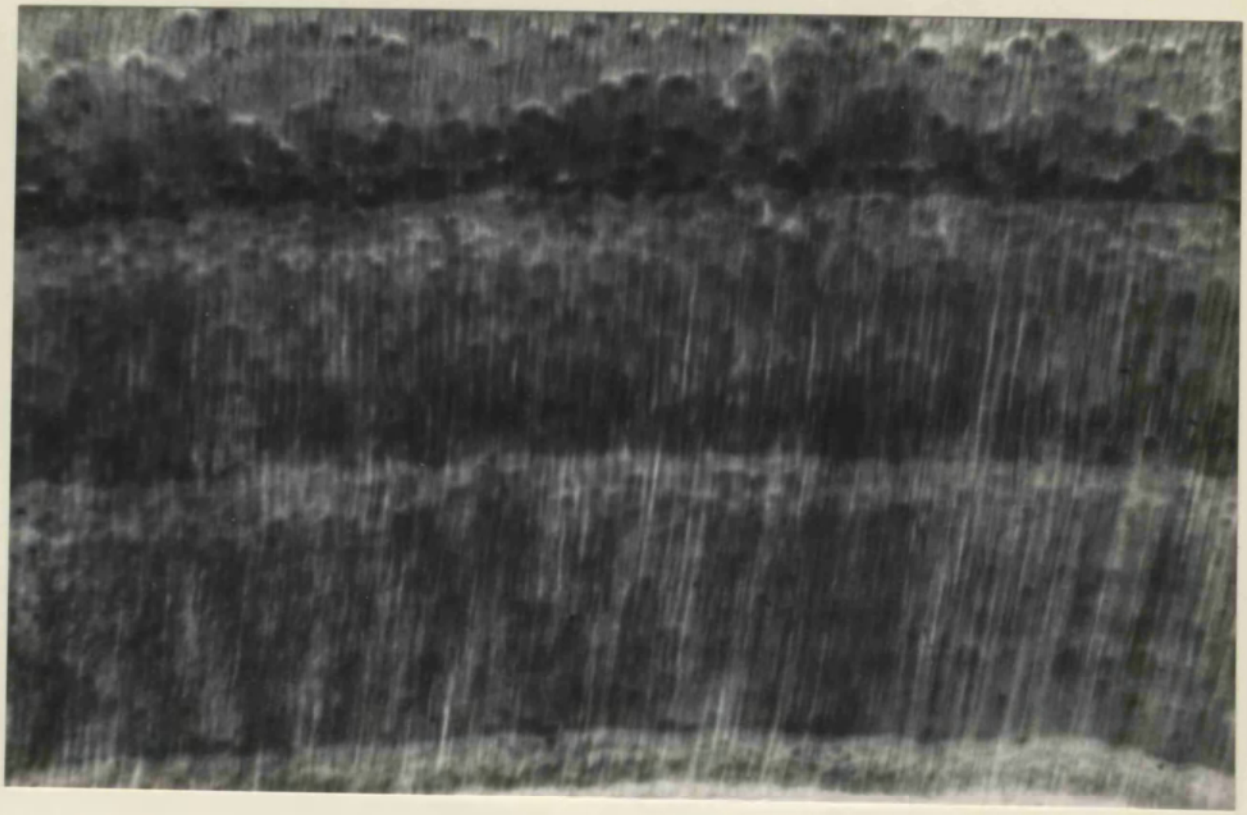

FIG. 2,38

FIG. 2,39

P A R T III

Tables 3,1-3,2

TABLE 3,1

CHEMICAL COMPOSITION OF HUMAN AMYLOID

Author	Protein	Polysaccharide
Hass (1942)	Protein	Sulphated MPS
Meyer (1947)		Sulphated MPS
Faber (1948)		Glucosamine increased
Bassiouni (1955)		Heparin-like
Wagner (1955)	Alpha 2 and beta globulin	MPS in same zones
Mellors and Ortega (1956)	Gamma globulin in capillary walls	
Vazquez and Dixon (1956)	Gamma globulin	
Larsen (1957)	Alpha 2 and beta globulin	MPS in same zones
	No migration	MPS like ground-substance
Braunstein and Buerger (1959)	Glycoprotein	Acidic MPS

MPS = Mucopolysaccharide

TABLE 3,2

PLASMA PROTEIN CHANGES IN HUMAN AND EXPERIMENTAL AMYLOIDOSIS

Author	Cause of amyloidosis	Source of material	Plasma protein changes
Faber (1948)	-	Man	Glucosamine increased
Gilliland et al, (1954)	-	Man	Alpha$_2$ and beta globulin increased
Gilliland and Stanton (1954)	-	Man	Low albumin/alpha$_2$ globulin ratio
Wagner (1955)	-	Man	Alpha$_2$ and beta globulin increased with increase in acidic MPS
Gellhorn et al, (1946)	L. donovani	Hamster	Albumin/globulin ratio decreased
Ada and Fulton (1948)	L. donovani	Hamster	Alpha globulin increased
Eklund and Reimann (1936)	Caseinate	Rabbit	Globulin increased
Perasalo and Latvalahti (1954)	Caseinate	Mouse, rat	Globulin increased
Teilum (1956)	Caseinate	Mouse	Gamma globulin increased
Giles and Calkins (1958)	Caseinate	Rabbit	Globulin increased; hexosamine increased
Dick and Leiter (1941)	Bacteria	Rabbit	Globulin increased
Bohle et al, (1950-51)	Nucleic acid	Rabbit	Gamma globulin increased

MPS = Mucopolysaccharide

P A R T III

Figs. 3,1 - 3,24

Fig. 3,1. - Early amyloid in glomerular tuft. Hamster. Haematoxylin and eosin. X 750.

Fig. 3,2. - Sulphur-35 distribution in glomerular tuft amyloid. The image is diffuse over endothelial cells and amyloid. Hamster. Autoradiograph, Kodak AR10. X 750.

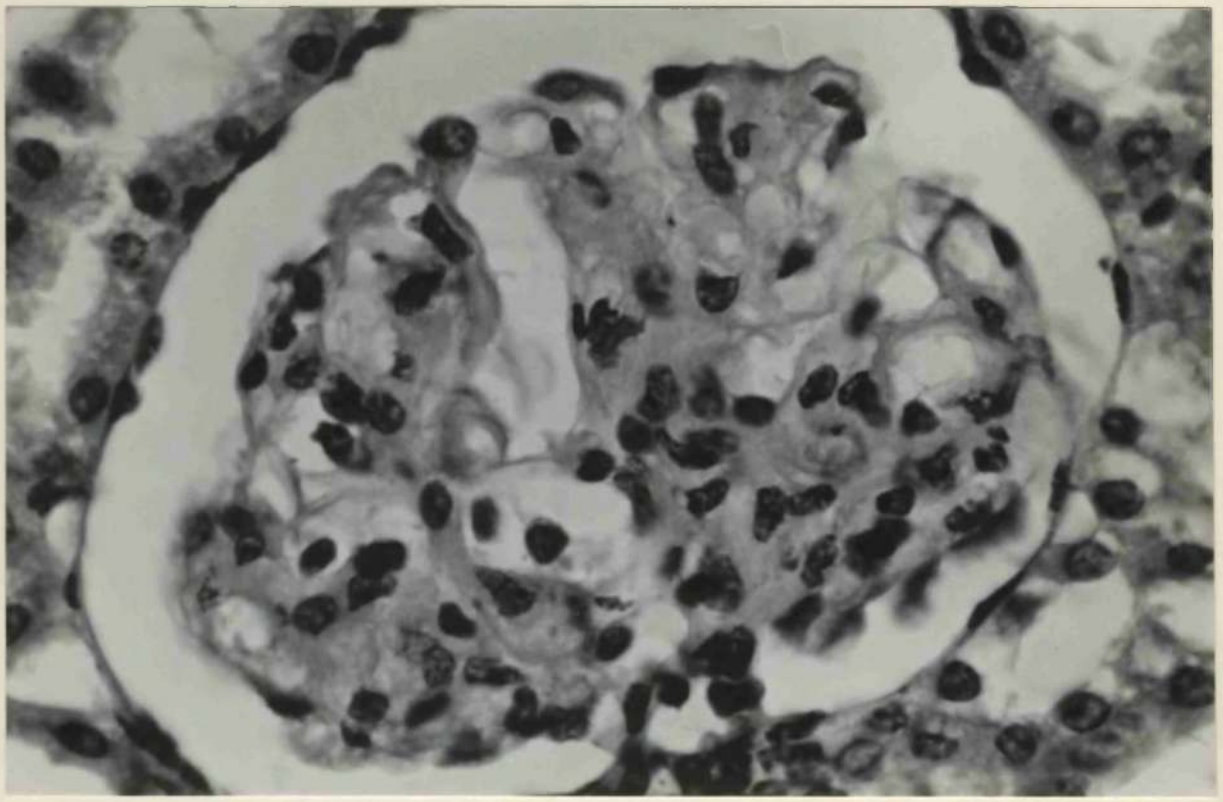

FIG. 3, 1

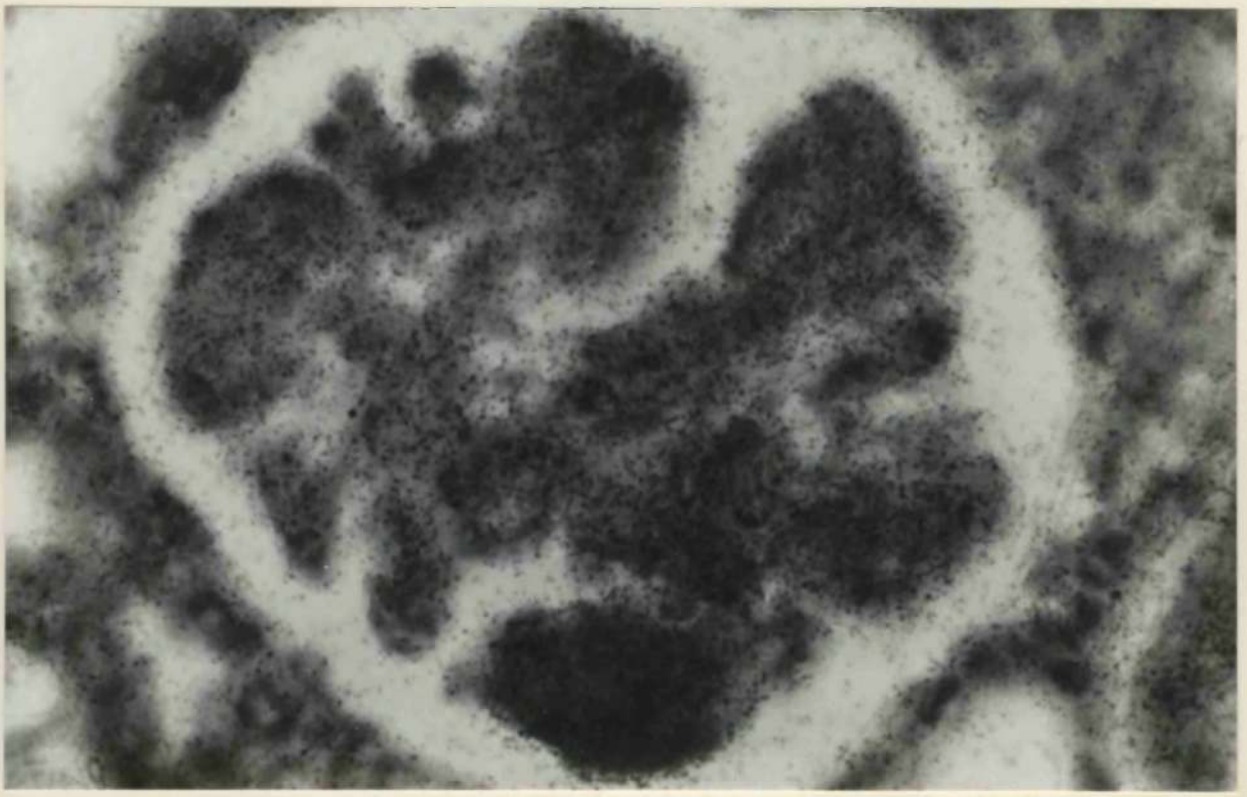

FIG. 3, 2

Fig. 3,3. - More extensive glomerular tuft amyloid with nodular appearance. Hamster. H. and E. X 950.

Fig. 3,4. - Radio-methionine distribution in glomerular tuft amyloid. The image is confined to the amyloid. It is not diffuse over endothelial cells and amyloid as in fig. 3,2. Hamster. Autoradiograph, Kodak AR10. X 470.

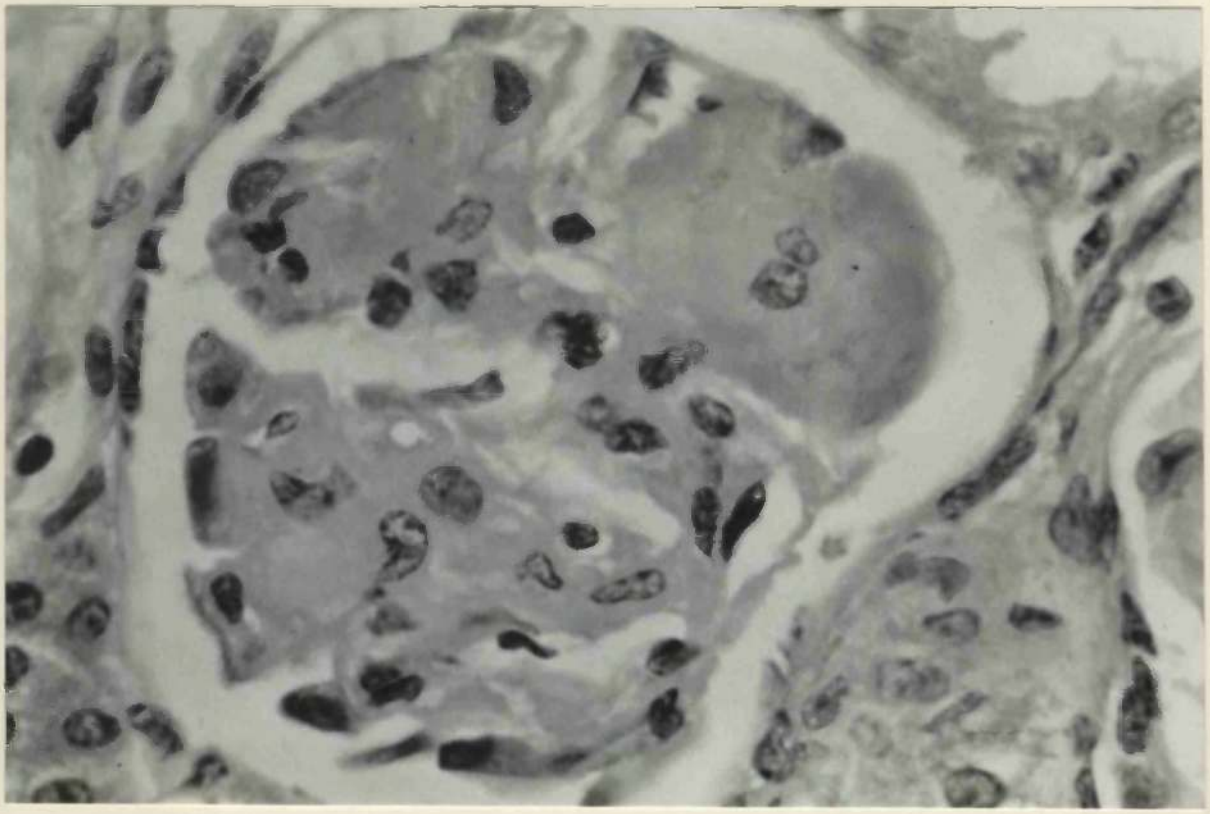

FIG.3,3

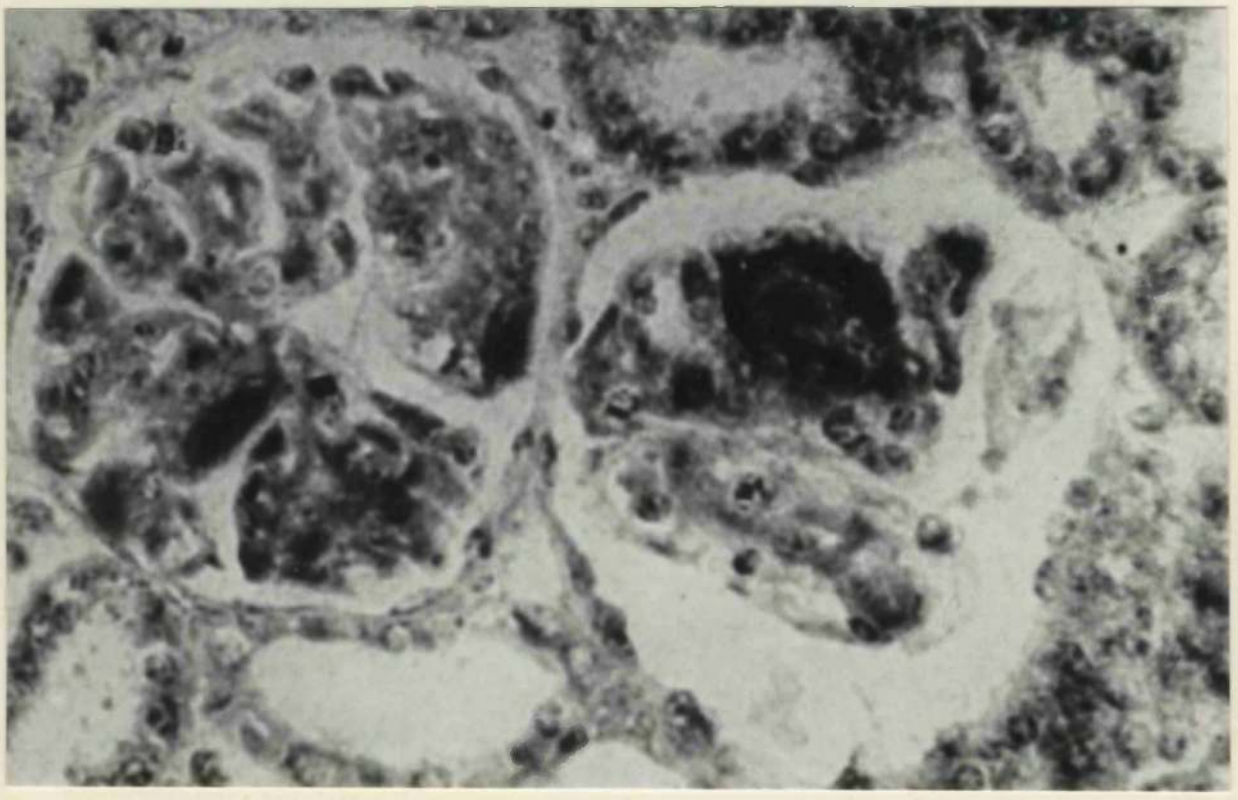

FIG3,4

Fig. 3,5. - Renal vein thrombosis in hamster with experimental amyloidosis. H. and E. X 20.

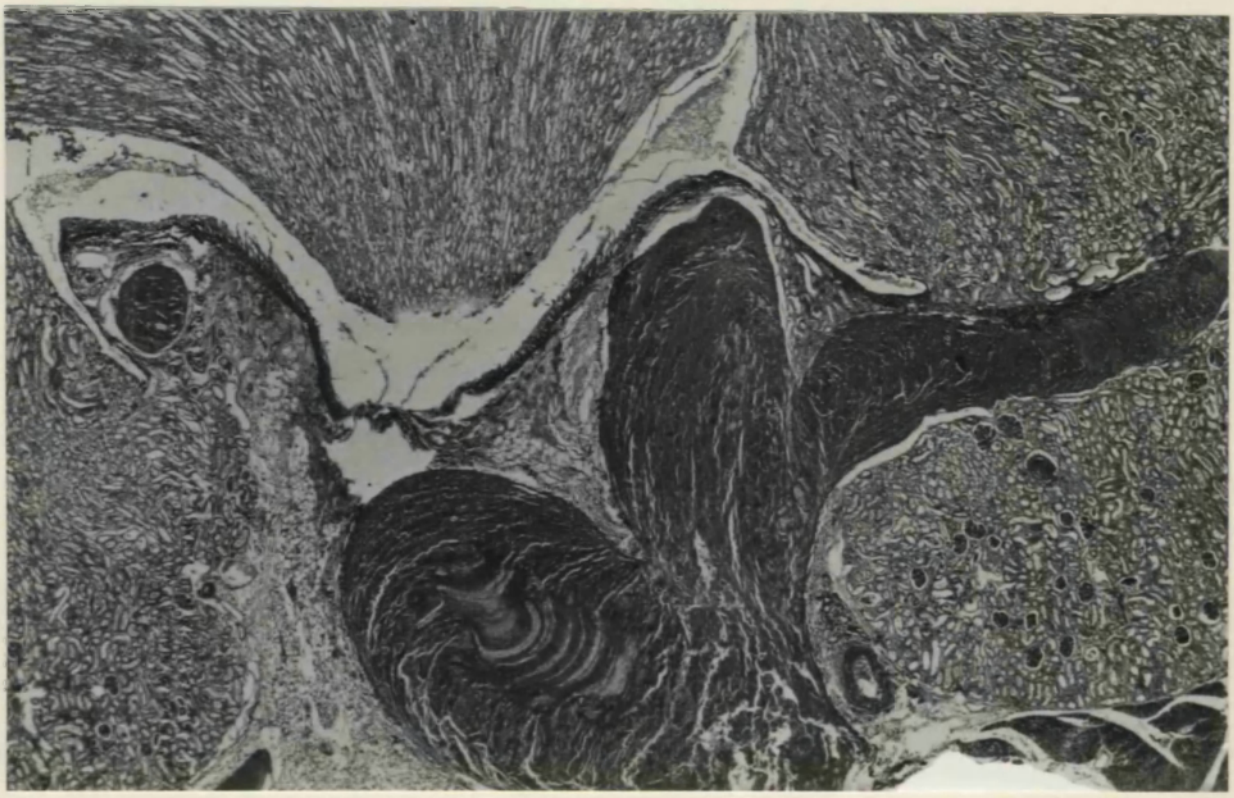

FIG. 3,5

Fig. 3,6. - Extension of perivascular amyloid into hepatic sinuses. Hamster. H. and E. X 135.

Fig. 3,7. - Radio-sulphate distribution in hepatic amyloid. A dark ring-image covers endothelium and extends over sub-endothelial amyloid. The image is lighter over the amyloid in the sinuses. Hamster. Autoradiograph, Kodak AR50. X 110.

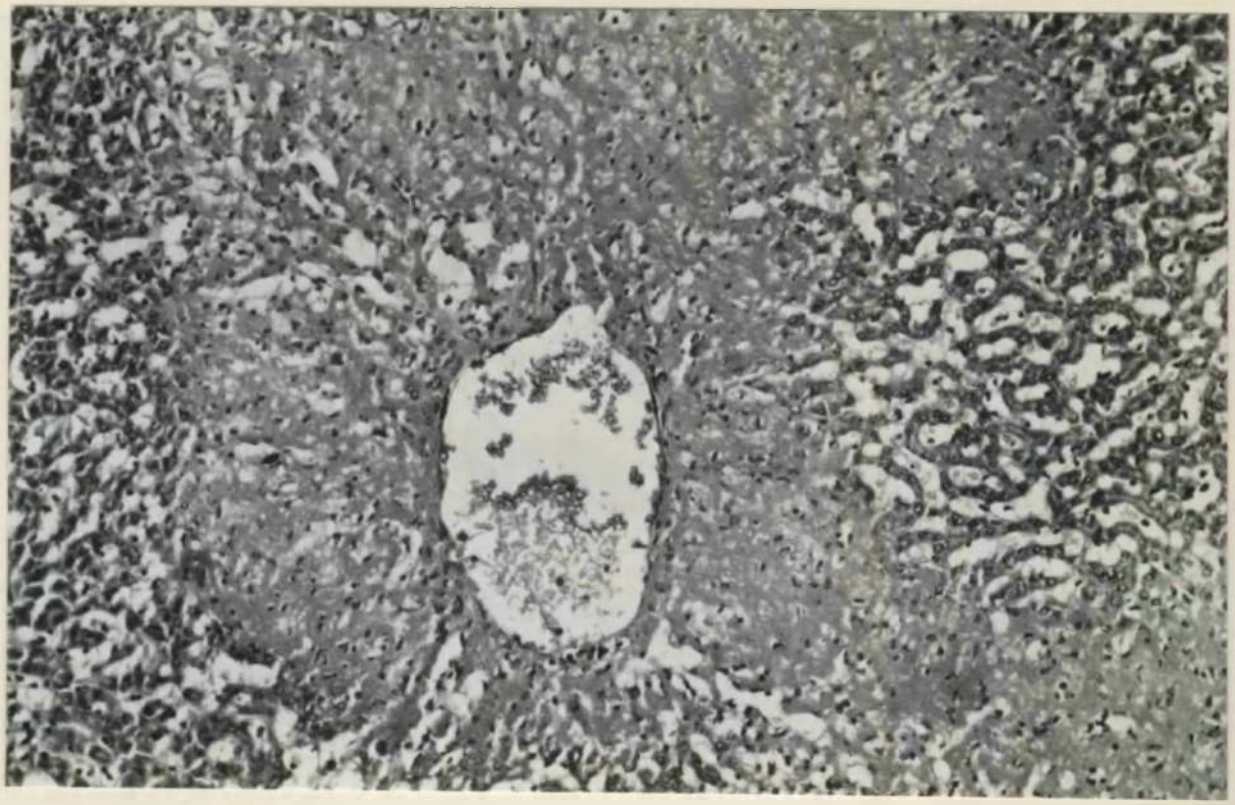

FIG.3,6

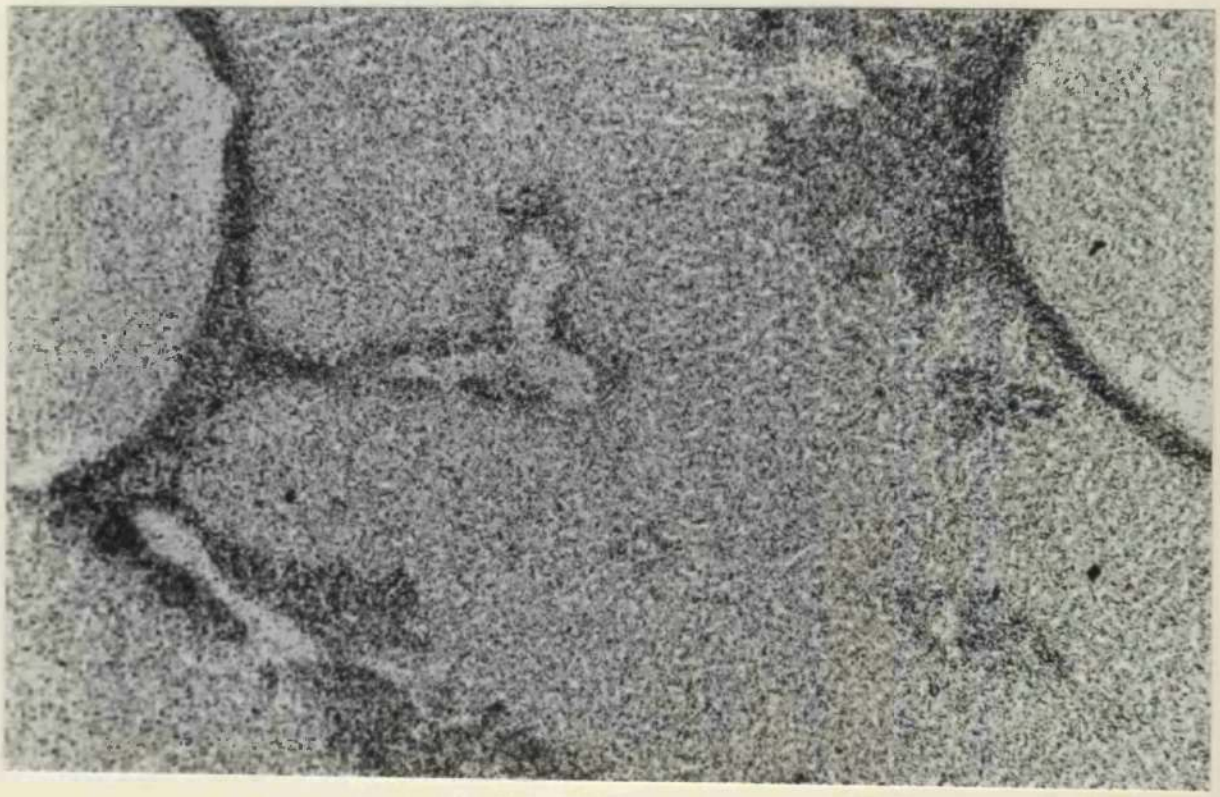

FIG.3,7

Fig. 3,8. - Perivascular amyloid in liver with associated cells of plasma series (lumen of vessel below). Hamster. H. and E. X 1000.

Fig. 3,9. - A clump of phagocytes in a hepatic vessel, and a laminated basophil body. Hamster. H. and E. X 1000.

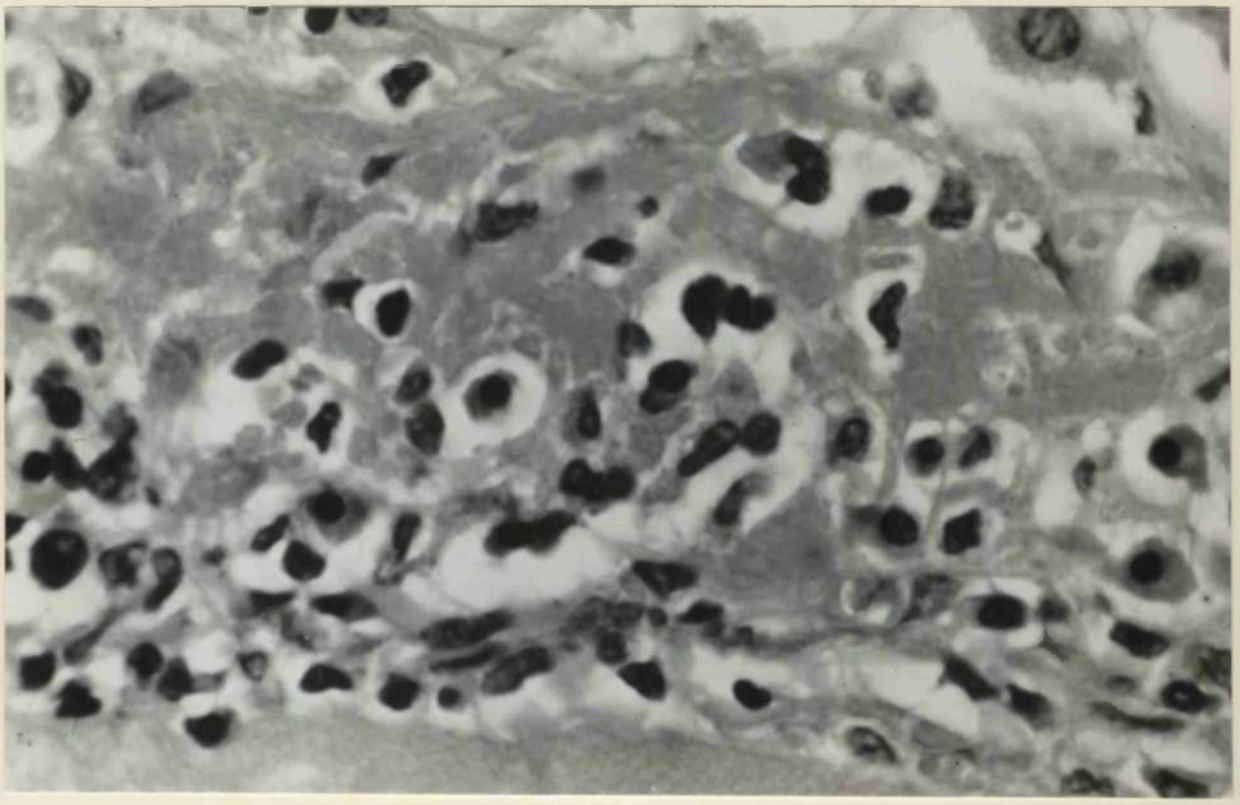

FIG.3,8

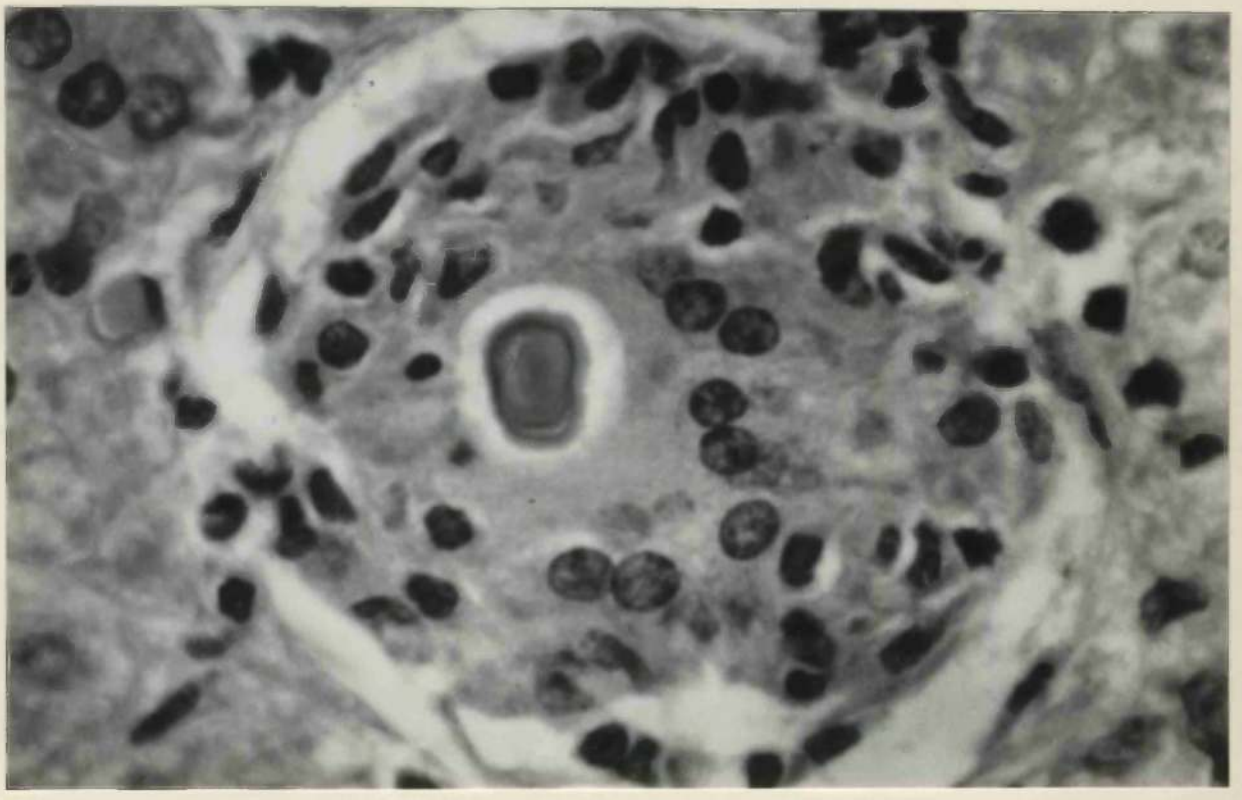

FIG.3,9

Fig. 3,10. - Early cirrhotic changes in hamster liver. H. and E. X 90.

Fig. 3,11. - Radio-sulphate distribution follows the pattern of the fibrous tissue in the cirrhotic liver. Hamster. Autoradiograph, Kodak AR50. X 90.

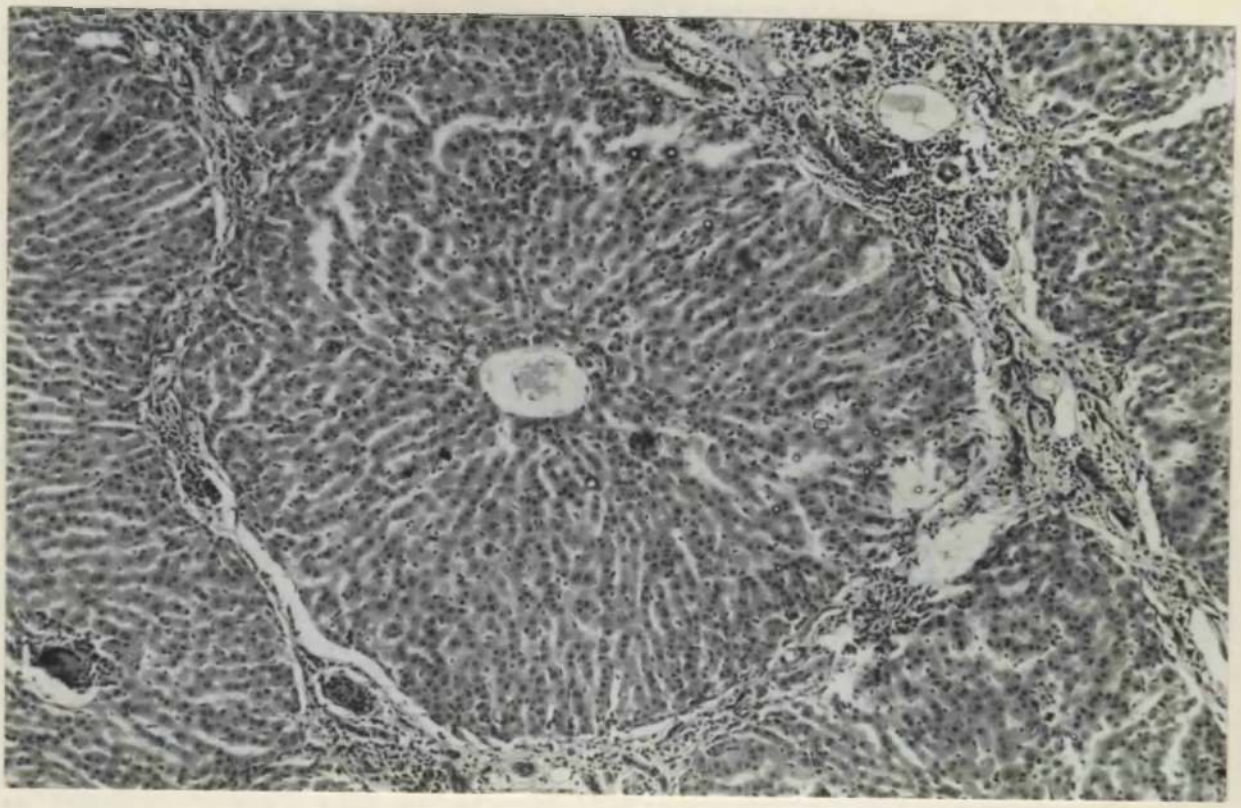

FIG. 3, 10

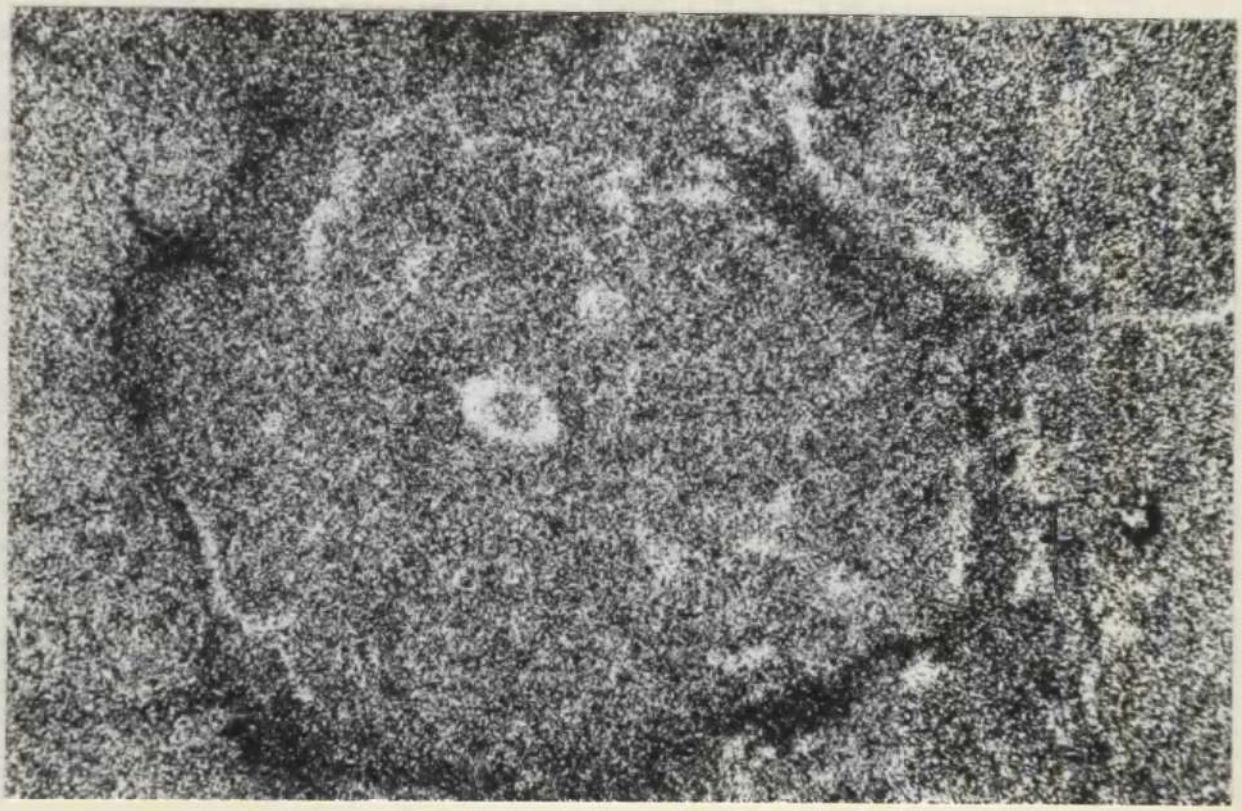

FIG. 3, 11

Fig. 3,12. - Early perifollicular amyloid in spleen. Hamster. H. and E. X 15.

Fig. 3,13. - Radio-sulphate incorporation in perifollicular amyloid in spleen. Hamster. Autoradiograph, Kodak AR50. X 15.

FIG. 3,12

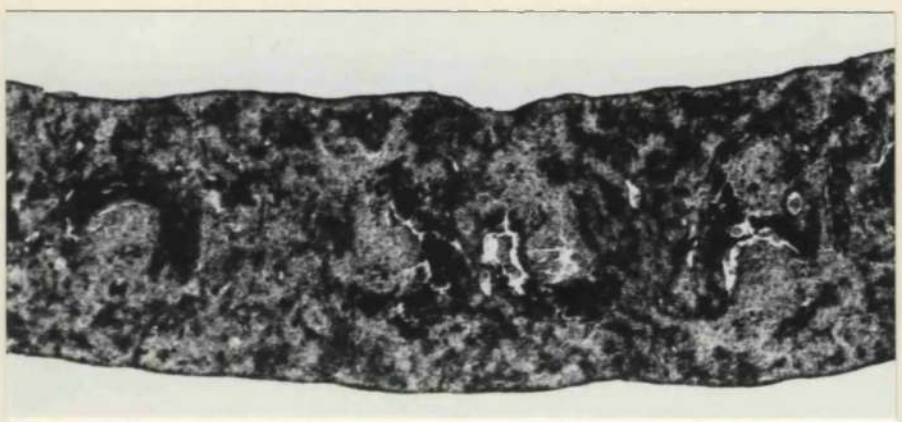

FIG. 3,13

Fig. 3,14. - Perivascular amyloid in spleen. Hamster. H. and E. X 850.

Fig. 3,15. - Radio-sulphate distribution in splenic perivascular amyloid. The image is a ring over endothelium and extends into surrounding amyloid. Hamster. Autoradiograph, Kodak AR50. X 750.

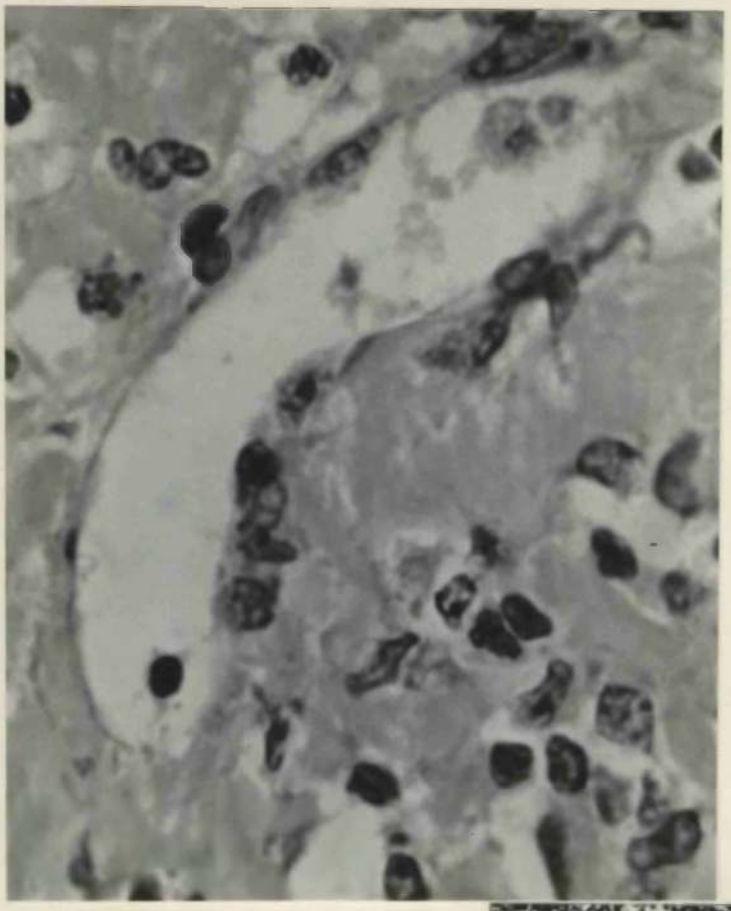

FIG. 3, 14

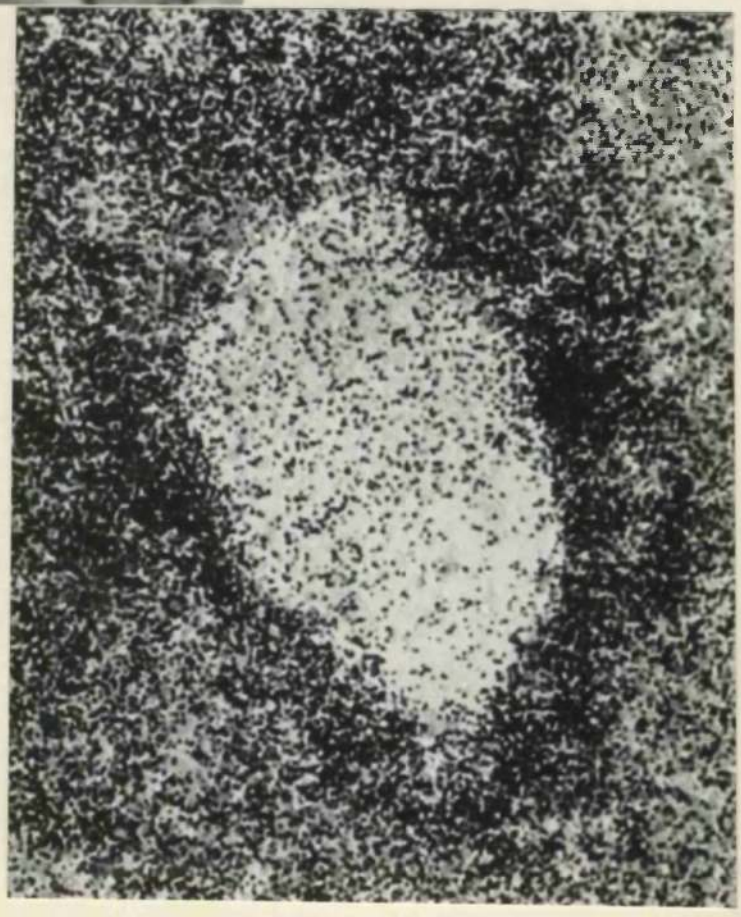

FIG. 3, 15

Fig. 3,16. - Plasma cell proliferation in splenic red pulp. No amyloid present. Mouse. Caseinate. Methyl green-pyronin. X 24.

Fig. 3,17. - Radio-sulphate uptake by plasma cell series in splenic red pulp. No amyloid present. Mouse. Caseinate. Autoradiograph, Kodak AR50. X 24.

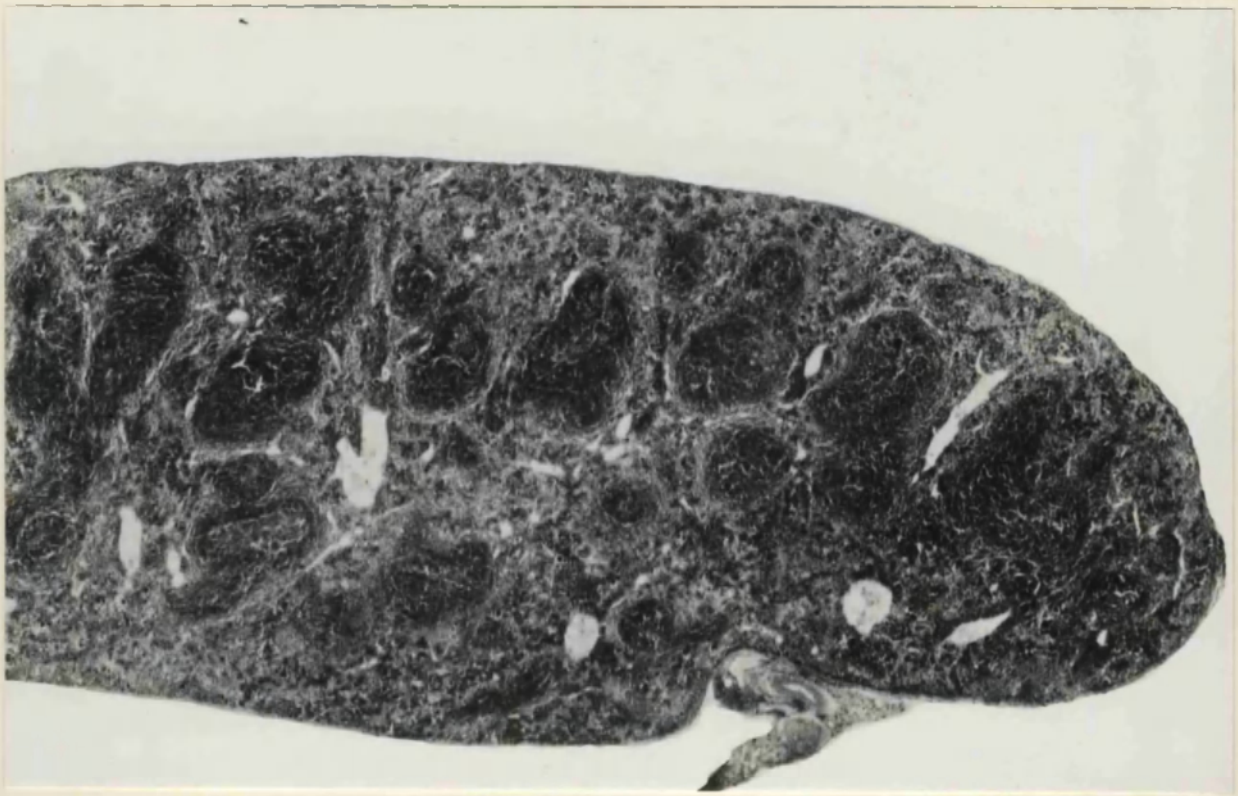

FIG. 3,16

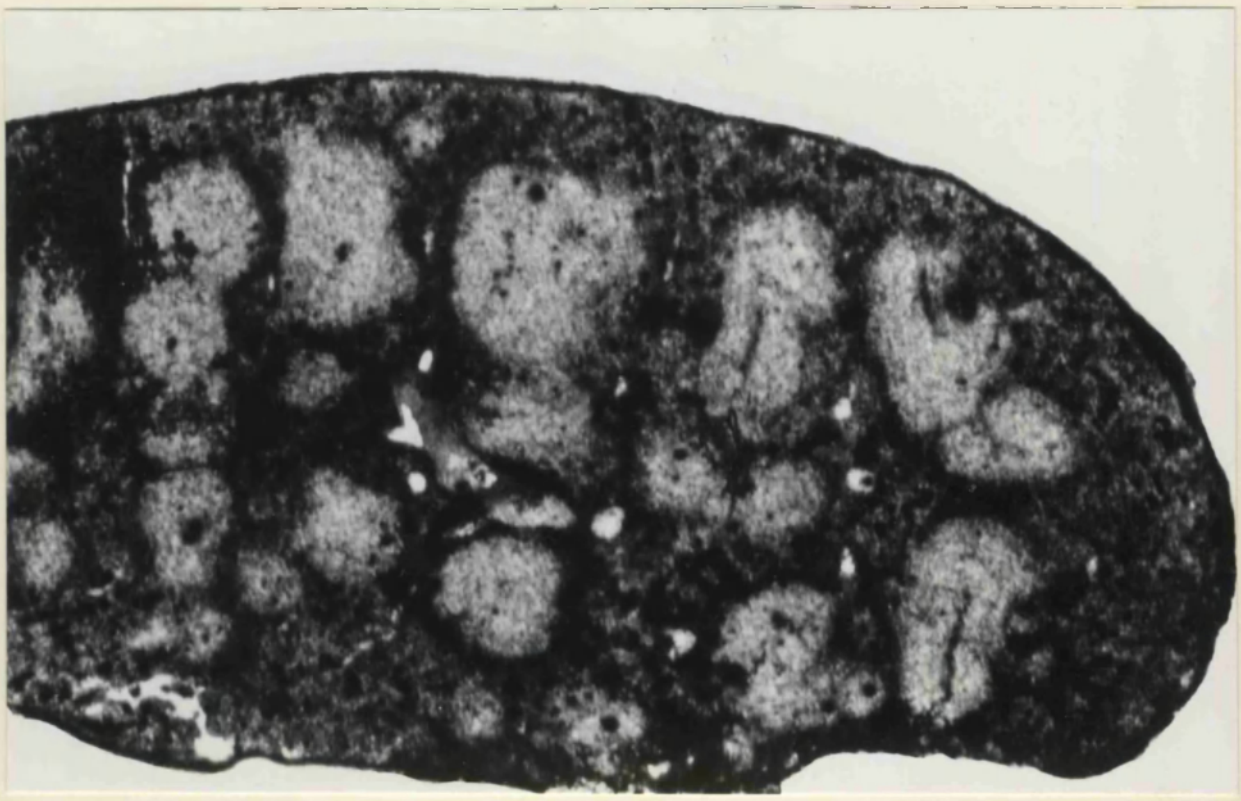

FIG. 3, 17

Fig. 3,18. - A clump of parasitised phagocytes in a splenic vascular space. Hamster. P.A.S. X 950.

Fig. 3,19. - Radio-methionine uptake by the clump of parasitised phagocytes is greater than by other splenic cells. Hamster. Autoradiograph, Kodak AR50. X 950.

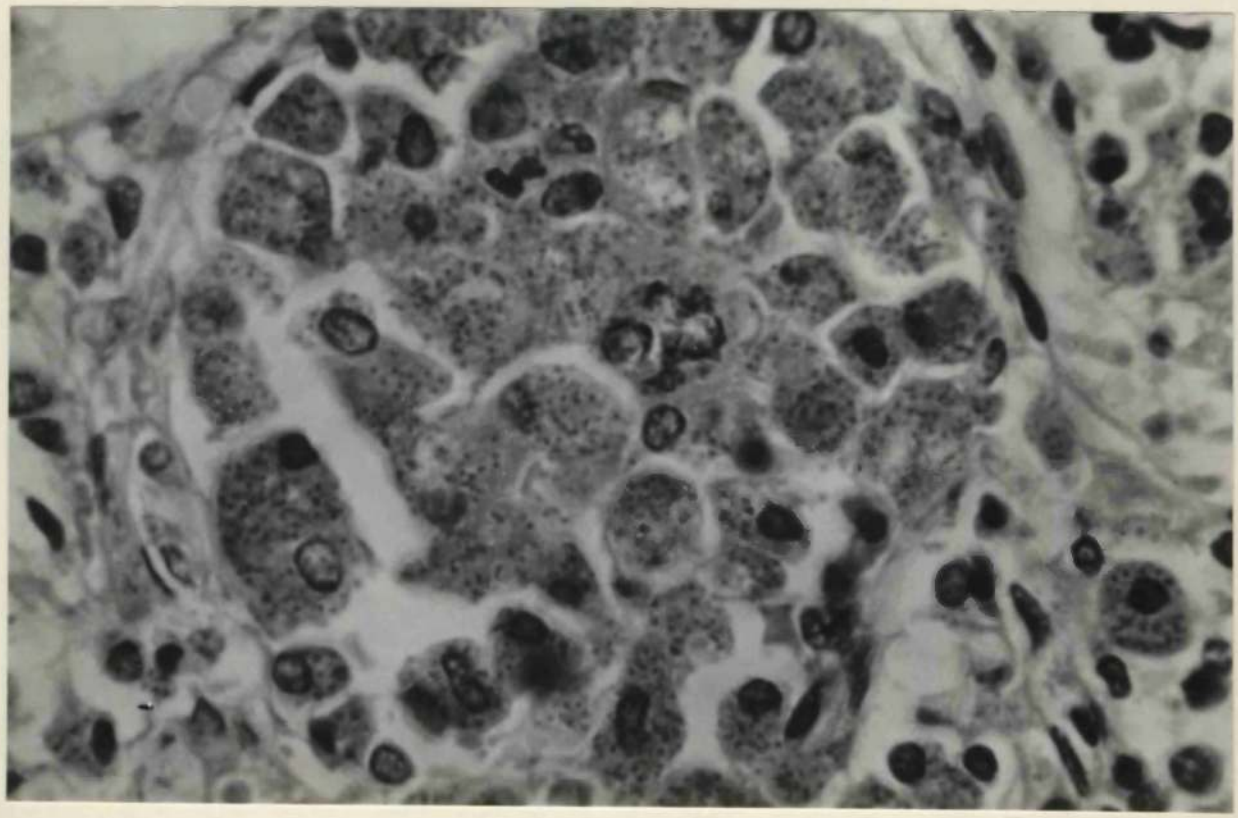

FIG. 3, 18

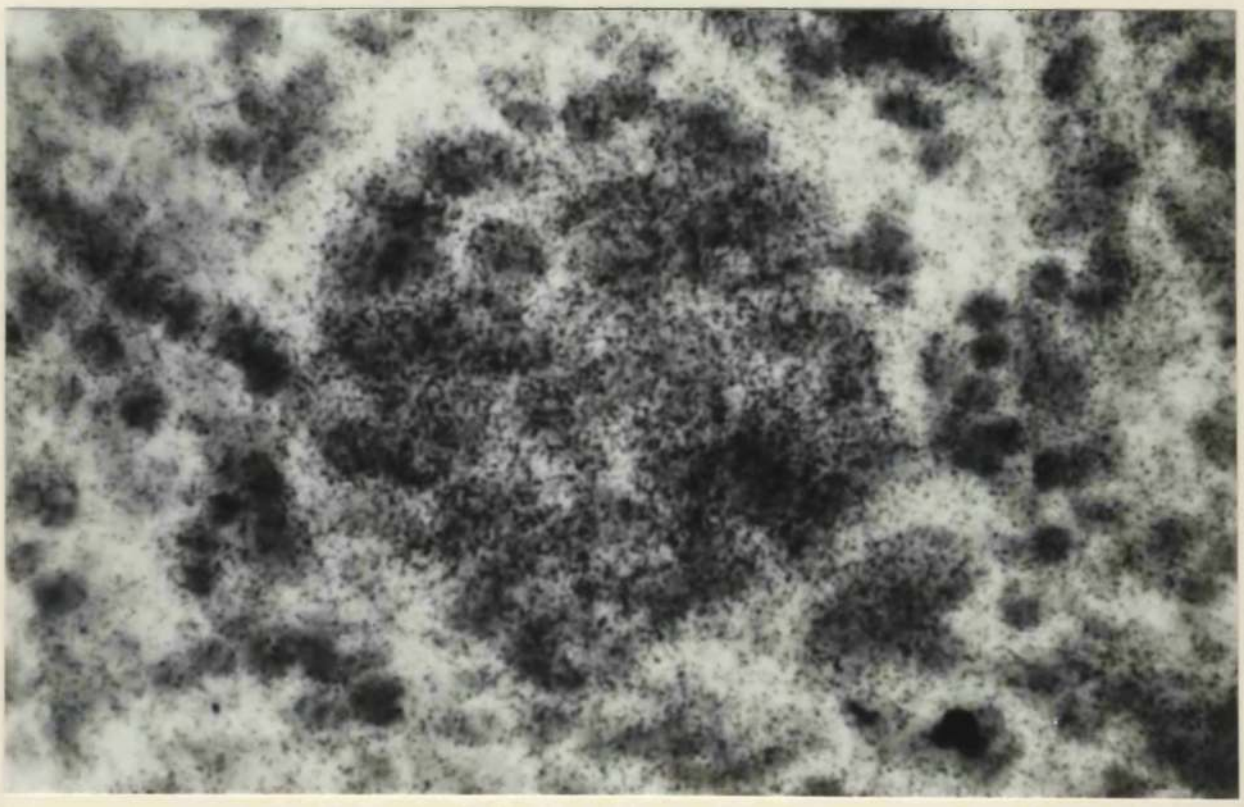

FIG. 3, 19

Fig. 3,20. - Amyloid infiltration in adrenal cortex (medulla below). Hamster. H. and E. X 250.

Fig. 3,21. - Radio-sulphate distribution in glomerular tuft amyloid. The image is confined to the tufts and is diffuse over endothelial cells and amyloid. Hamster. Autoradiograph, Kodak AR50. X 160.

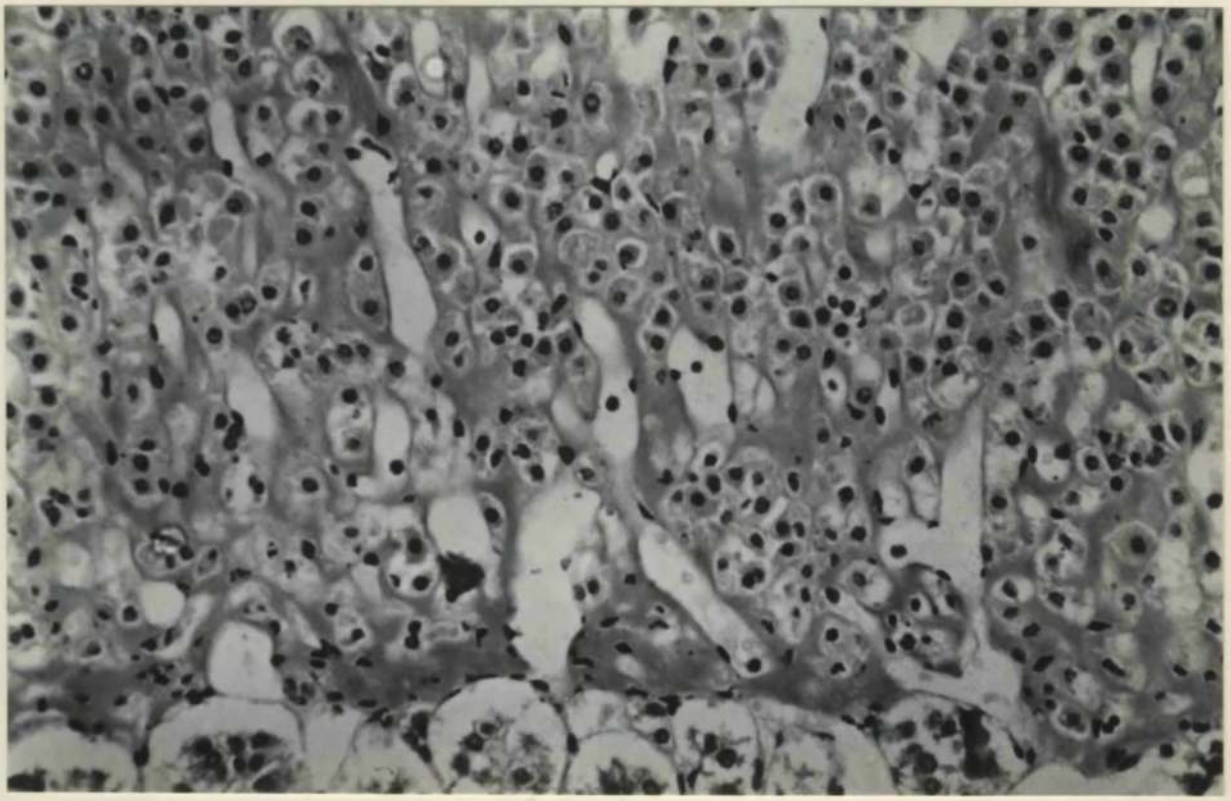

FIG.3,20

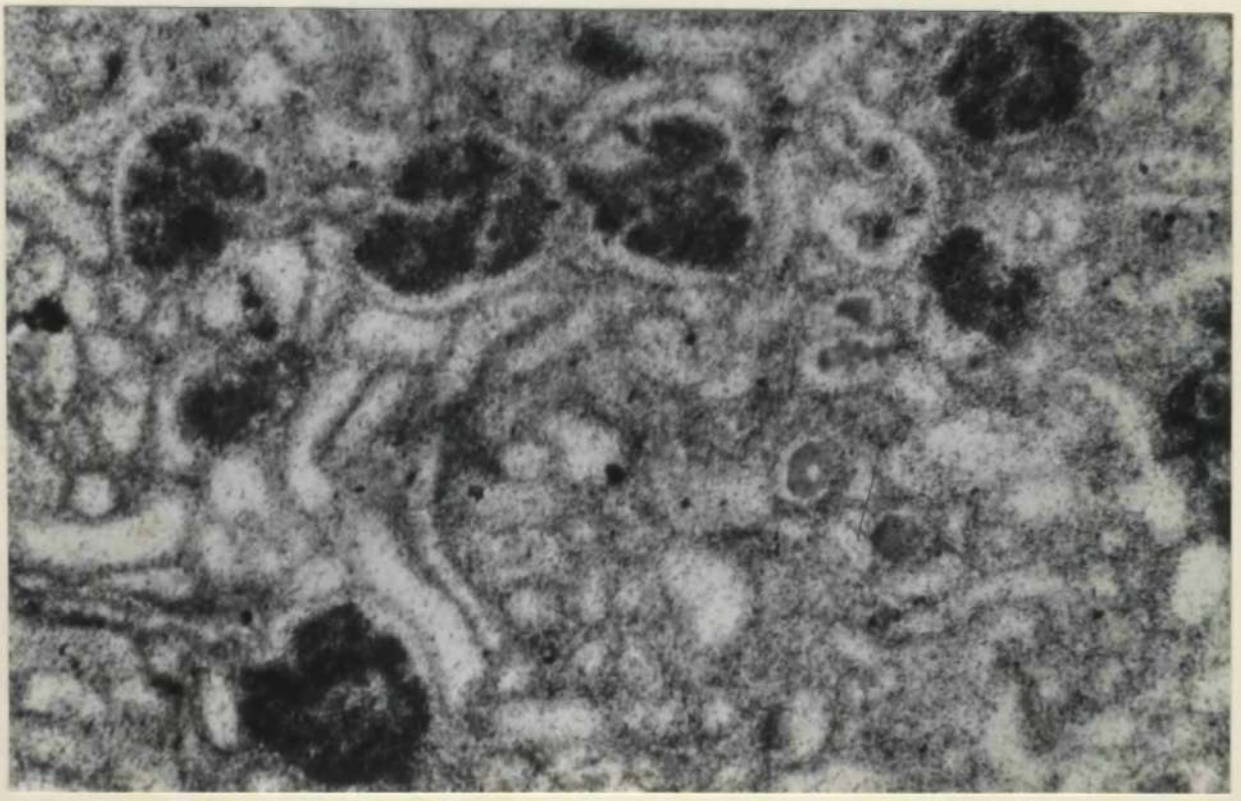

FIG.3,21

Fig. 3,22. - Radio-methionine distribution in hepatic amyloid. There is no ring-image as in fig. 3,7. The image is diffuse over amyloid and hepatic parenchyma. Hamster. Autoradiograph, Kodak AR50. X 120.

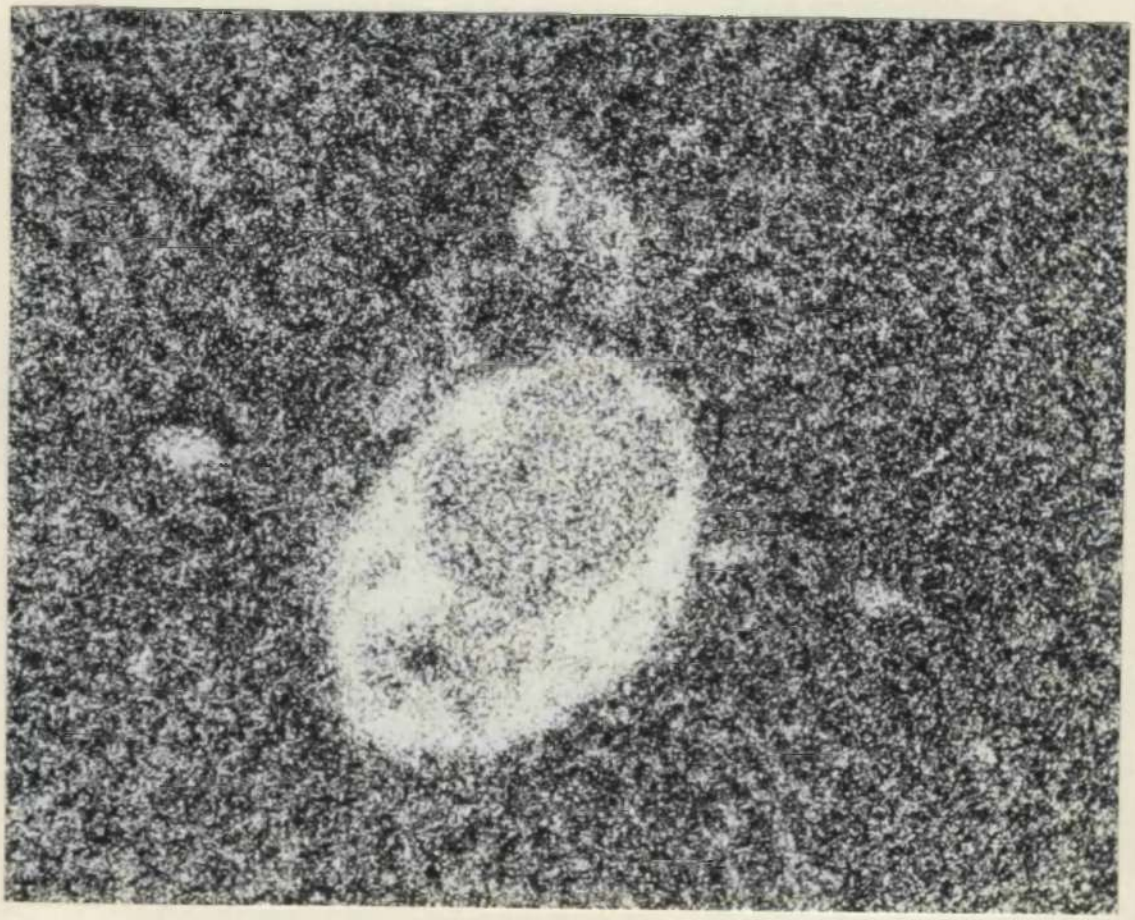

FIG.3,22

Fig. 3,23. - Perifollicular amyloid in spleen. Dilated vascular spaces are evident. Hamster. H. and E. X 90.

Fig. 3,24. - Radio-methionine is present in perifollicular amyloid in spleen, but there is no evidence of endothelial ring-image around vascular spaces as in fig. 3,15. Hamster. Autoradiograph, Kodak AR50. X 90.

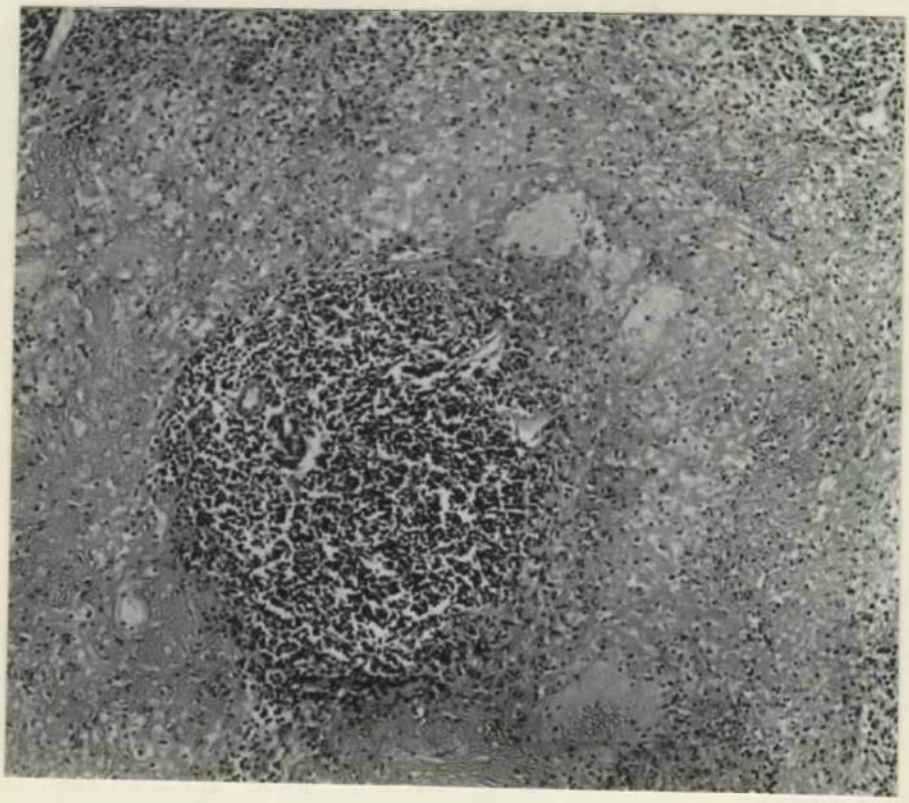

FIG.3,23

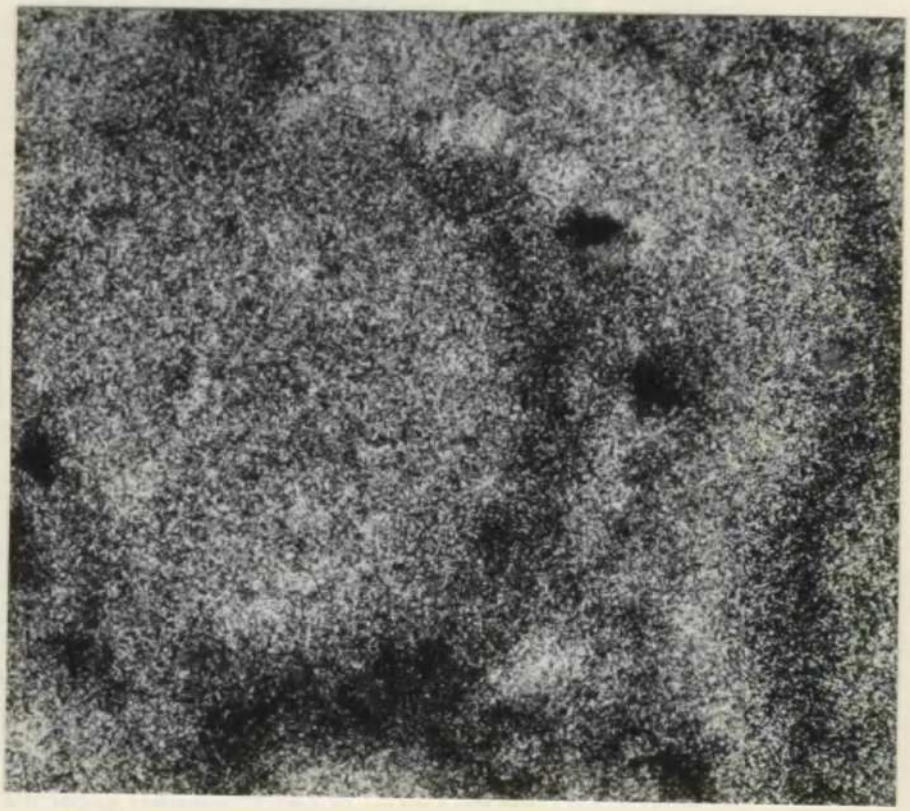

FIG.3,24

P A R T IV

Tables 4,1 - 4,12

TABLE 4.1

ANALYSIS OF 159 PATHOLOGICAL THYROID GLANDS

Disease	Cases	Male	Female
Adenoma	30	4	26
Carcinoma	24	5	19
Non-toxic nodular goitre	31	3	28
Toxic nodular goitre	5	0	5
Diffuse colloid goitre	3	1	2
Cyst	10	2	8
Dyshormonogenetic goitre	20	8	12
Graves' disease	9	1	8
Hashimoto's thyroiditis	27	2	25

TABLE 4,2

UTILISATION OF IODINE-131 IN THYROID ADENOMA

Case No.	Sex	Age	Type of tumour	Uptake of iodine-131
5762/55	F	55	3	+
4366/56	F	47	4	0
4697/56	F	42	3	0
5011/56	F	29	3	++
383/57	F	31	2	++
851/57	F	23	2	0
4989/57	F	29	2	+
5276/57	M	-	4	+
5282/57	F	57	1	0
323/58	F	50	2	+
1452/58*	F	30	3	++
1696/58	F	24	6	+
2119/58	M	-	1	0
2124/58	F	37	3	++
4853/58	F	-	2	++
5117/58	F	44	3	+
5251/58	M	52	3	0

++ means more active than peripheral thyroid, + means active but to lesser degree than peripheral thyroid. 0 means inactive. Tumour types: 1, trabecular; 2, microfollicular; 3, simple follicular; 4, macrofollicular; 5, papillary; 6, mixed papillary-follicular.
*clinically toxic.

continued on next page.

TABLE 4,2 (continued)

UTILISATION OF IODINE-131 IN THYROID ADENOMA

Case No.	Sex	Age	Type of tumour	Uptake of iodine-131
111/59	F	30	3	+
628/59**	F	59	1	0
650/59	M	60	4	+
870/59**	F	60	5,6	0
3129/59	F	25	3	+
4656/59	F	49	3	++
4905/59	F	17	2	++
187/60	F	32	4	0
5661/60	F	-	3	0
253/61	F	-	1	0
559/61**	F	60	2,3	++
1695/61	F	28	2	0
6243/61	F	19	1	0

++ means more active than peripheral thyroid; + means active but to lesser degree than peripheral thyroid; 0 means inactive. Tumour types: 1, trabecular; 2, microfollicular; 3, simple follicular; 4, macrofollicular; 5, papillary; 6, mixed papillary-follicular. **multiple tumours including Hurthle-cell type in 870/59.

TABLE 4.3

UTILISATION OF IODINE-131 IN THYROID ADENOMA

Type of tumour	Cases	Image intensities		
		++	+	0
Follicular	26	7	8	11
trabecular	4	-	-	4
microfollicular	7	3	2	2
simple	11	4	4	3
macrofollicular	4	-	2	2
Papillary-follicular	1	-	1	-
Multiple	3	1	-	2

++ means more active than peripheral thyroid;
+ means active but to lesser degree than peripheral thyroid; 0 means inactive.

TABLE 4.4

UTILISATION OF IODINE-131 IN THYROID CARCINOMA

Case No.	Sex	Age	Type of tumour	Uptake of iodine-131
4780/54	F	76	2	0
246/55	F	62	6	0
3145/55	F	71	4	0
926/56	F	48	4	0
1119/56	M	54	5	0
1283/57	F	28	1	0
1518/57	F	10	1	0
1734/57	F	62	4	0
2380/57	F	32	1	o
2393/57	F	36	5	0
4906/57	M	-	2	0
3467/58*	F	11	3	+
587/59	F	32	2	0
1096/59	F	61	1	+
1330/59	F	-	4	0
3310/59	F	-	3	0
5365/59	F	64	4	0
423/60	F	79	4	0
4597/60	M	62	5	0

0 means no uptake; + means active but to lesser degree than normal thyroid. Tumour types: 1, follicular; 2, papillary; 3, mixed papillary-follicular; 4, small-cell; 5, giant-cell; 6, Hürthle-cell.

*dyshormonogenetic goitre with cancer.

continued on next page

TABLE 4,4 (continued)

UTILISATION OF IODINE-131 IN THYROID CARCINOMA

Case No.	Sex	Age	Type of tumour	Uptake of iodine-131
1837/61	F	45	2	0
1999/61	F	42	4	0
3929/61	M	48	4	0
4786/61**	M	45	3	+
5672/61	F	34	3	0

0 means no uptake; + means active but to lesser degree than normal thyroid. Tumour types: 1, follicular; 2, papillary; 3, mixed papillary-follicular; 4, small-cell; 5, giant-cell; 6, Hürthle-cell. **Uptake in primary and secondary.

TABLE 4,5

UTILISATION OF IODINE-131 IN THYROID CARCINOMA

Type of tumour	Cases	Image intensities	
		+	0
Differentiated	13	3	10
follicular	4	1	3
papillary	4	-	4
papillary-follicular	4	2	2
Hürthle-cell	1	-	1
Undifferentiated	11	-	11
small-cell	8	-	8
giant-cell	3	-	3

0 means no uptake: + means active but to lesser degree than normal thyroid.

TABLE 4.6

DETAILS OF NON-TOXIC NODULAR GOITRE CASES

Case No.	Sex	Age	Thyroidectomy Specimen
732/55	F	54	1-7cm. nodules
2277/55	F	34	55 g.
3753/55	F	51	70 g.
2580/56	F	42	280 g.
4560/56	F	36	150 g.
647/57	F	50	1-4cm. nodules
3019/57	F	44	60 g.
5143/57	F	48	50 g.
1134/58	F	60	50 g.
2457/58	F	48	-
2599/58	F	40	100 g.
4412/58	F	56	25 g.
4669/58	F	53	200 g.
2579/59	F	51	20 g.
2895/59	F	46	12 g.
3240/59	F	38	20 g.
3383/59	F	44	30 g.
3453/59	F	41	400 g.

continued on next page

TABLE 4,6 (continued)

DETAILS OF NON-TOXIC NODULAR GOITRE CASES

Case No.	Sex	Age	Thyroidectomy Specimen
3602/59	M	52	-
4690/59	F	59	60 g.
4963/59	M	34	100 g.
5586/59	F	56	1-2.5cm. nodules
302/60	F	46	80 g.
1104/60	F	31	90 g.
3859/60	F	35	77 g.
4691/60	F	47	90 g.
5436/60	F	-	120 g.
5840/60	F	-	40 g.
2961/61	F	55	50 g.
3957/61	M	65	110 g.
4535/61	F	56	160 g.

TABLE 4,7

DETAILS OF TOXIC NODULAR GOITRE CASES

Case No.	Sex	Age	Thyroidectomy Specimen
934/54	F	28	55 g.
1491/54	F	42	120 g.
5323/55	F	35	90 g.
1341/56	F	51	320 g.
1540/56	F	50	75 g.

TABLE 4,8

DETAILS OF DIFFUSE COLLOID GOITRE CASES

Case No.	Sex	Age	Thyroidectomy Specimen
4854/58	F	-	35 g.
1850/59	M	32	100 g.
2551/59	F	36	20 g.

TABLE 4,9

DETAILS OF THYROID CYST CASES

Case No.	Sex	Age	Classification
5038/55	F	-	Degenerative
4413/56	F	77	"
5144/57	F	38	"
198/58	F	49	"
3394/58	F	34	"
4244/58	F	35	"
5063/58	M	50	"
2438/59	F	49	"
1782/61	M	17	Thyroglossal
4010/61	F	32	Degenerative

TABLE 4,10

DETAILS OF CASES OF DYSHORMONOGENETIC GOITRE

Case No.	Sex	Age	Thyroidectomy Specimen	Defect	Clinical
2727/55	F*	18	530 g. nodular	4	Euthyroid
4114/55	F*	21	170 g. "	4	Hypothyroid
1282/57	F	22	65 g. "	–	Cretin
1487/57	F***	15	215g. "	1	Euthyroid, deaf
3001/57	M	10	160 g. "	4	Euthyroid
1337/58	F*	19	560 g. "	4	Euthyroid
3467/58	F°	11	100 g. "	2	Euthyroid
4191/58	F	18	100 g. "	2	Cretin
4448/58	F***	19	225 g. "	1	Euthyroid, deaf
4604/58	M**	10	60 g. "	4	Euthyroid
4791/58	F	18	230 g. "	1	Euthyroid
2401/59	F	14	35 g. "	1	—
4164/59	F	16	70 g., diffuse	4	—
5327/59	M	—	38 g. nodular	–	—
303/60	M**	5½	20 g. diffuse	4	—
4738/60	M	16	0.5 g. biopsy	2	—
822/61	M	8mth.	Biopsy, diffuse	3	Cretin
1155/61	M	15	Biopsy	1	—
2396/61	M	8	60 g. nodular	4	—
239/62	F	15	80 g. "	2	Hypothyroid

Defects: 1, binding defect; 2, impaired dehalogenase activity, 3, coupling defect; 4, abnormal iodoprotein.

*sisters; **brothers; ***sisters; °carcinoma case.

TABLE 4,11

UTILISATION OF IODINE-131 IN GRAVES' DISEASE

Case No.	Sex	Age	Clinical	Uptake of iodine-131
4974/55	F	32	Thiouracil	0
2224/56	M	41	"	+
5321/57	F*	44	—	+
3764/58	F	20	Thiouracil	+
3976/58	F	29	—	+
1967/59	F	24	Thiouracil	+
4505/59	F	33	Carbimazol	+
P.M. 67/59	F	56	12 mc I131	++
P.M. E.30/59	F	68	10 mc I131	+

0, no image; +, image in all colloid present;
++, image in colloid and epithelial cells
*diffuse thyroiditis of Hashimoto type

TABLE 4.12

DETAILS OF HASHIMOTO'S THYROIDITIS CASES

Case no.	Sex	Age	Thyroidectomy Specimen
1530/55	F	-	150 g.
3849/55	F	55	245 g.
1778/56	M	60	120 g.
3049/56	F	52	125 g.
274/57	F	54	50 g.
586/57	F	45	35 g.
723/57	F	44	45 g.
1280/57	F	43	60 g.
1445/57	F	52	70 g.
2262/57	F	47	20 g.
3678/58	F	60	120 g.
3790/58	F	46	90 g.
4023/58	F	57	40 g.
4677/58	F	51	300 g.
4755/58	F	56	120 g.
5376/58	F	53	150 g.
759/59	F	53	125 g.
2699/59	F	63	—
3799/59	F	60	60 g.
4420/59	F	37	60 g.
4623/59	F	—	40 g.
4566/60	F	—	60 g.
4969/60	F	58	85 g.
1205/61	F	54	290 g.
3113/61	F	52	—
5470/61	F	39	55 g.
5725/61	M	60	140 g.

P A R T IV

Figs. 4.1 - 4.59

Fig. 4,1. - Case 559/61. Adenoma showing a predominantly follicular structure with, at one pole, a homogenous group of medium size follicles. Haematoxylin and eosin. X 19.

Fig. 4,2. - Case 559/61. Corresponding autoradiograph. The adenoma shows utilisation of iodine-131 in colloid of microfollicles. The images are more intense than in the surrounding thyroid tissue. The group of medium size follicles at one pole is inert. Kodak AR50. X 19.

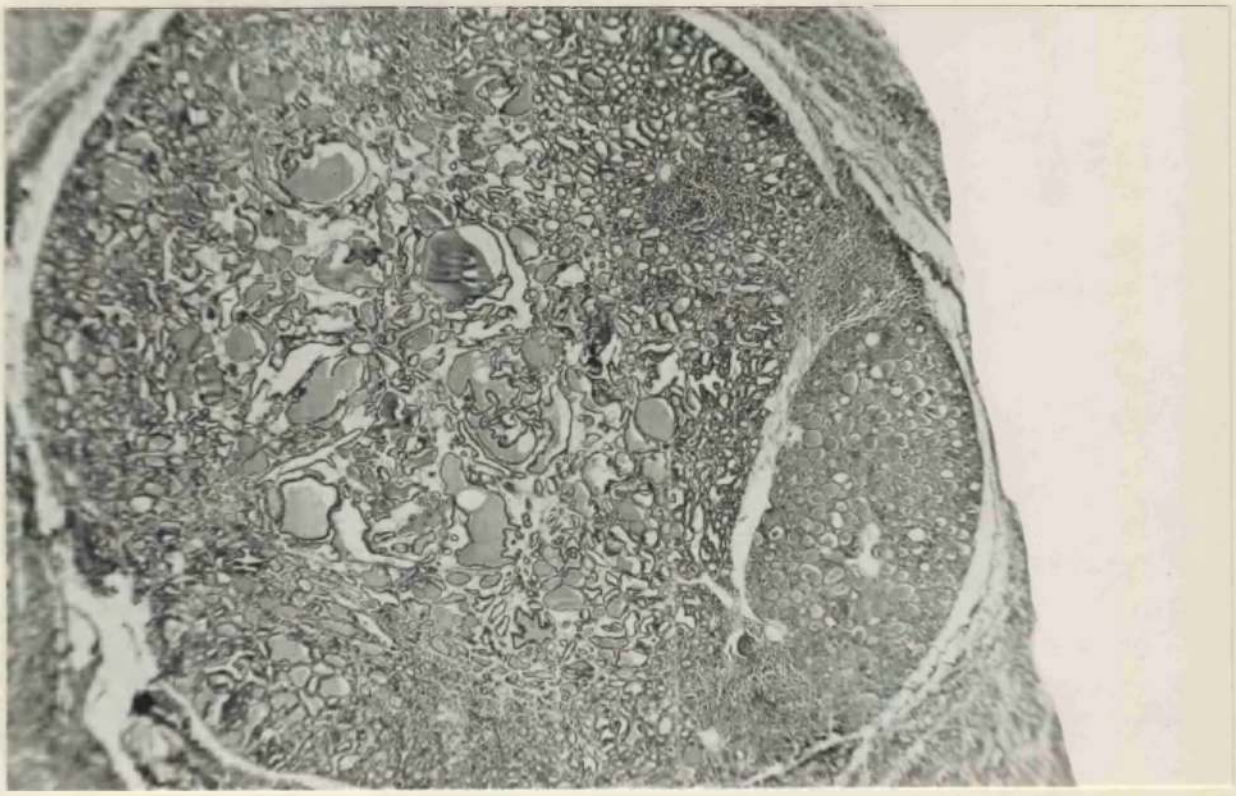

FIG.4,1

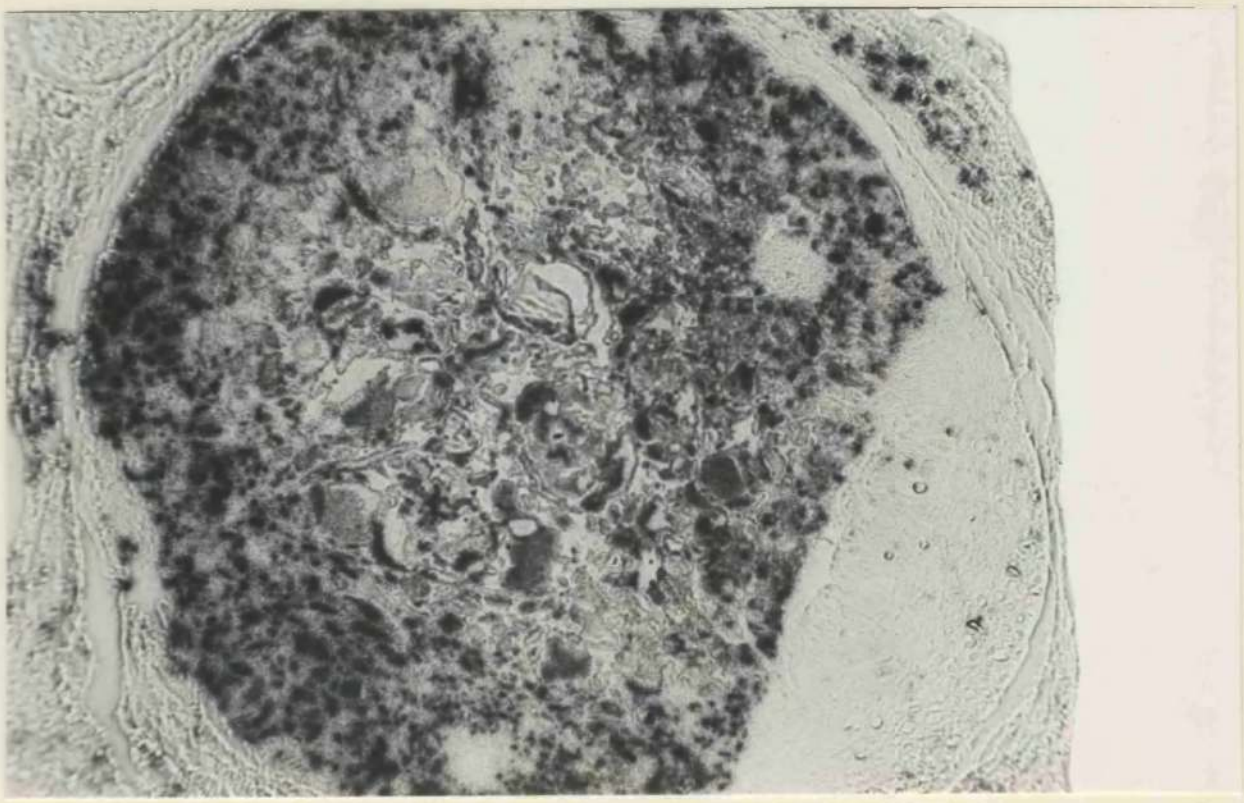

FIG.4,2

Fig. 4,3. - Case 2124/58. Simple follicular adenoma showing histological evidence of functional activity. H. and E. X 95.

Fig. 4,4. - Case 2124/58. Corresponding autoradiograph shows clear evidence of iodine-131 incorporation in colloid in the adenoma. Kodak AR50. X 95.

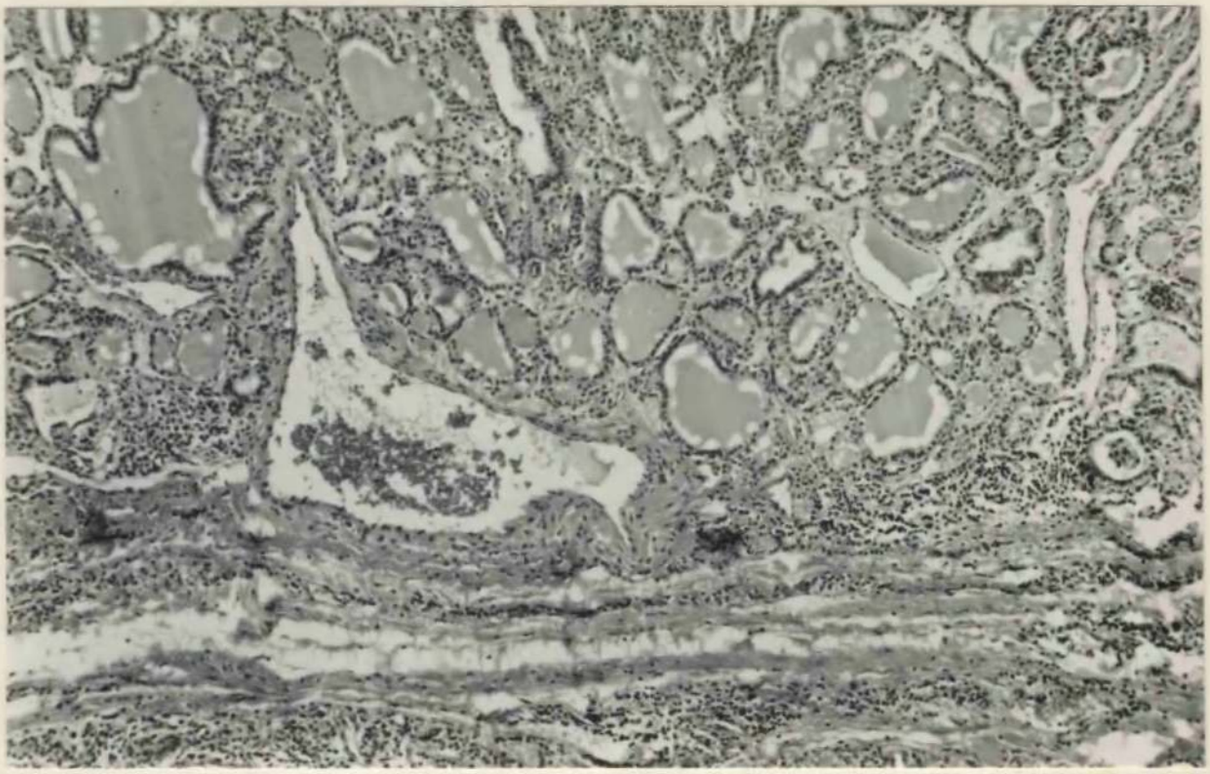

FIG. 4,3

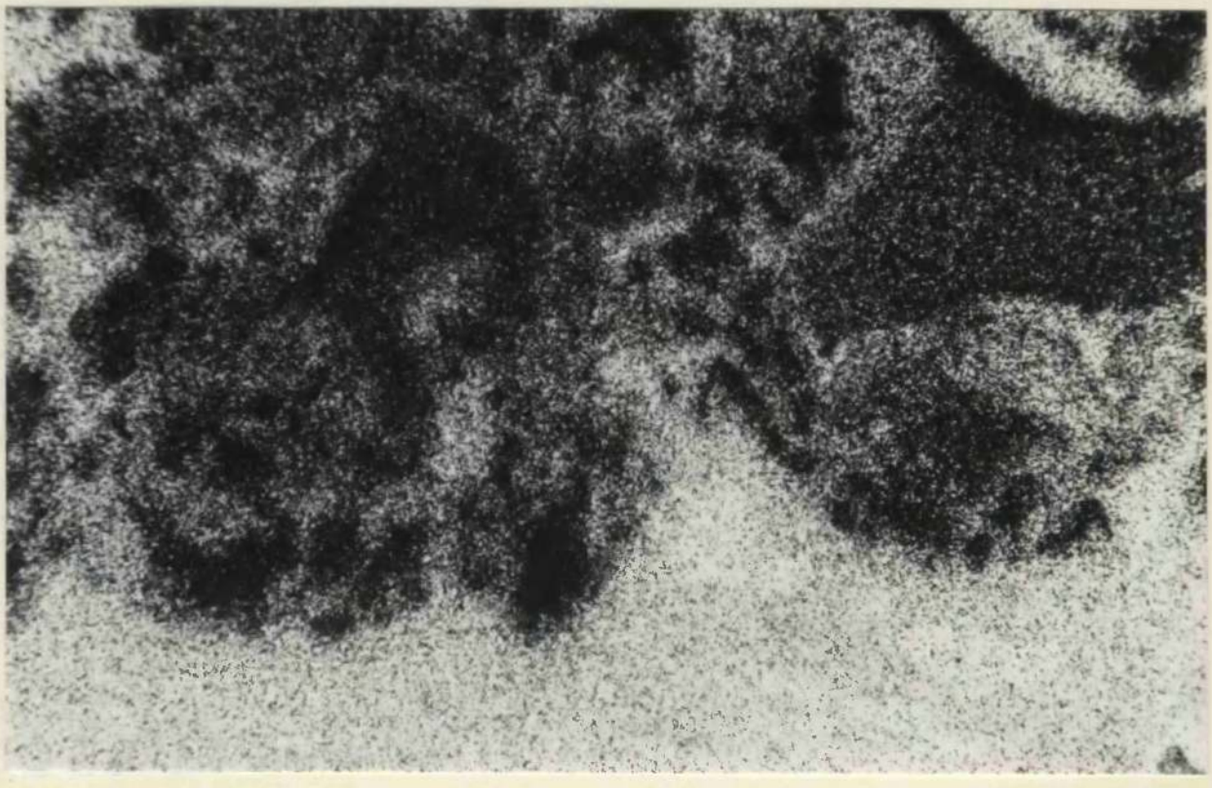

FIG. 4,4

Fig. 4,5. - Case 253/61. A trabecular adenoma showing occasional small follicles. H. and E. X 30.

Fig. 4,6. - Case 253/61. Corresponding autoradiograph. The trabecular adenoma is inactive. Iodine-131 is present only in colloid in occasional microfollicles and the image intensity is less than that in the follicles of the surrounding thyroid tissue. Kodak AR50. X 30.

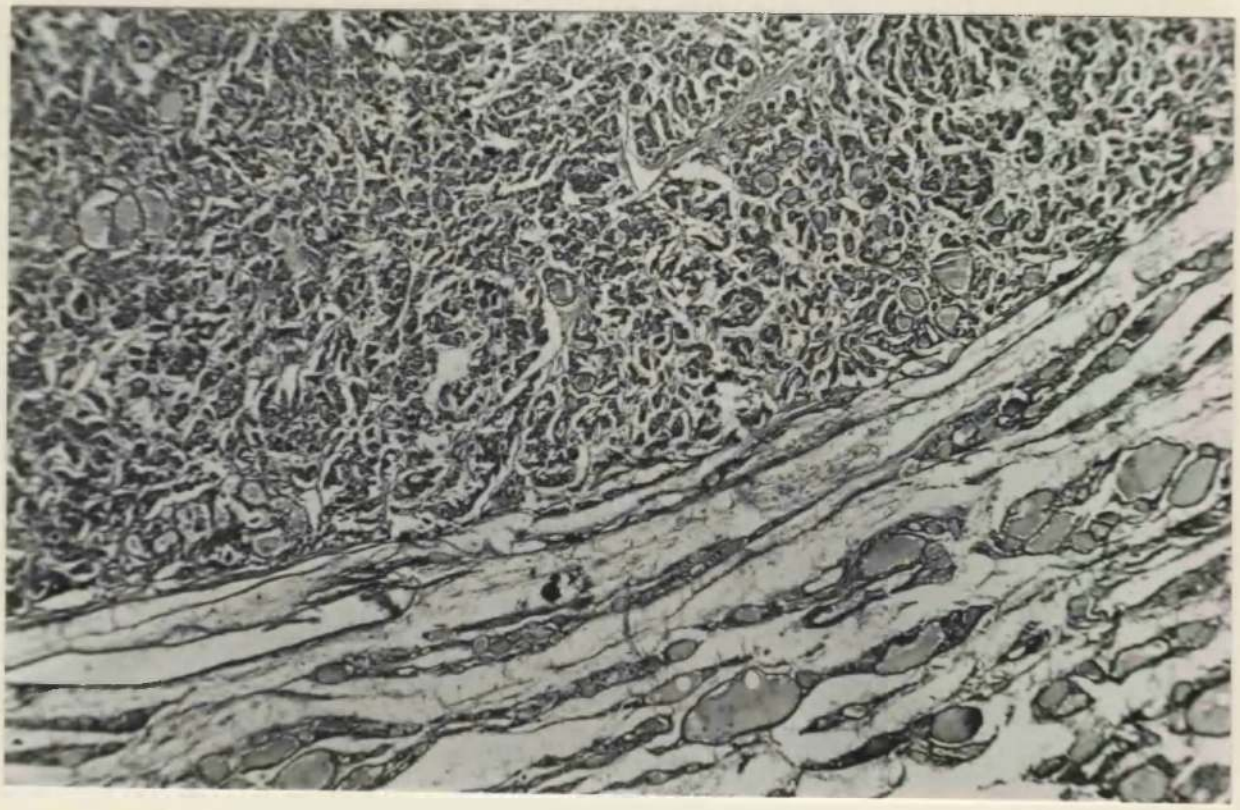

FIG.4,5

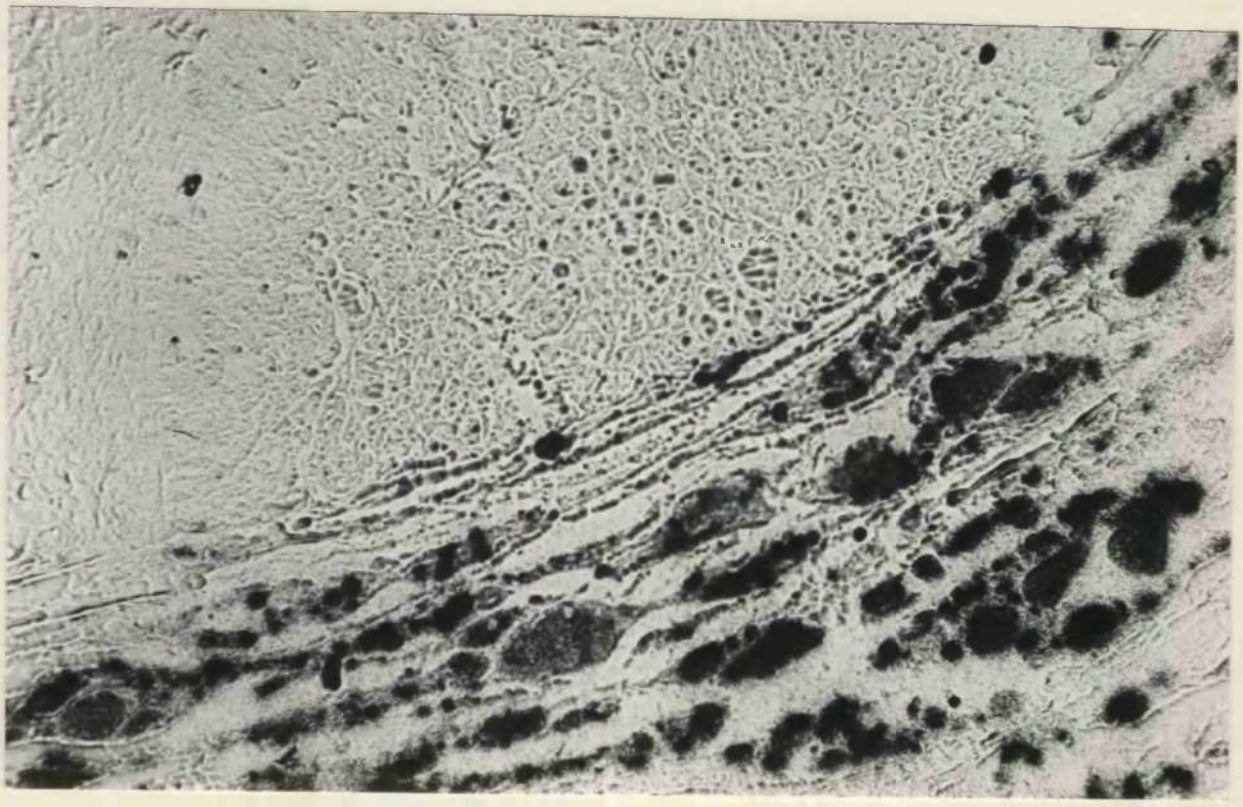

FIG.4,6

Fig. 4,7. - Case 4786/61. Differentiated carcinoma showing mixed papillary-follicular structure. H. and E. X 95.

Fig. 4,8. - Case 4786/61. Corresponding autoradiograph. Iodine-131 is present only in the colloid of the follicular part of the tumour. Kodak AR50. X 95.

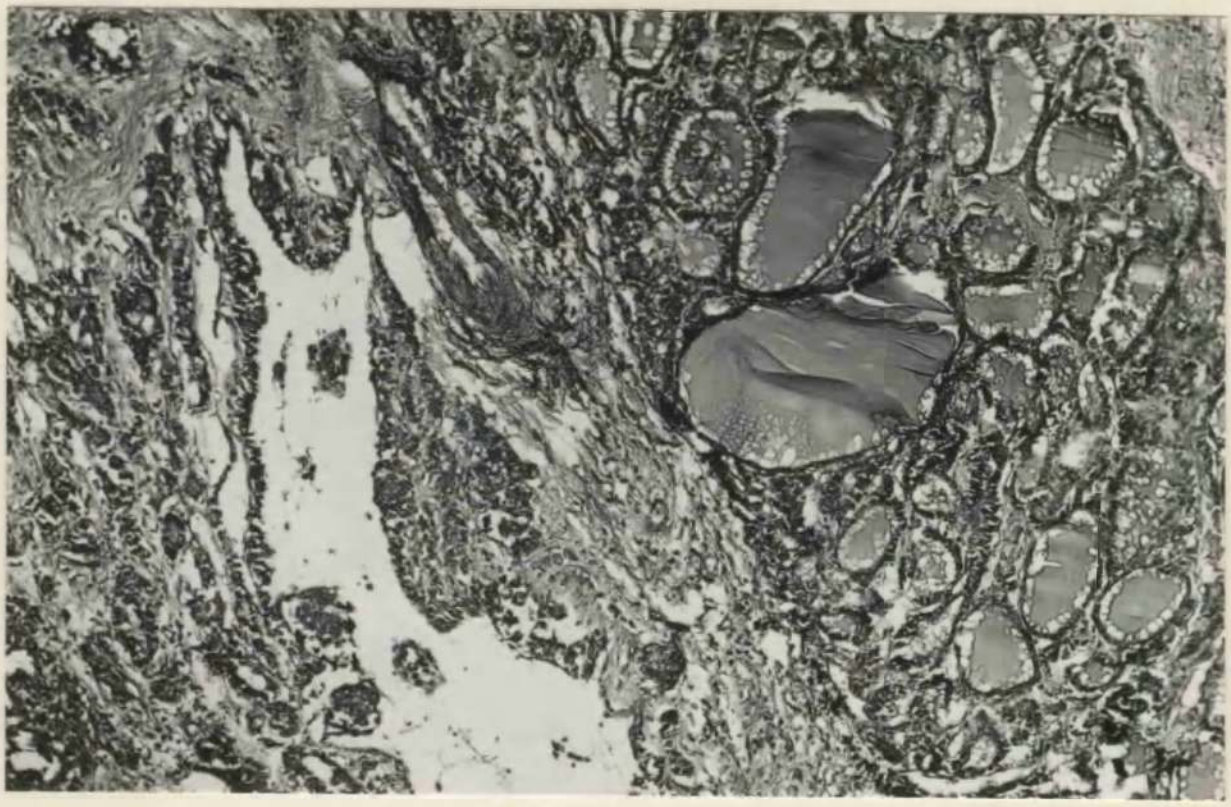

FIG.4,7

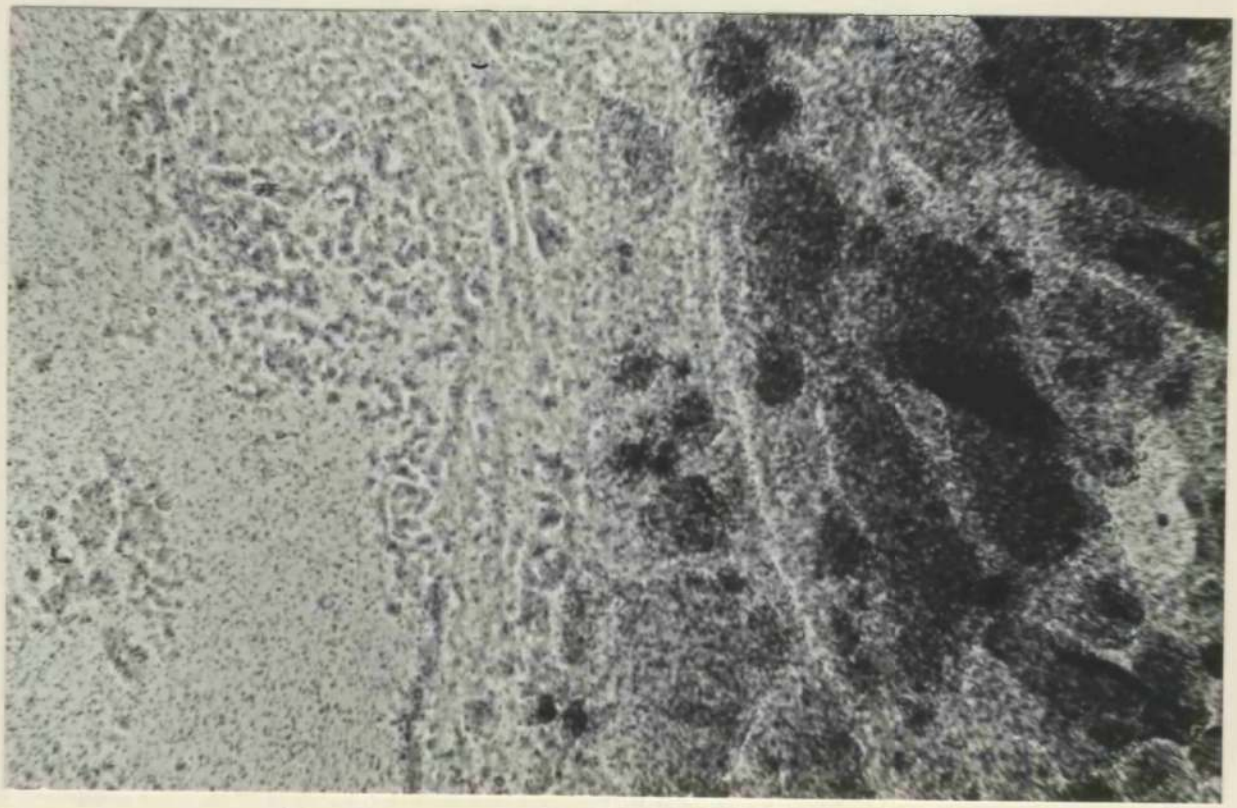

FIG.4,8

Fig. 4,9. - Case 4786/61. Secondary deposit of mixed papillary-follicular carcinoma in a lymph node. H. and E. X 10.

Fig. 4,10. - Case 4786/61. Only the follicular part of the secondary tumour shows colloid-bound iodine-131. Autoradiograph. Kodak AR50. X 37.

FIG.4,9

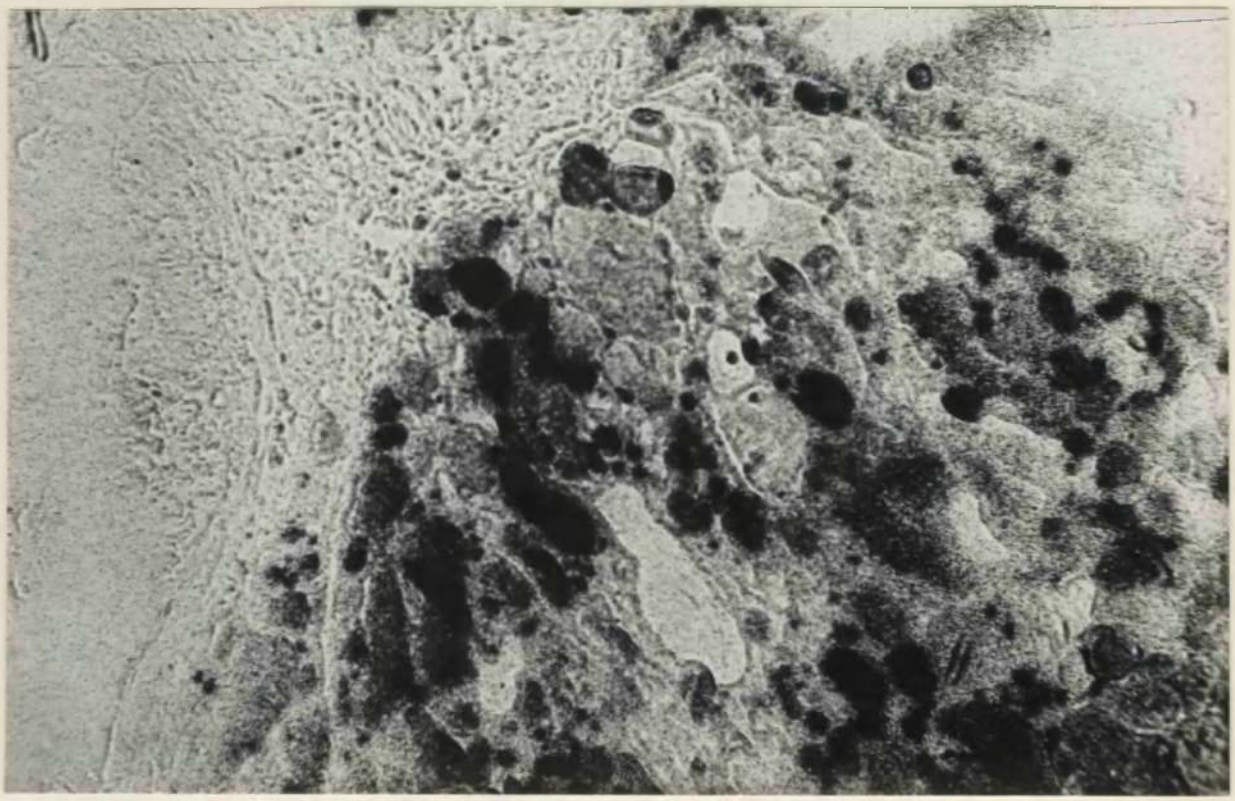

FIG.4,10

Fig. 4,11. - Case 2380/57. Follicular carcinoma invading normal thyroid tissue. H. and E. X 95.

Fig. 4,12. - Case 2380/57. Corresponding autoradiograph shows no evidence of iodine-131 in the tumour. The normal thyroid tissue shows a dense image in colloid. Kodak AR50. X 95.

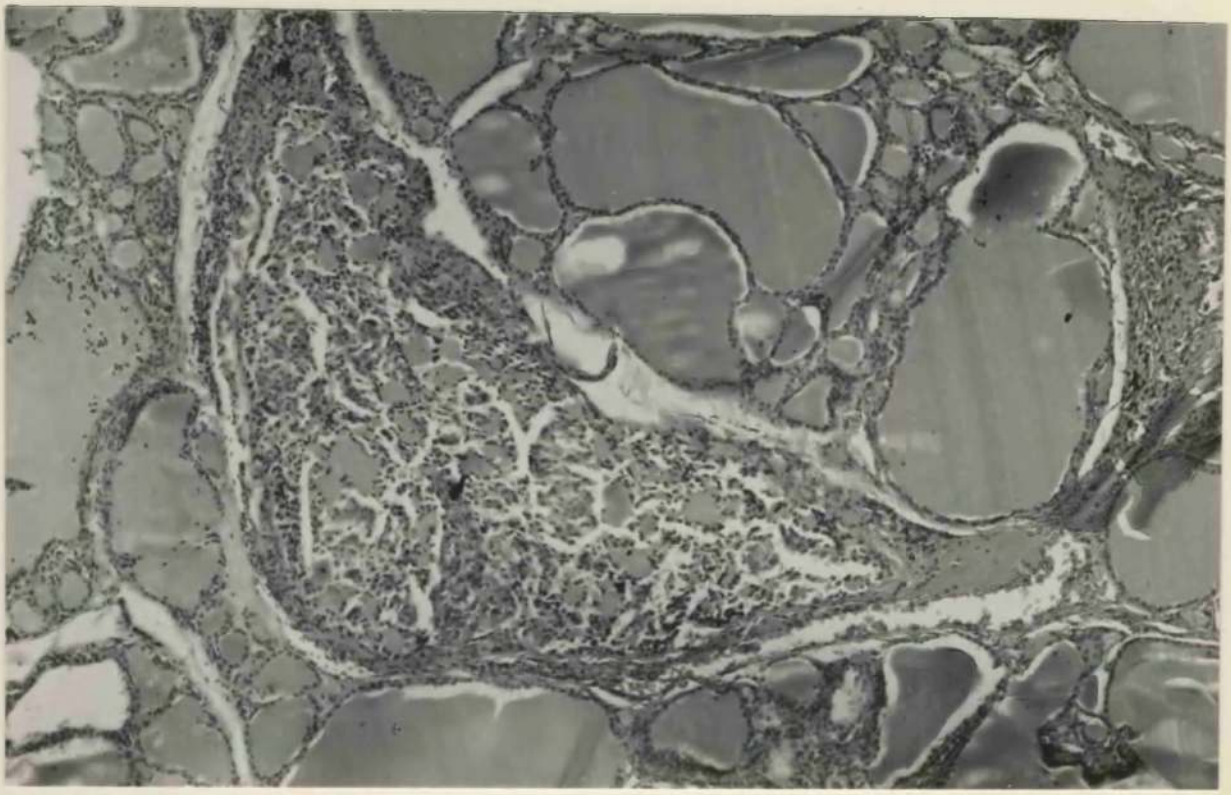

FIG.4,11

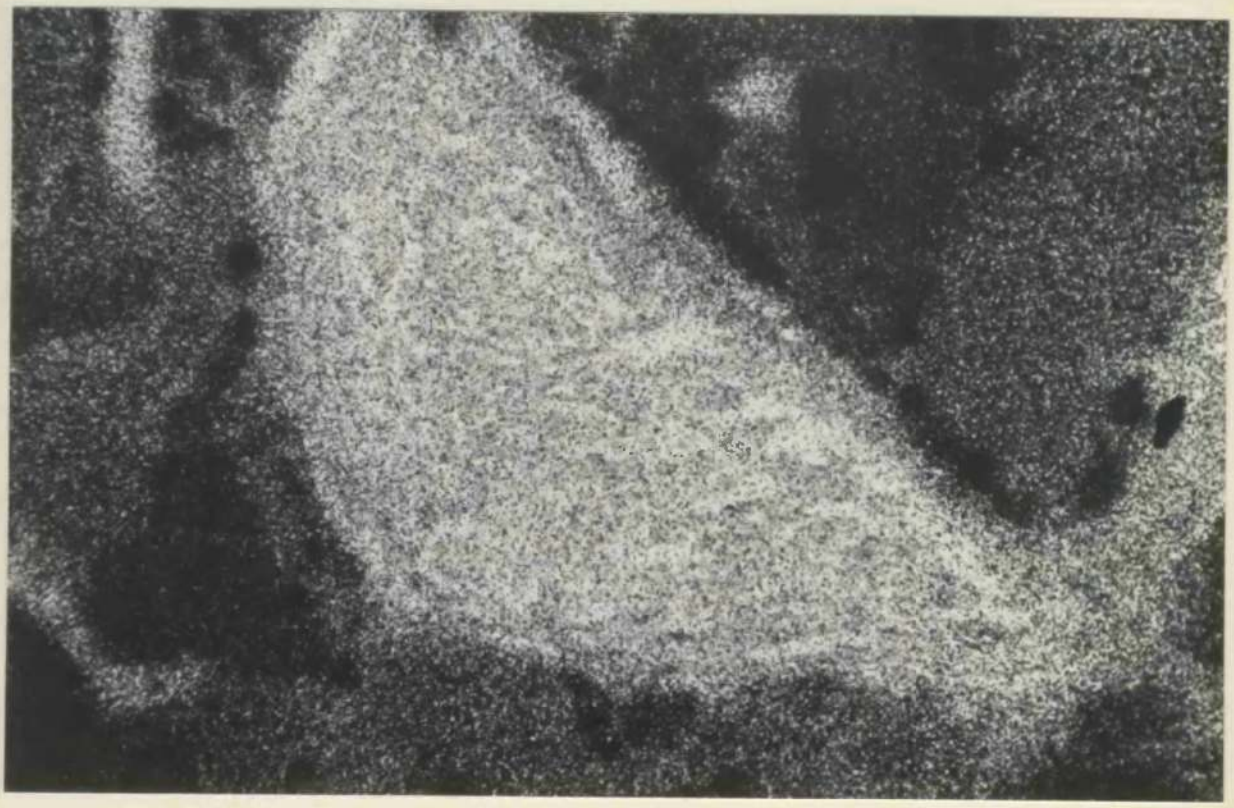

FIG.4,12

Fig. 4.13. - Case 1837/61. Small papillary carcinoma of thyroid. H. and E. X 16.

Fig. 4.14. - Case 1837/61. Corresponding autoradiograph shows the tumour to be quite inactive. The surrounding normal thyroid tissue contains bound iodine-131 in colloid. Kodak AR50. X 16.

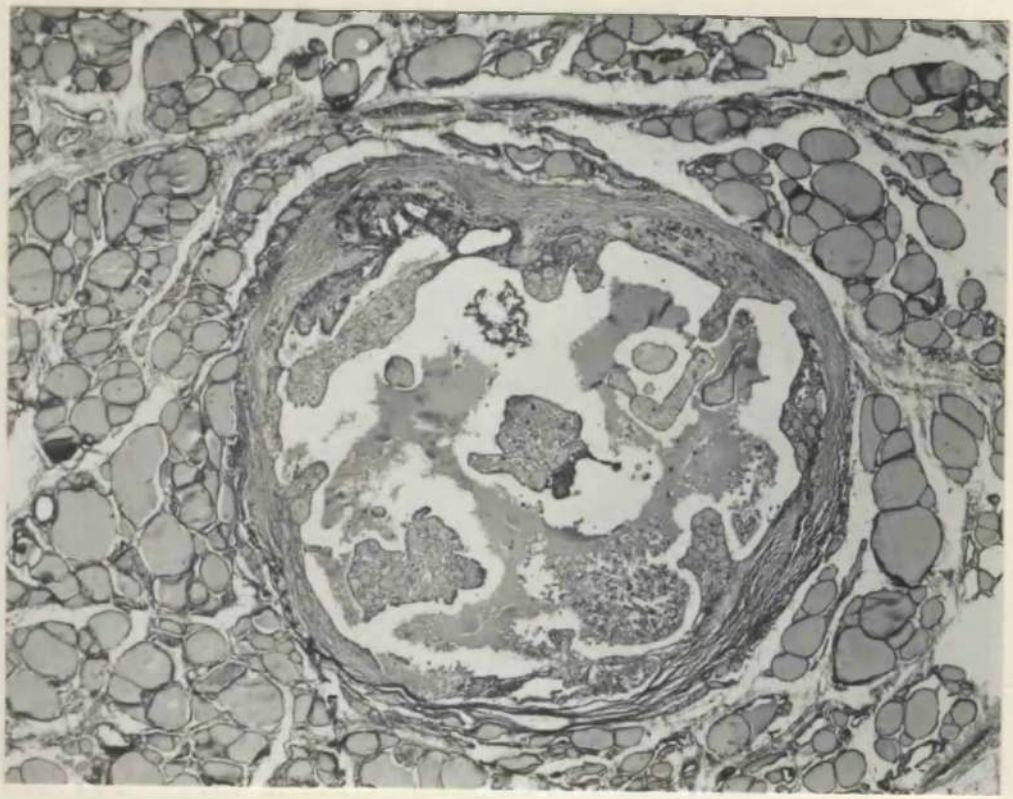

FIG.4,13

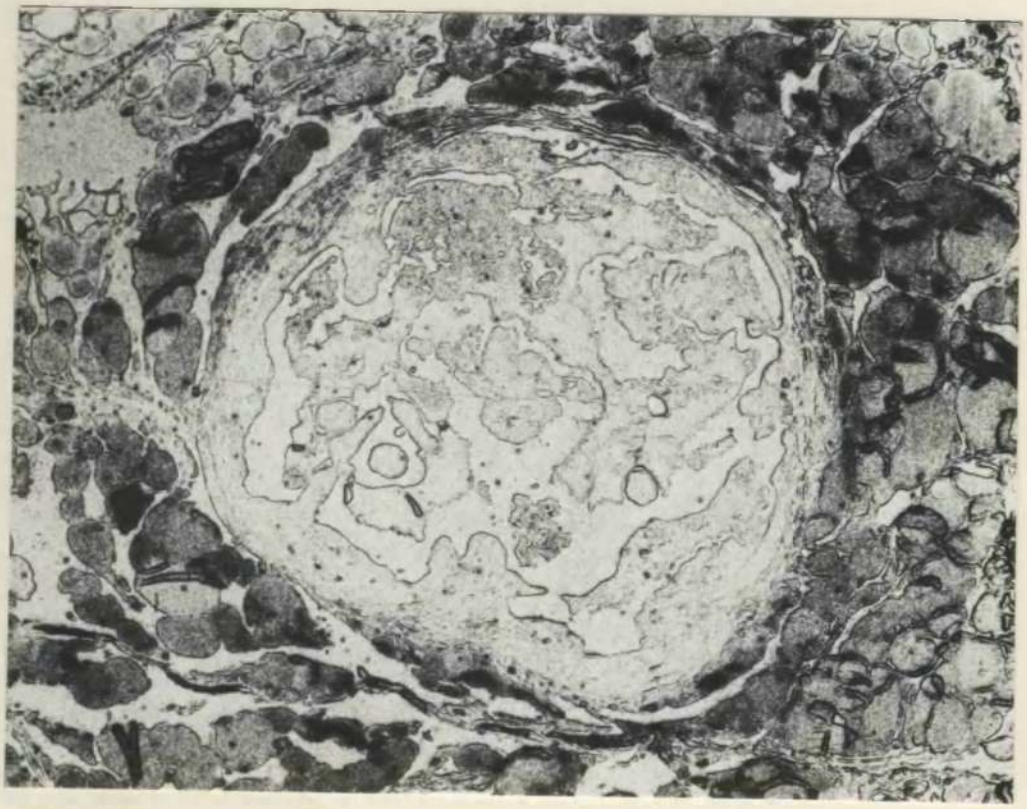

FIG.4,14

Fig. 4,15. - Case 1999/61. Undifferentiated small-cell carcinoma invading normal thyroid. H. and E. X 18.

Fig. 4,16. - Case 1999/61. Corresponding autoradiograph shows no utilisation of iodine-131 by the tumour. Dense colloid-images are present in the normal thyroid tissue. Kodak AR50. X 18.

FIG.4,15

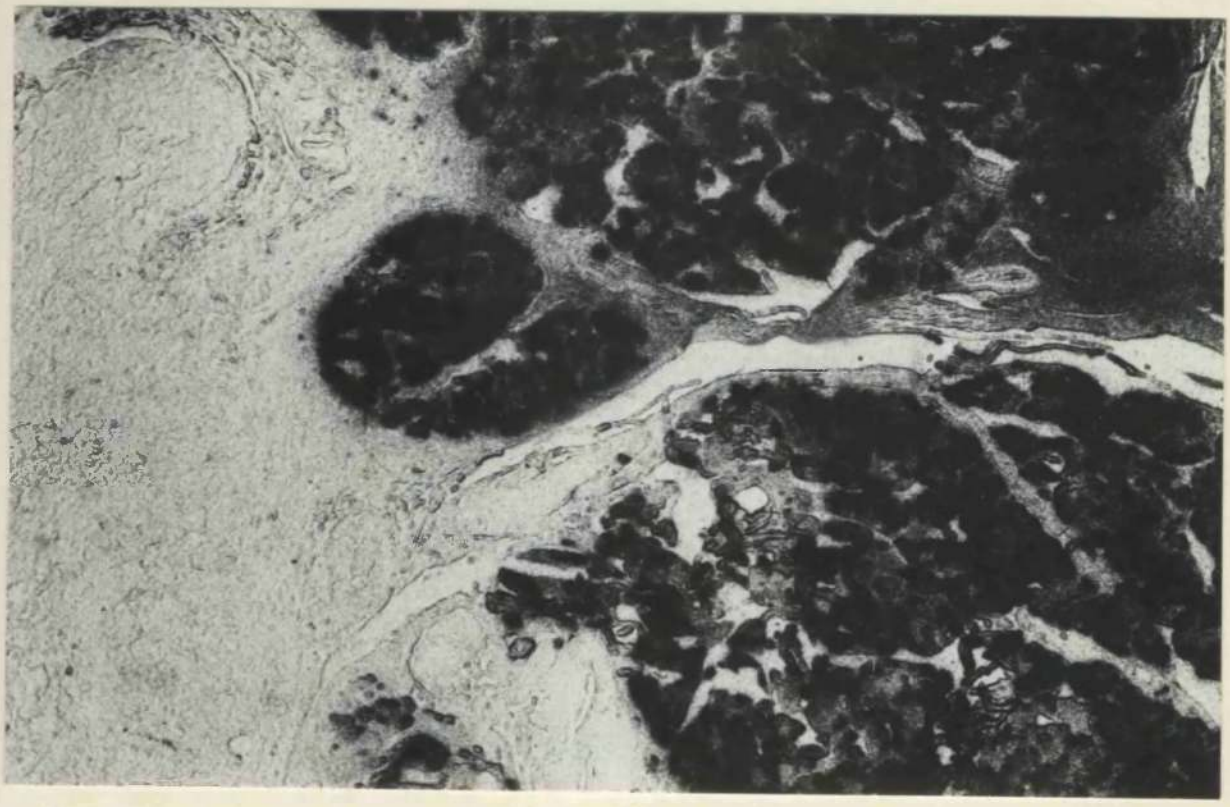

FIG.4,16

Fig. 4,17. - Case 2393/57. Giant-cell carcinoma of thyroid (top) and Hashimoto's thyroiditis (bottom). H. and E. X 75.

Fig. 4,18. - Case 2393/57. Corresponding autoradiograph. No iodine-131 is present in the tumour (top). The distribution of radio-iodine in Hashimoto's thyroiditis is in colloid in follicles lined by Askanazy-cells. Kodak AR50. X 95.

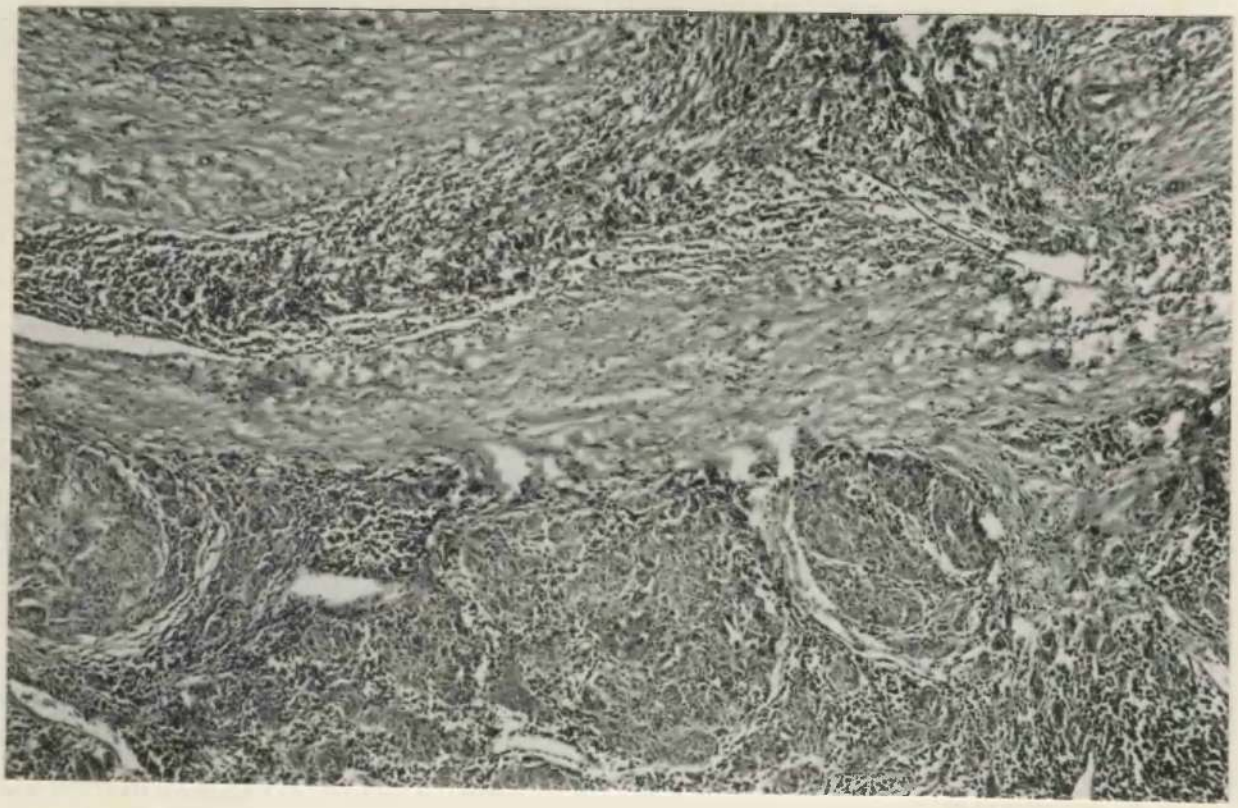

FIG.4,17

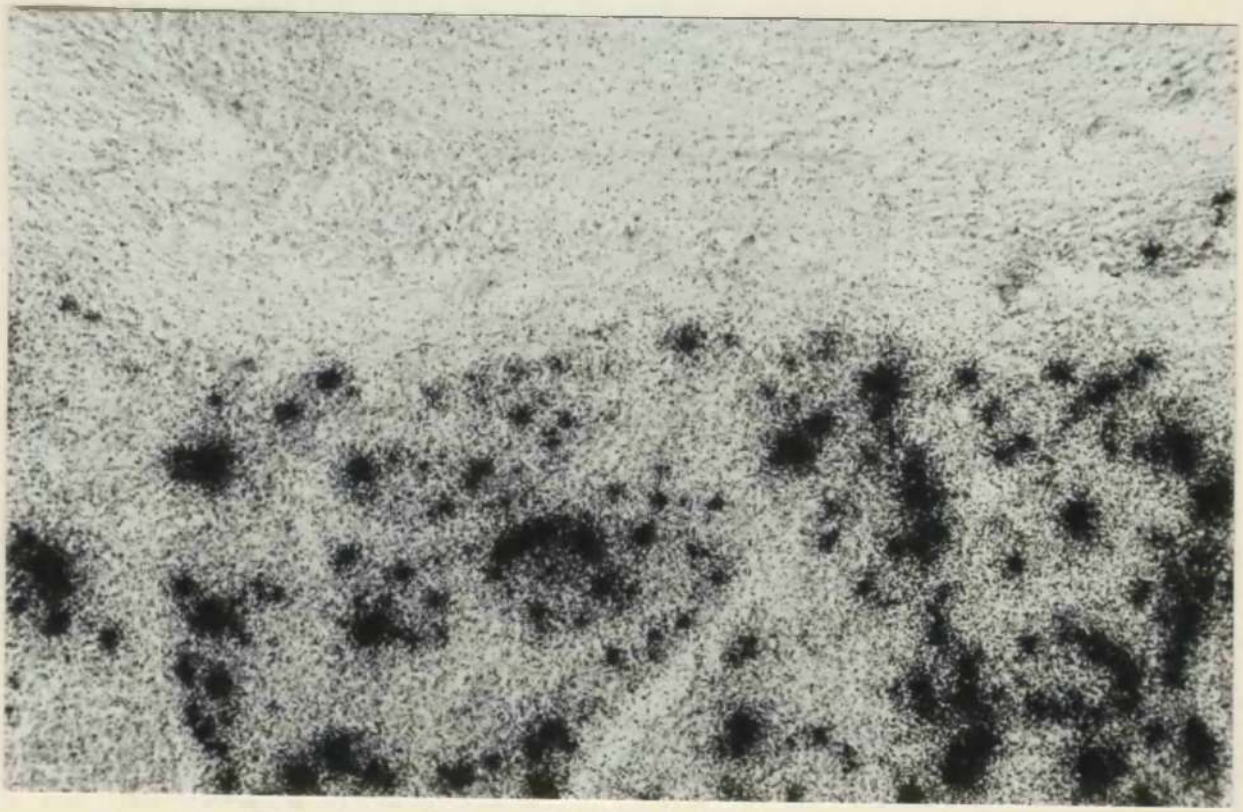

FIG.4,18

Fig. 4.19. - Case 2393/57. Higher power view of the undifferentiated giant-cell carcinoma. H. and E. X 300.

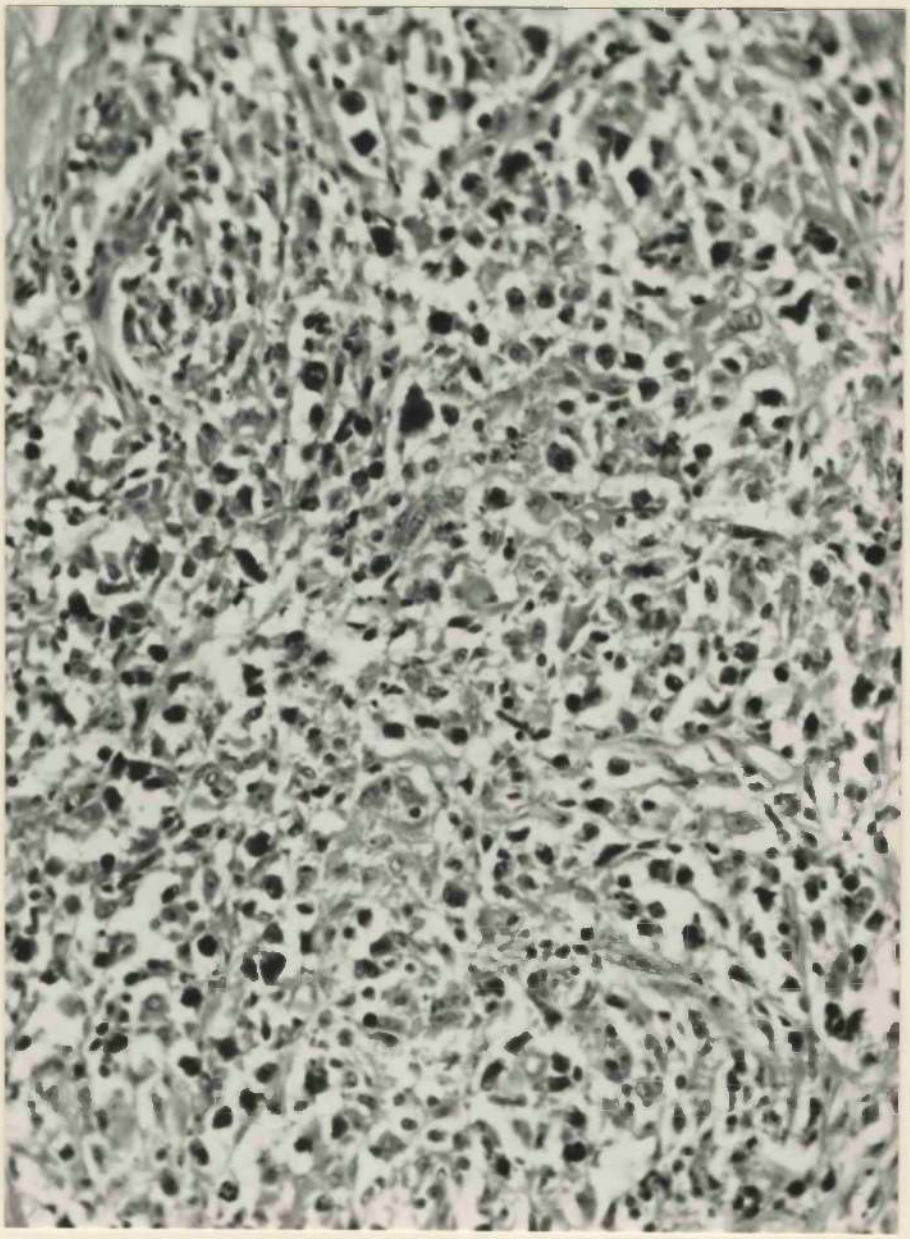

FIG.4,19

Fig. 4,20. - Case 1734/57. A field of focal thyroiditis of Hashimoto type related to an undifferentiated small-cell carcinoma (not shown). H. and E. X 230.

Fig. 4,21. - Case 1734/57. Corresponding autoradiograph. Colloid-bound iodine-131 is present in follicles lined by Askanazy-cells. Kodak AR10. X 230.

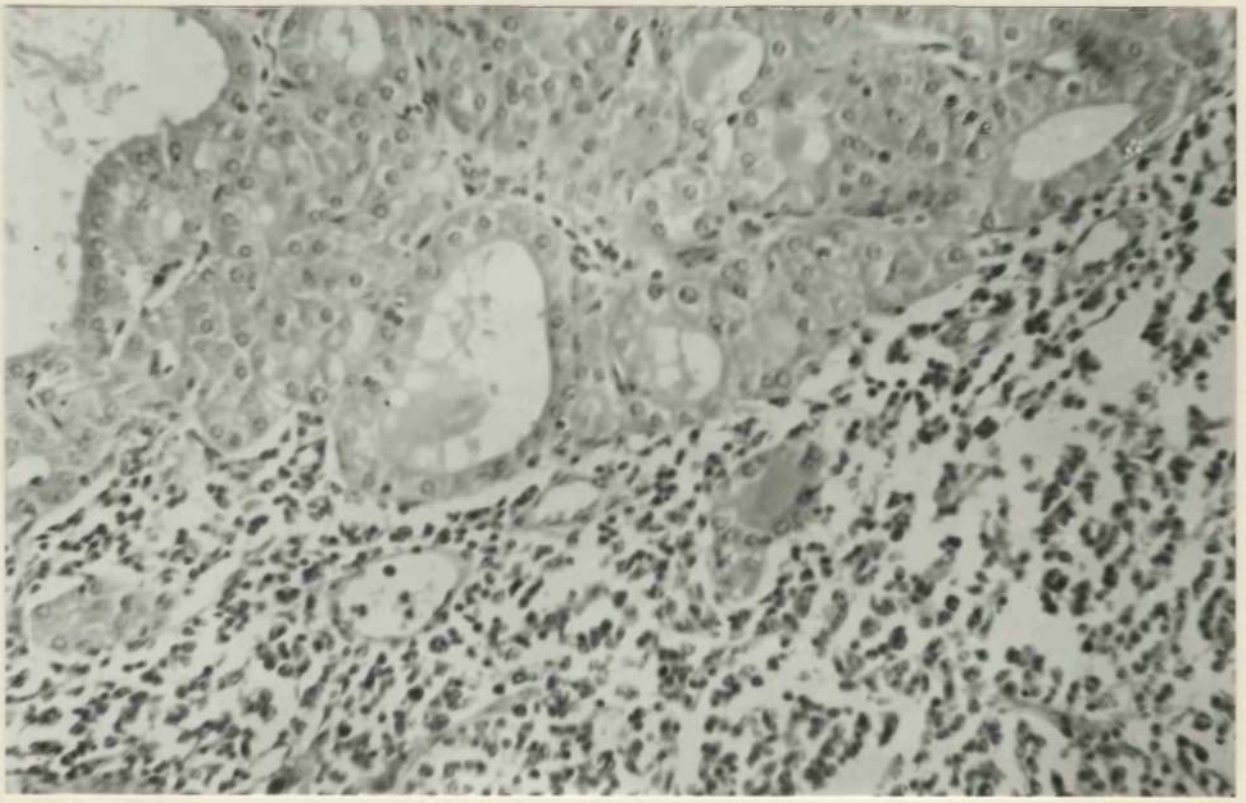

FIG.4,20

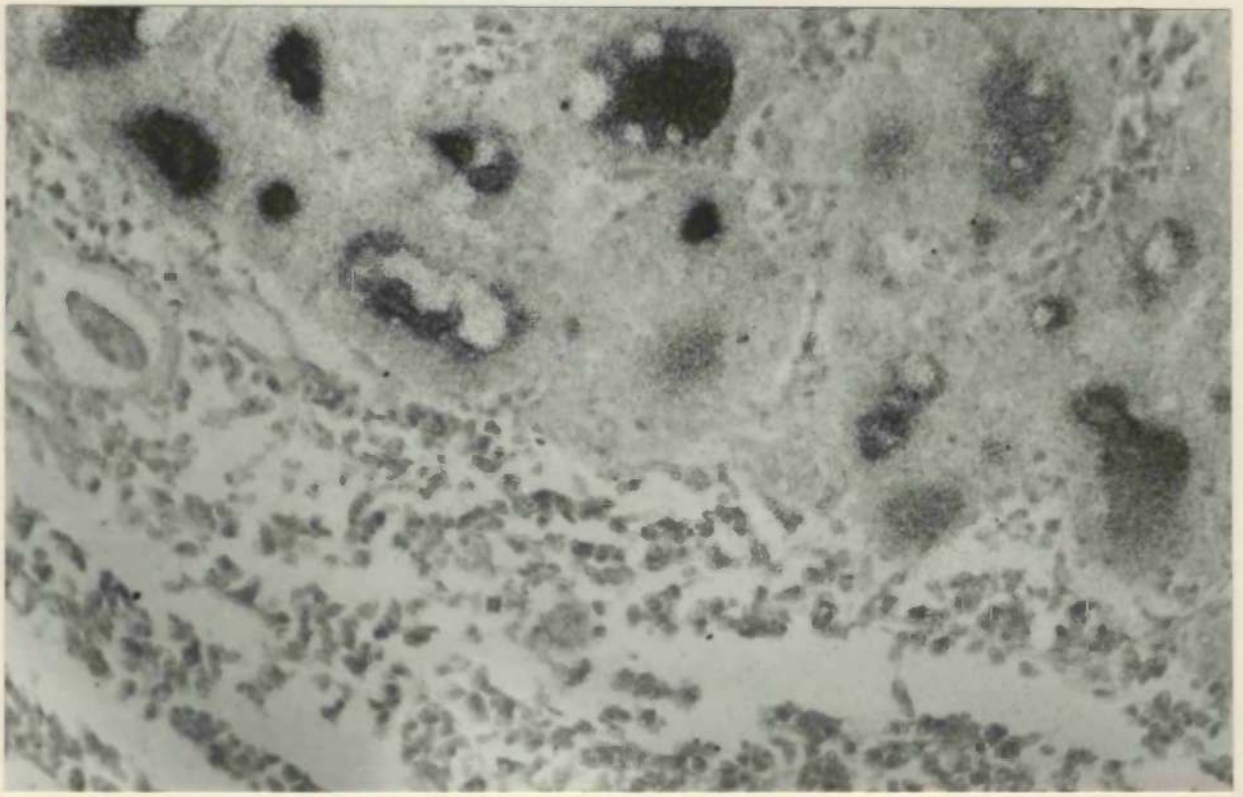

FIG.4,21

Fig. 4,22. - Case 302/60. Non-toxic nodular goitre. Parts of 3 nodules are shown in the autoradiograph. The nodules are similar in structure and the distribution-patterns of iodine-131 are also similar. The image is most intense in colloid in microfollicles and least intense in colloid in macrofollicles. Kodak AR50. X 30.

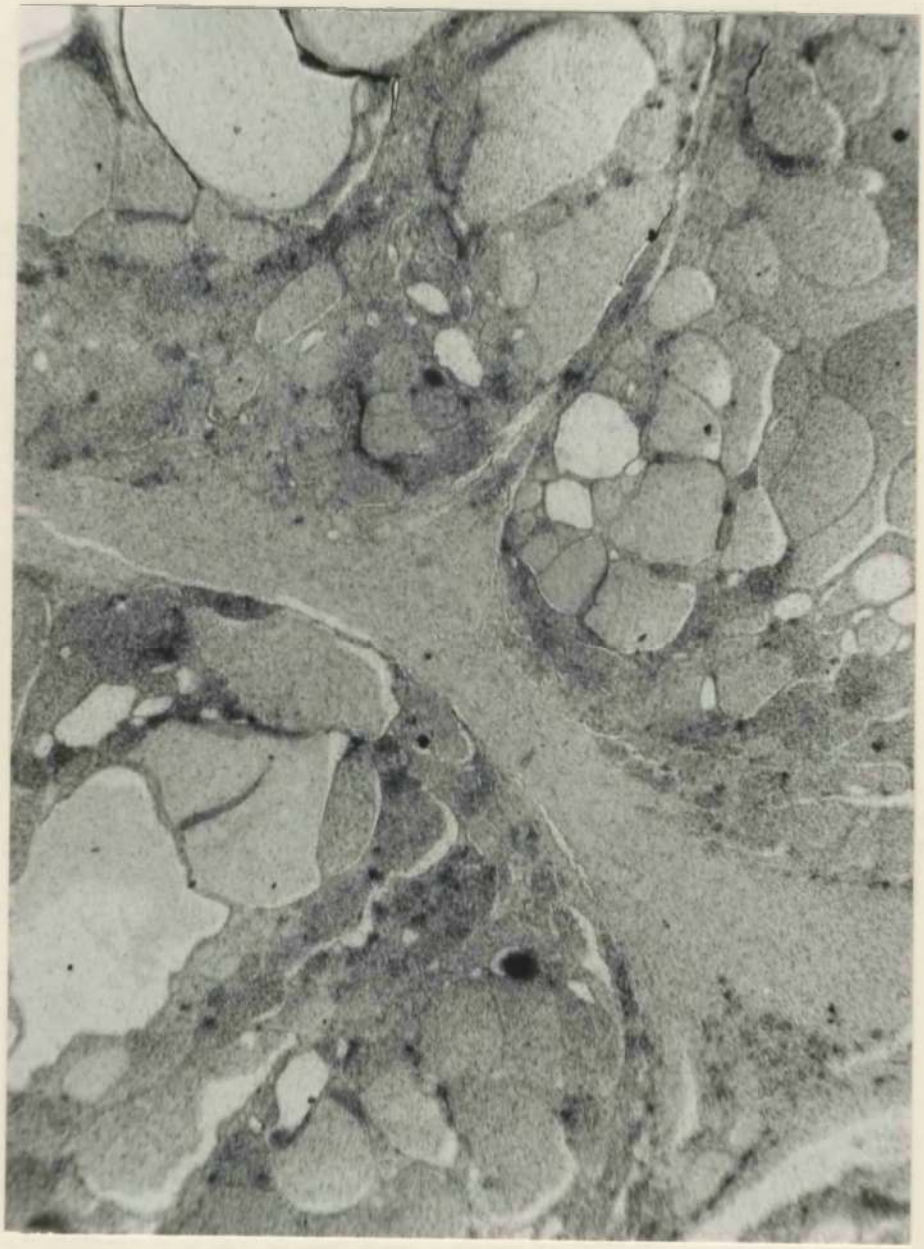

FIG.4, 22

Fig. 4.23. - Case 3753/55. Non-toxic nodular goitre. Higher power view to show image intensity greatest in smallest follicles of a nodule. Autoradiograph, Kodak AR50. X 250.

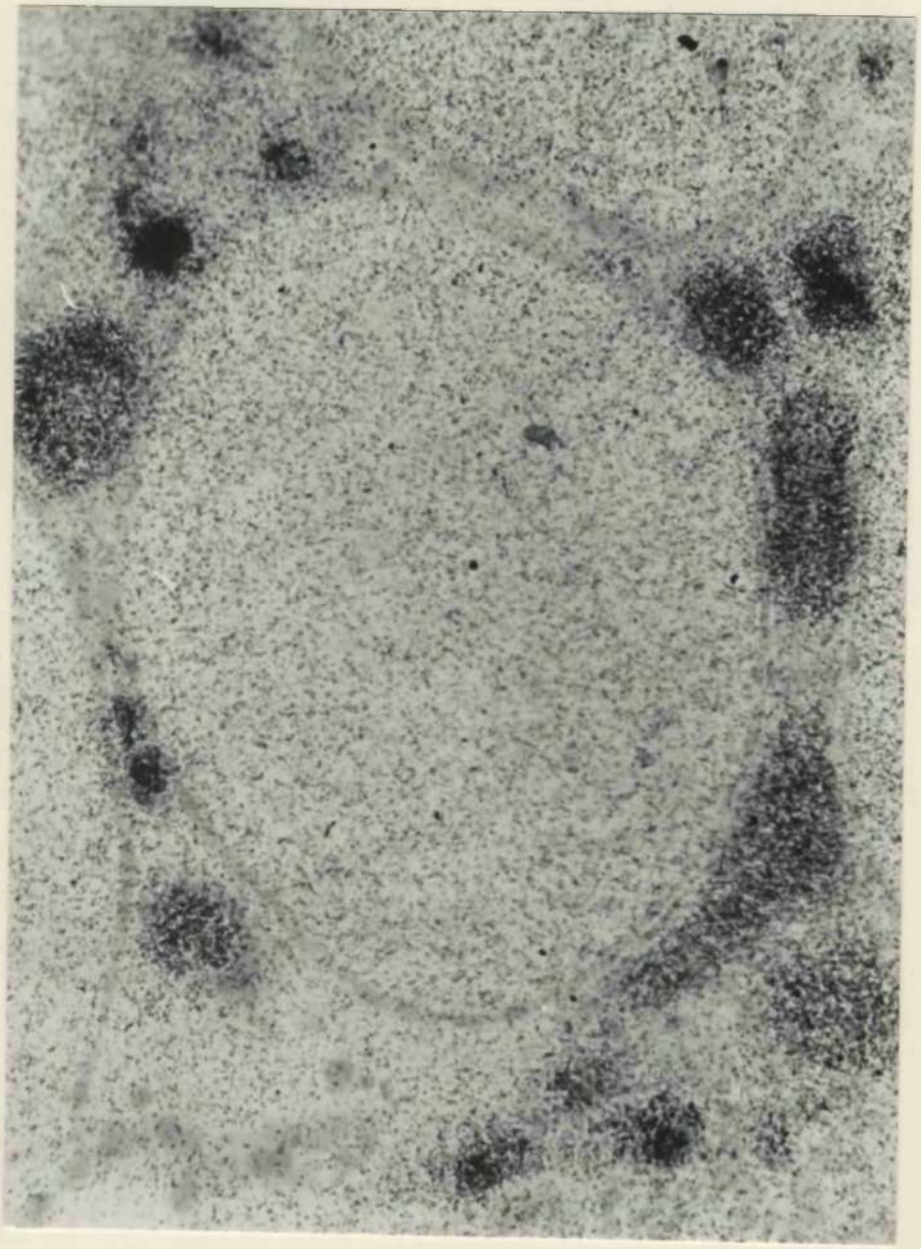

FIG. 4, 23

Fig. 4,24. - Case 3753/55. Non-toxic nodular goitre. Autoradiograph shows a nodule which is considerably more active than the internodular tissue and other nodules. Kodak AR10. X 50.

Fig. 4,25. - Case 1134/58. Non-toxic nodular goitre. Another example of a hot nodule. Autoradiograph, Kodak AR50. X 30.

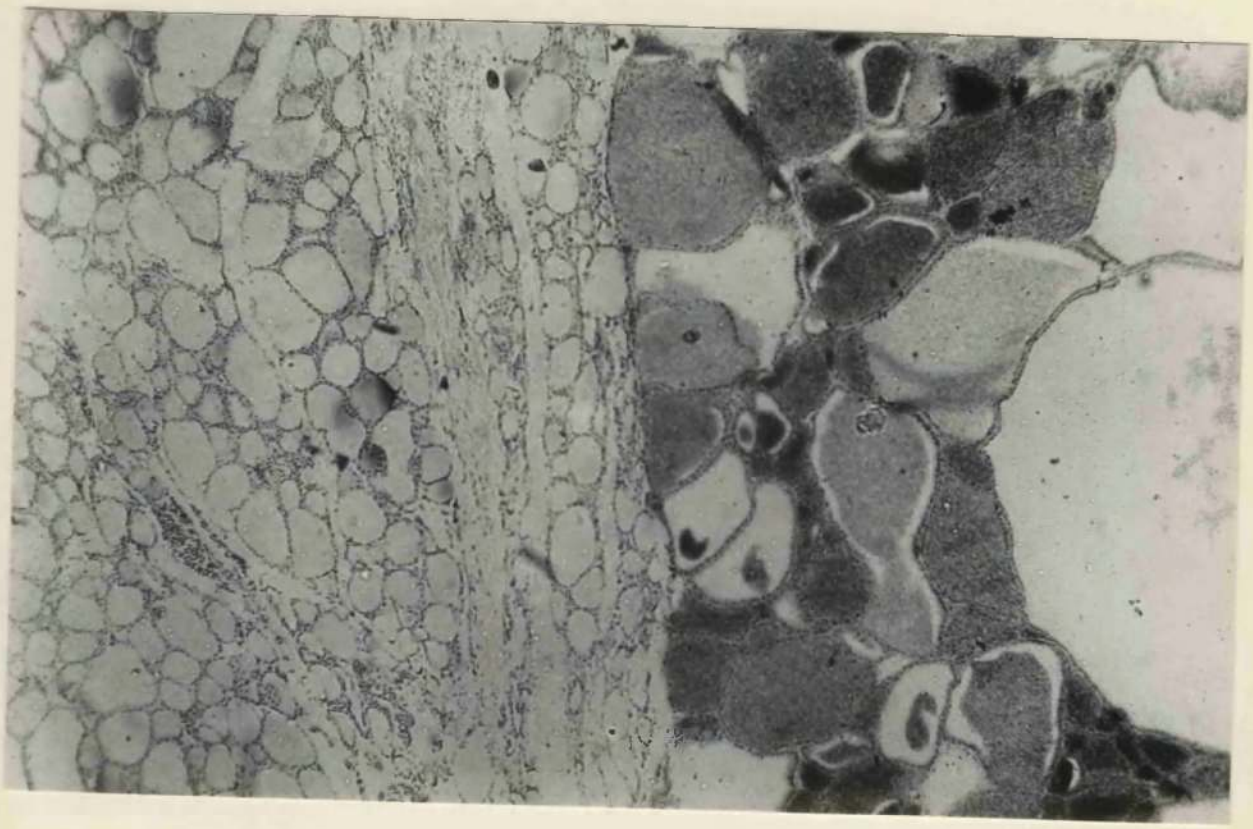

FIG.4,24

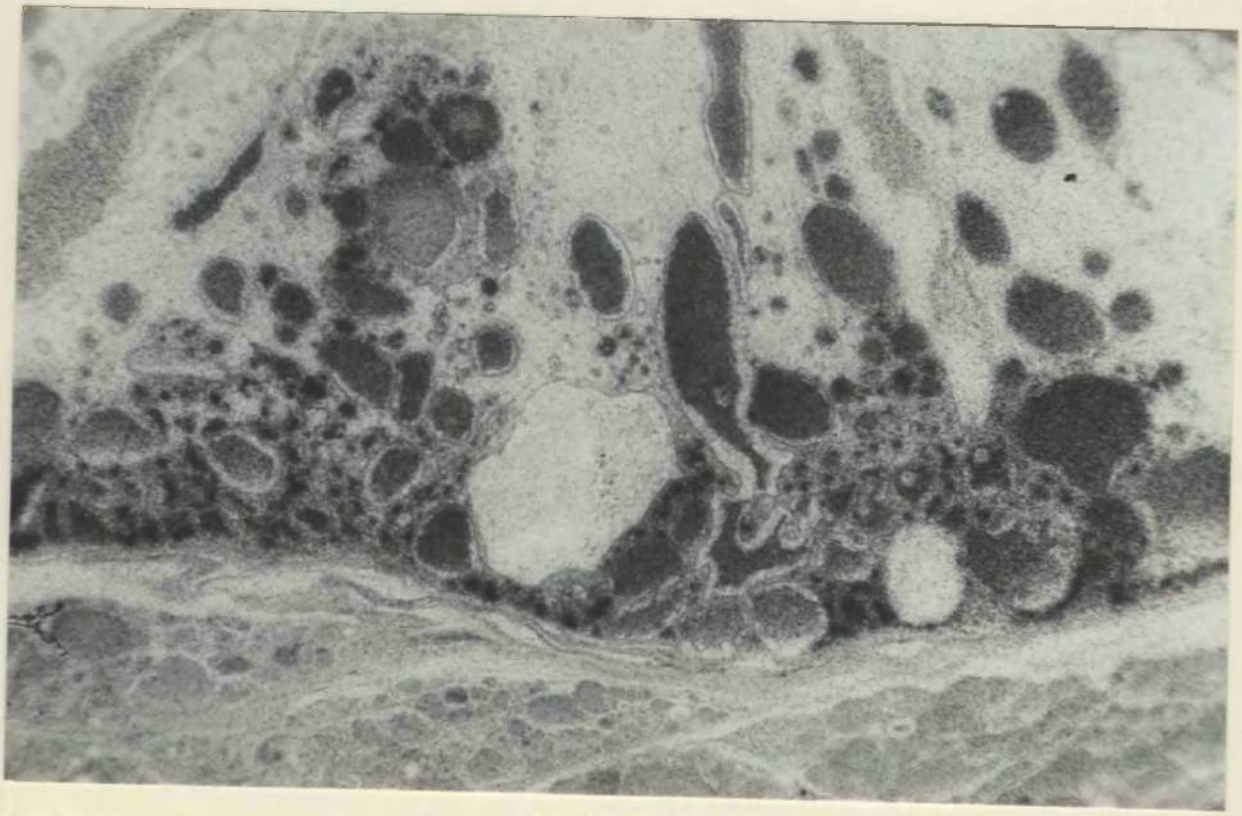

FIG.4,25

Fig. 4,26. - Case 4413/56. Non-toxic nodular goitre. A small nodule which is considerably less active than the internodular tissue. In this case the resected portion of thyroid also contained a cyst. Autoradiograph, Kodak AR50. X 50.

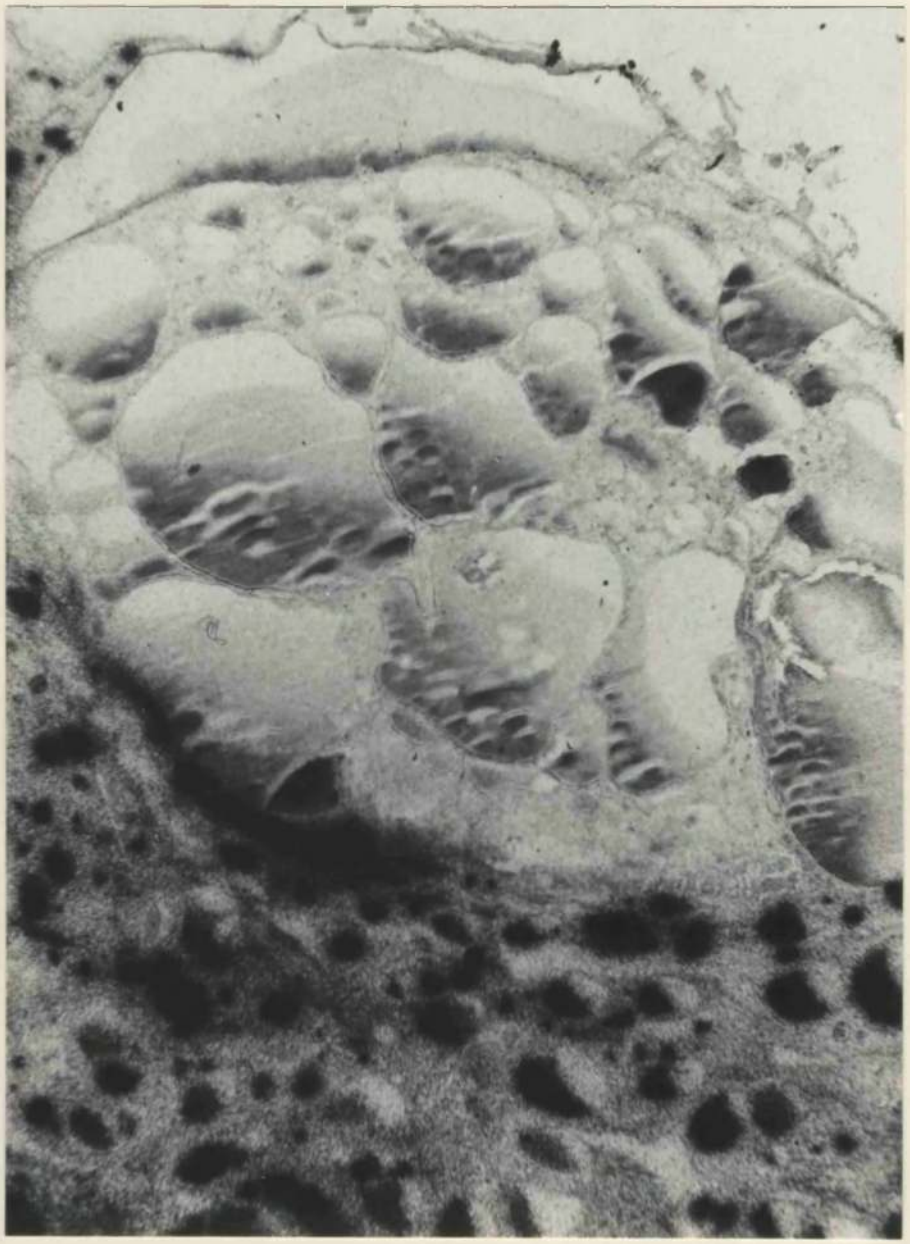

FIG. 4,26

Fig. 4,27. - Case 2580/56. Non-toxic nodular goitre. This nodule is predominantly micro-follicular. H. and E. X 30.

Fig. 4,28. - Case 2580/56. Corresponding autoradiograph shows abundant colloid-bound iodine-131 in hot nodule. Kodak AR50. X 30.

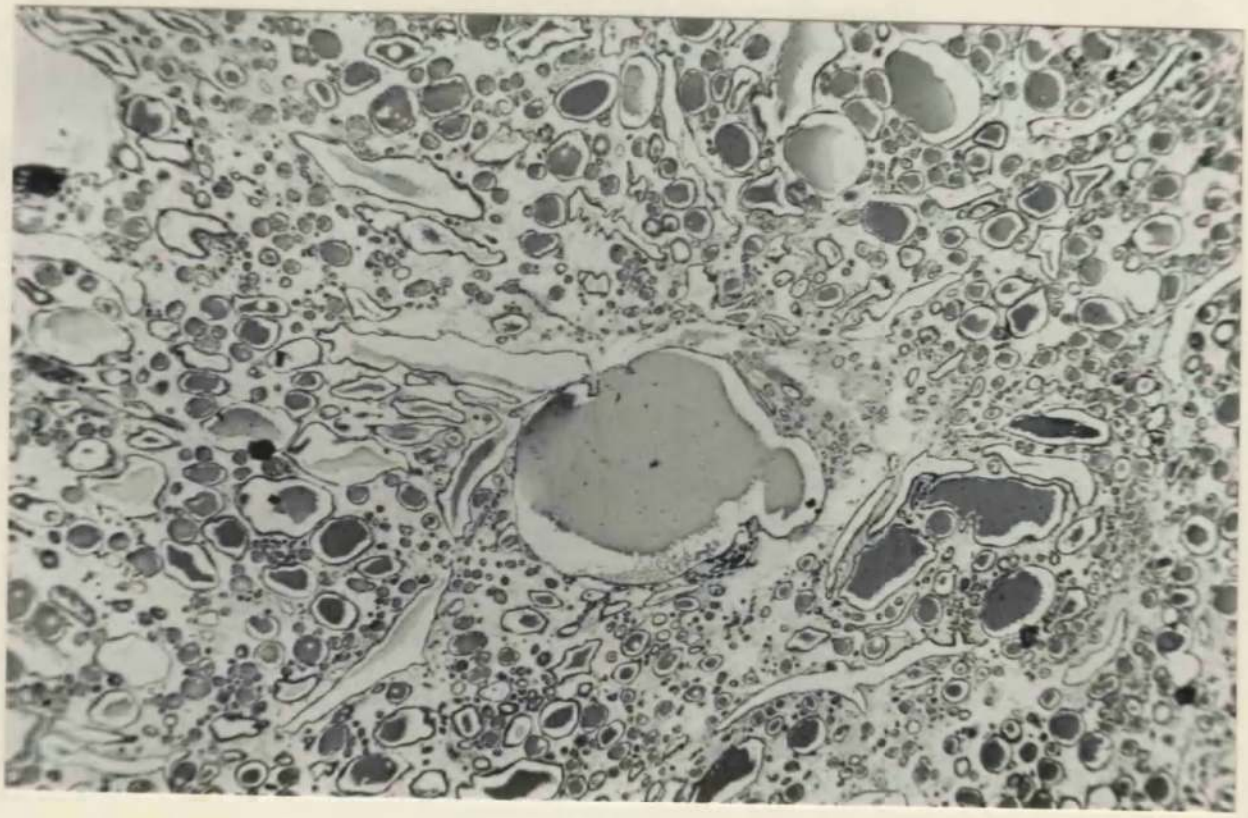

FIG. 4,27

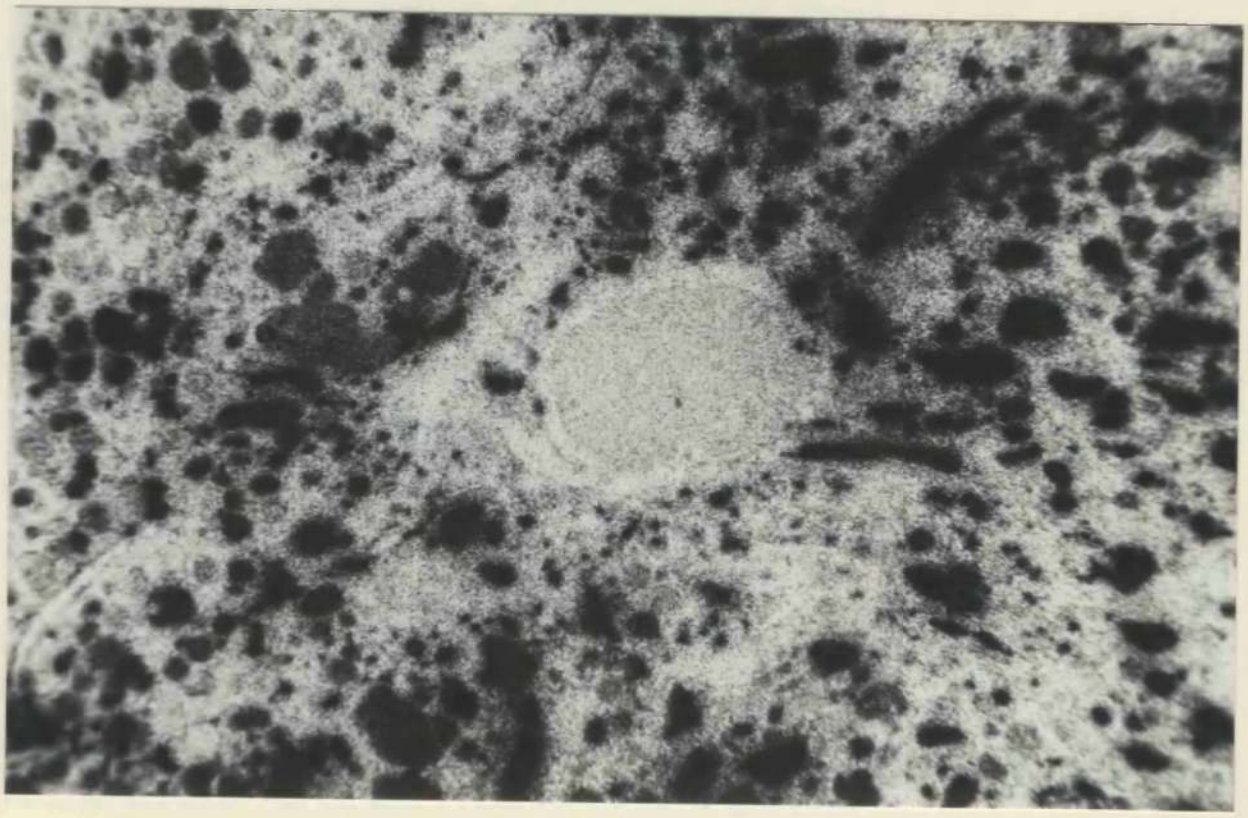

FIG. 4,28

Fig. 4,29. - Case 934/54. Toxic nodular goitre. Iodine-131 is present in microfollicular areas in the internodular tissue. Autoradiograph, Kodak AR10. X 300.

Fig. 4,30. - Case 1850/59. Diffuse colloid goitre. Iodine-131 utilisation is very limited. An occasional follicle contains more heavily labelled colloid. Autoradiograph, Kodak AR50. X 95.

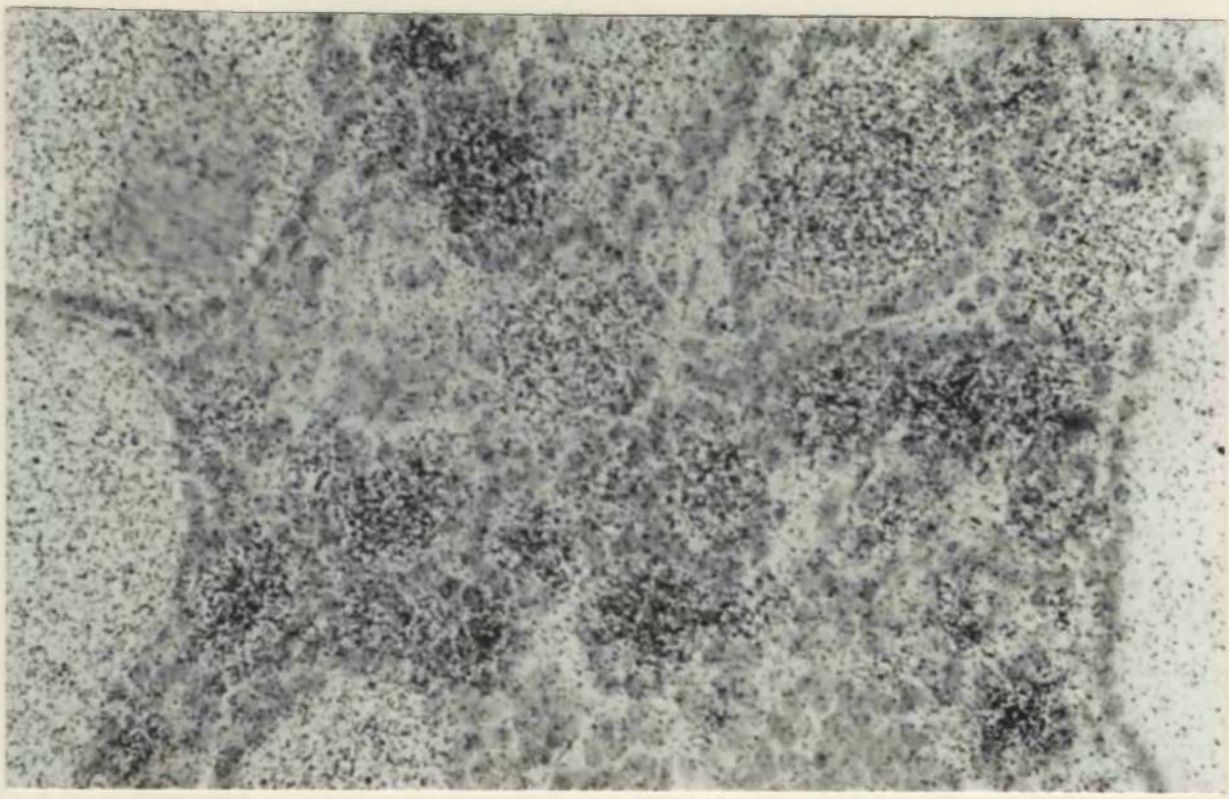

FIG.4,29

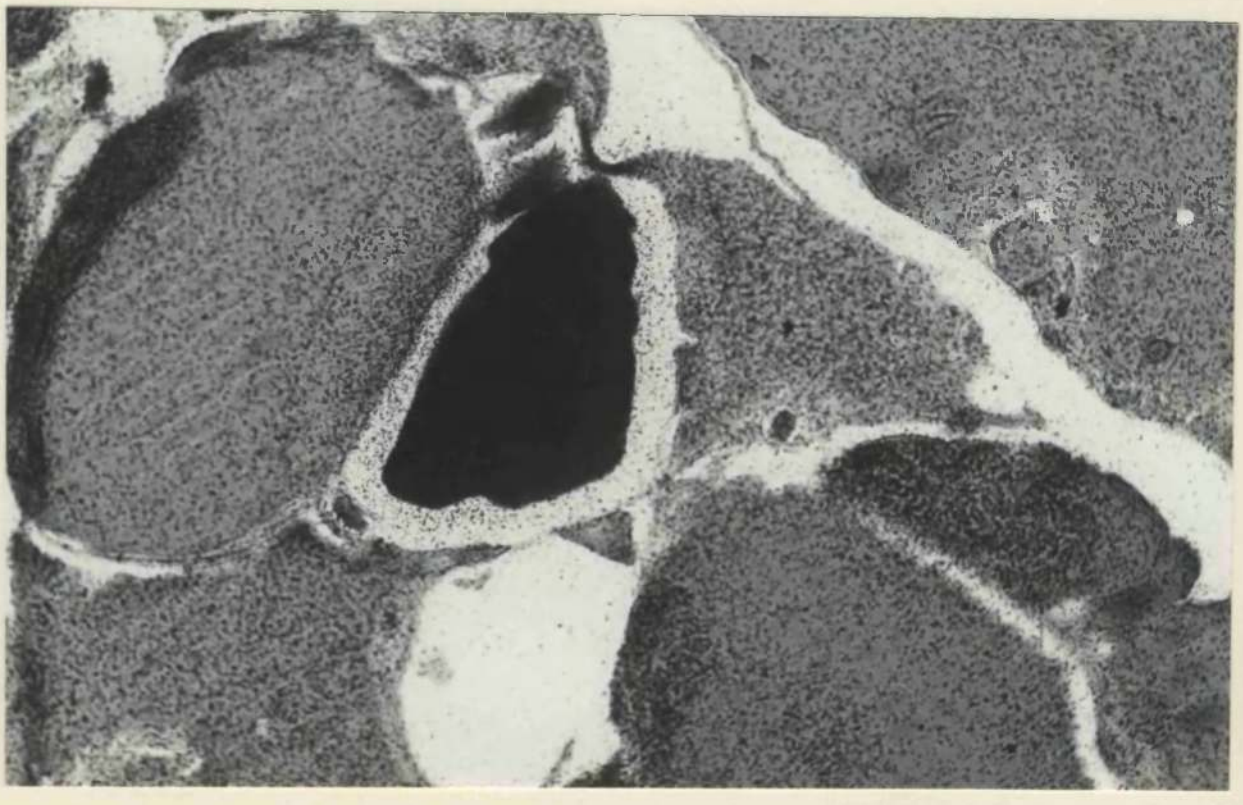

FIG.4,30

Fig. 4,31. - Case 5038/55. Thyroid cyst. Thyroid tissue adherent to the cyst wall (bottom) shows a nodular structure and iodine-131 distribution-pattern is similar to that seen in non-toxic nodular goitre. Autoradiograph, Kodak AR10. X 12.

Fig. 4,32. - Case 1782/61. Autoradiograph of normal thyroid tissue resected with thyroglossal cyst. All colloid present contains iodine-131. The image is more intense in the smaller follicles. Kodak AR50. X 95.

FIG.4,31

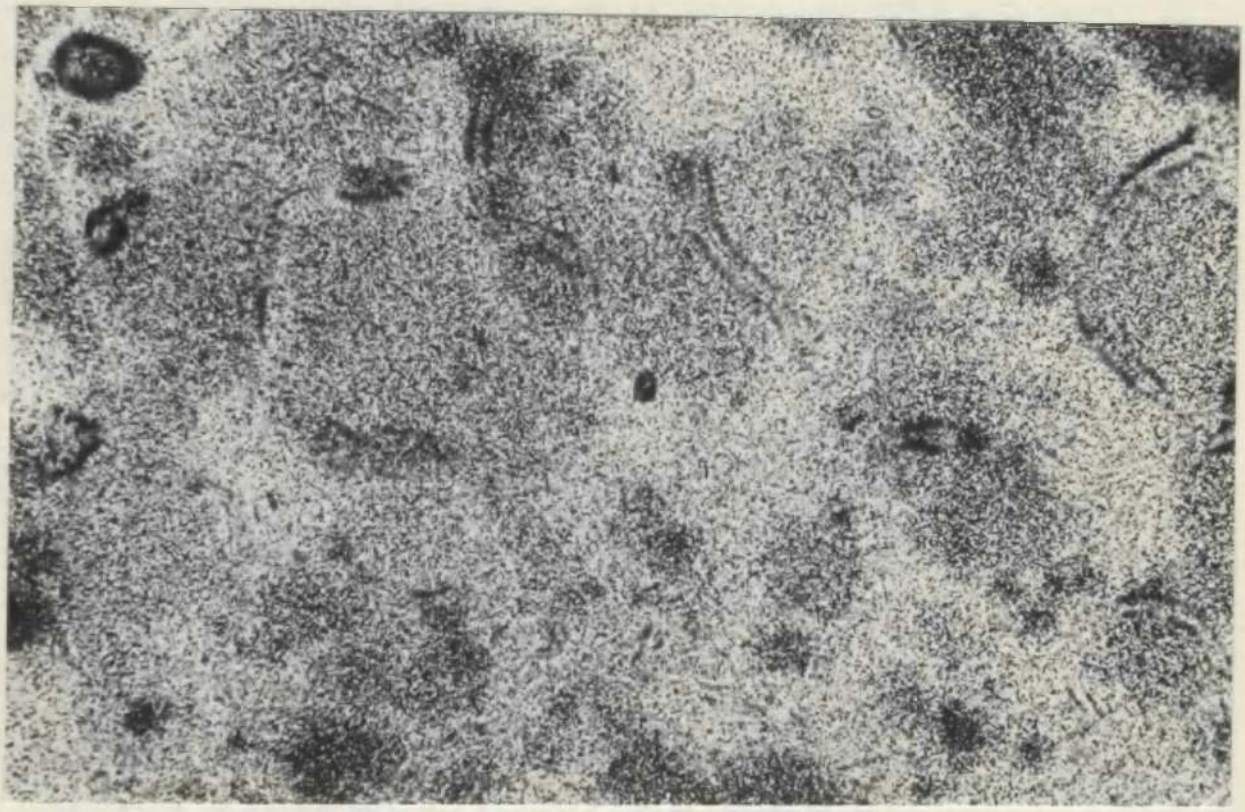

FIG.4,32

Fig. 4,33. - Case 239/62. Dyshormonogenetic goitre - impaired dehalogenase activity. The internodular tissue shows a marked degree of cellular pleomorphism. This is also seen in diffuse goitres due to dyshormonogenesis. H. and E. X 230.

Fig. 4,34. - Case 3001/57. Dyshormonogenetic goitre - abnormal iodoprotein. Columns of epithelial cells in capsule mimic the appearance of malignant invasion. H. and E. X 230.

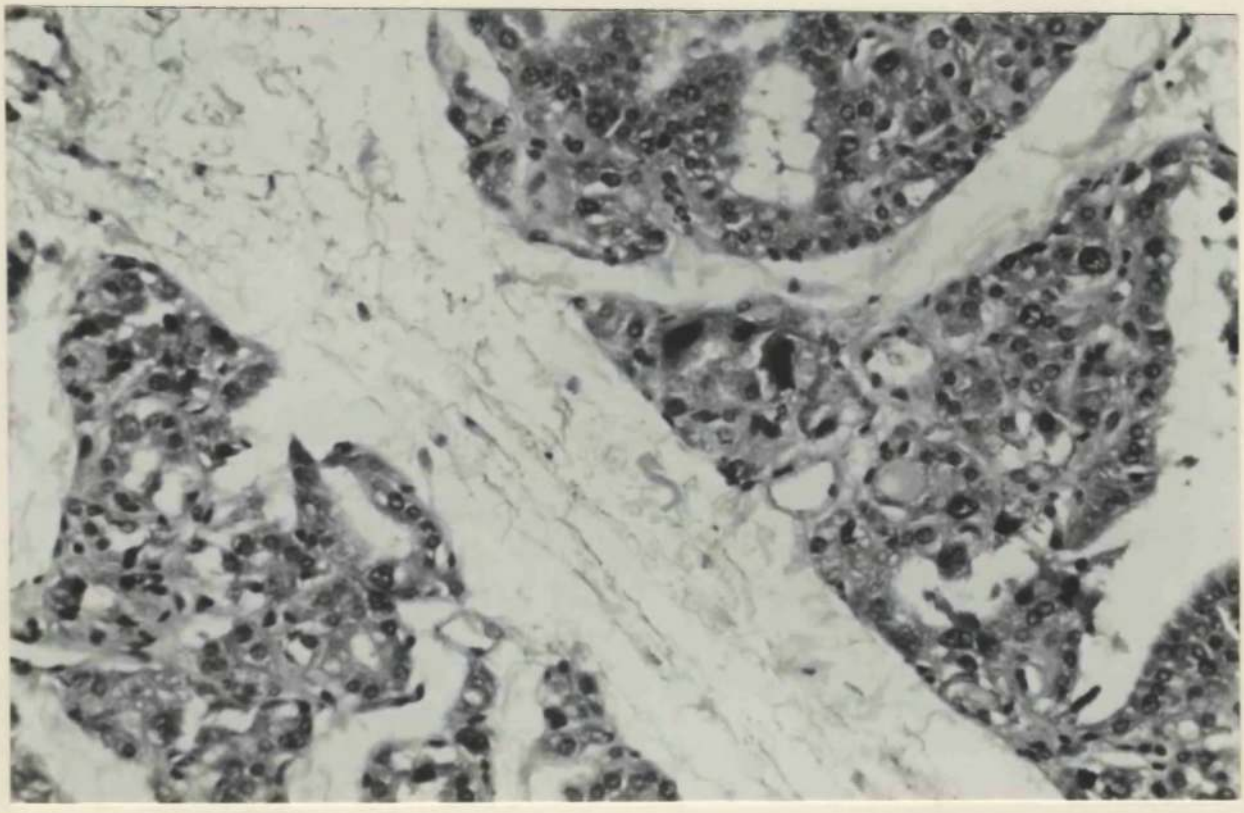

FIG.4,33

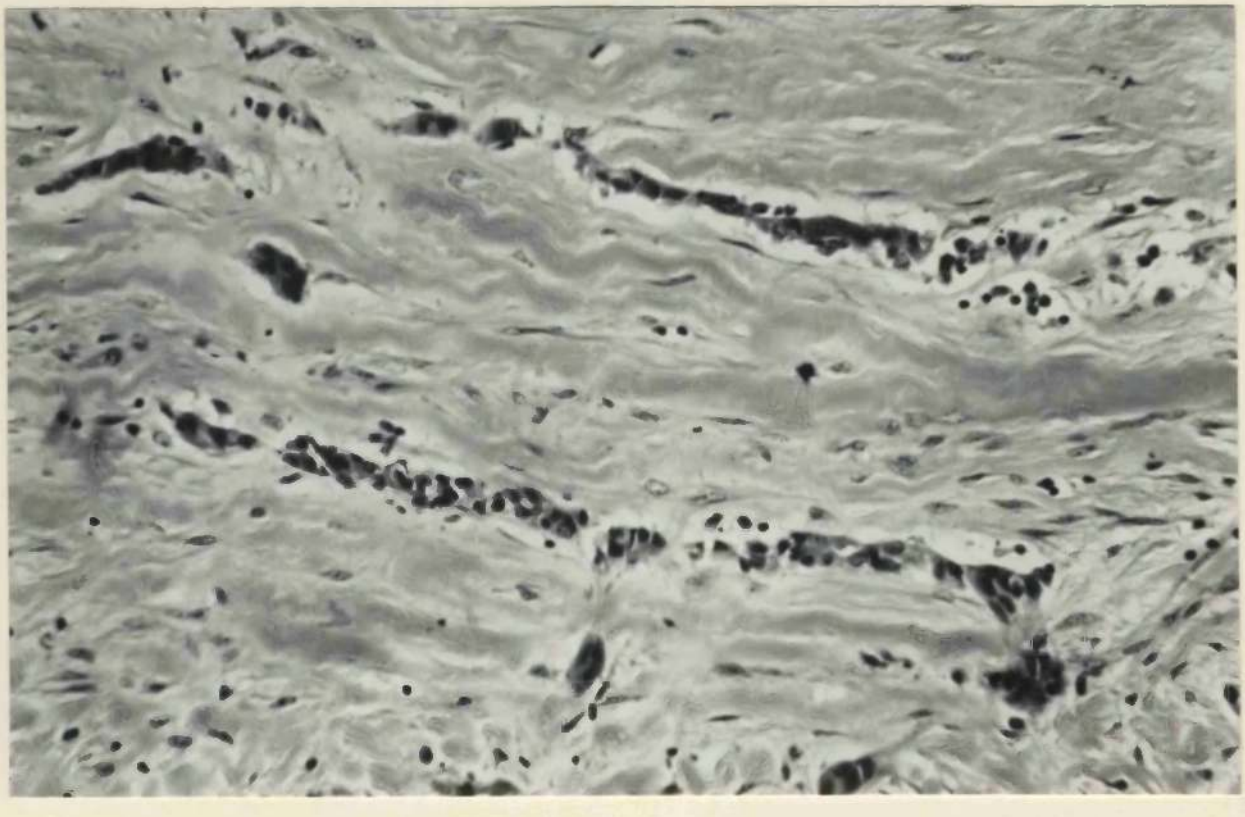

FIG.4,34

Fig. 4,35. - Case 3467/58. Dyshormonogenetic goitre - impaired dehalogenase activity. Carcinoma is present in this case with evidence of invasion of vessel walls. H. and E. X 290.

Fig. 4,36. - Case 303/60. Dyshormonogenetic goitre - abnormal iodoprotein. A diffuse goitre in which there is evidence of epithelial utilisation of iodine-131. Autoradiograph, Kodak AR50. X 140.

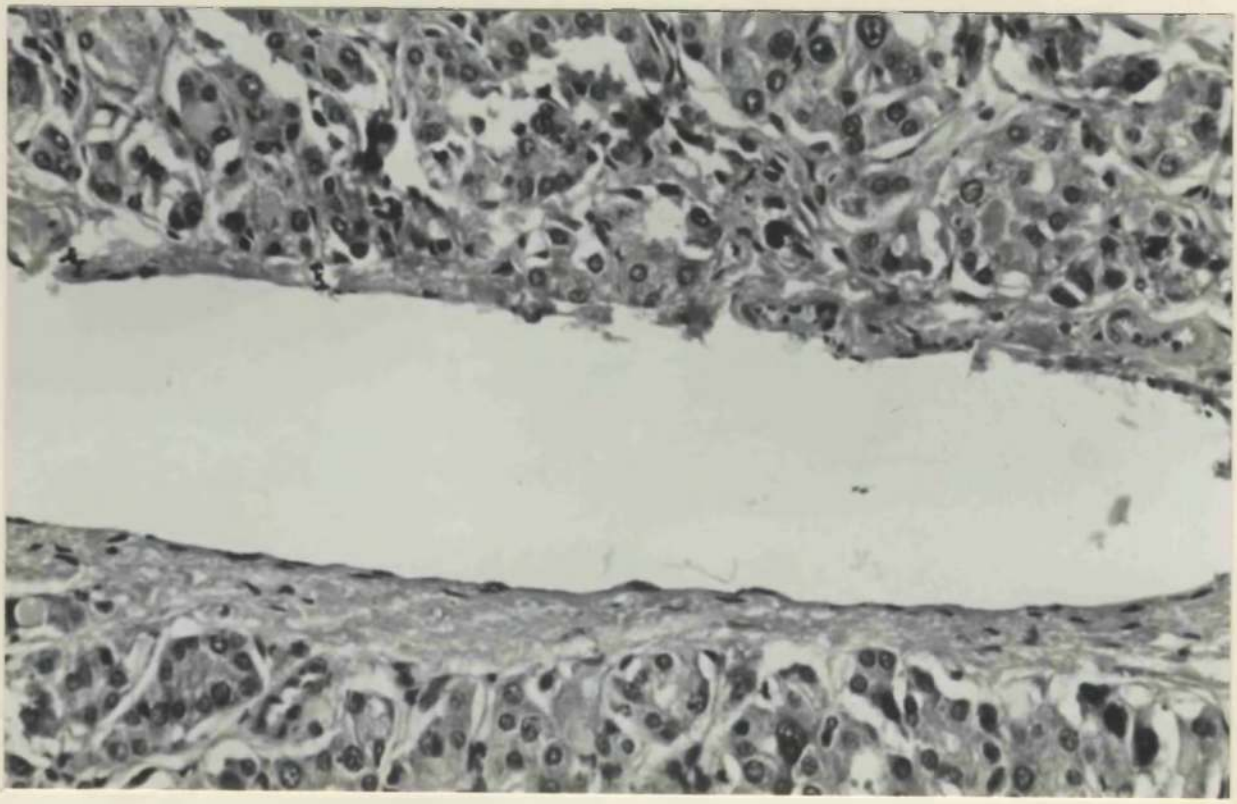

FIG.4.35

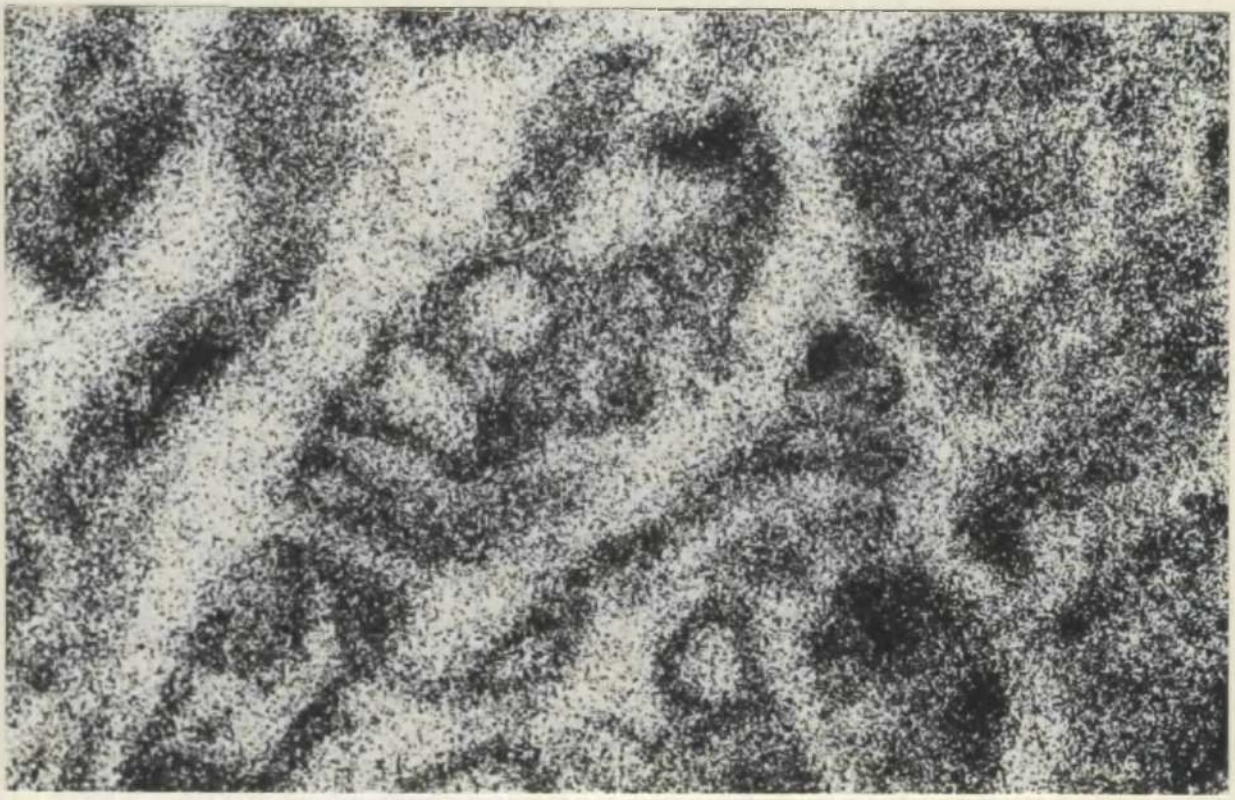

FIG.4.36

Fig. 4,37. - Case 3001/57. Dyshormonogenetic goitre - abnormal iodoprotein. Two inactive trabecular nodules. In contrast the internodular tissue shows diffuse epithelial utilisation of iodine-131 with more intense image over occasional microfollicles. Autoradiograph, Kodak AR10. X 20.

Fig. 4,38. - Case 4191/58. Dyshormonogenetic goitre - impaired dehalogenase activity. Iodine-131 is present in the follicular nodule: the trabecular nodule is inactive. A diffuse epithelial image is seen in the internodular tissue. There is very little colloid. Autoradiograph, Kodak AR10. X 15.

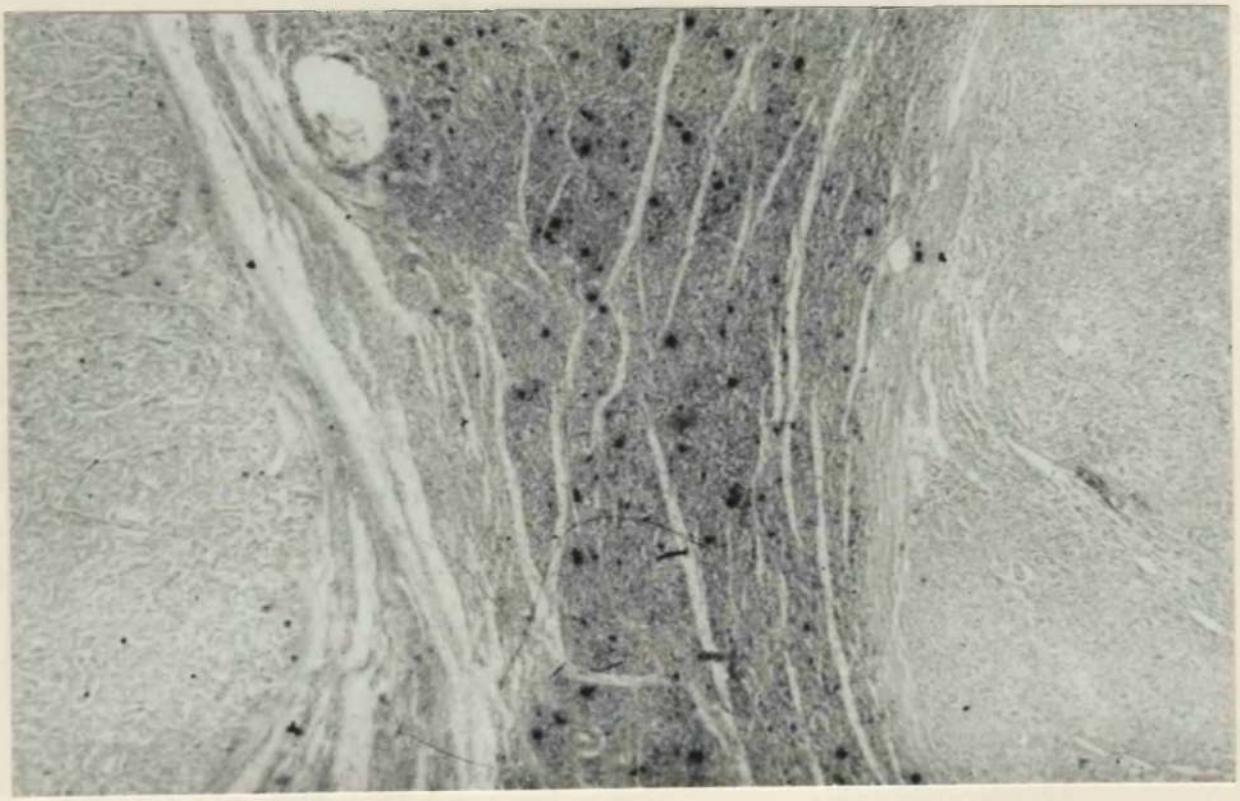

FIG. 4,37

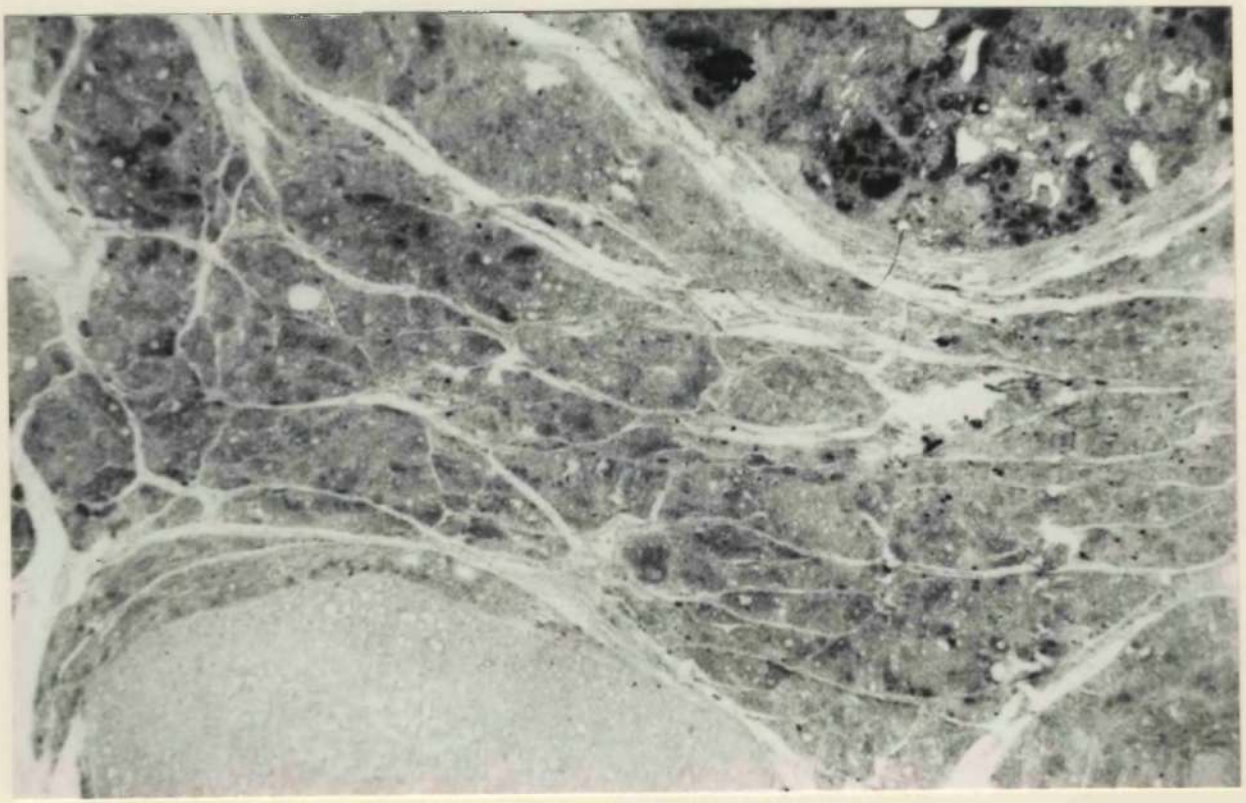

FIG. 4,38

Fig. 4,39. - Case 3001/57. Dyshormonogenetic goitre - abnormal iodoprotein. A small developing nodule is shown with iodine-131 in microfollicles. The microfollicular concentration of the isotope is greater than the epithelial concentration in the inter-nodular tissue. Autoradiograph, Kodak AR10. X 20.

Fig. 4,40. - Case 4114/55. Dyshormonogenetic goitre - abnormal iodo-protein. Autoradiograph shows image in compressed follicles between inactive lung-like nodule and trabecular-microfollicular nodule with microfollicular content of iodine-131. Kodak AR10. X 19.

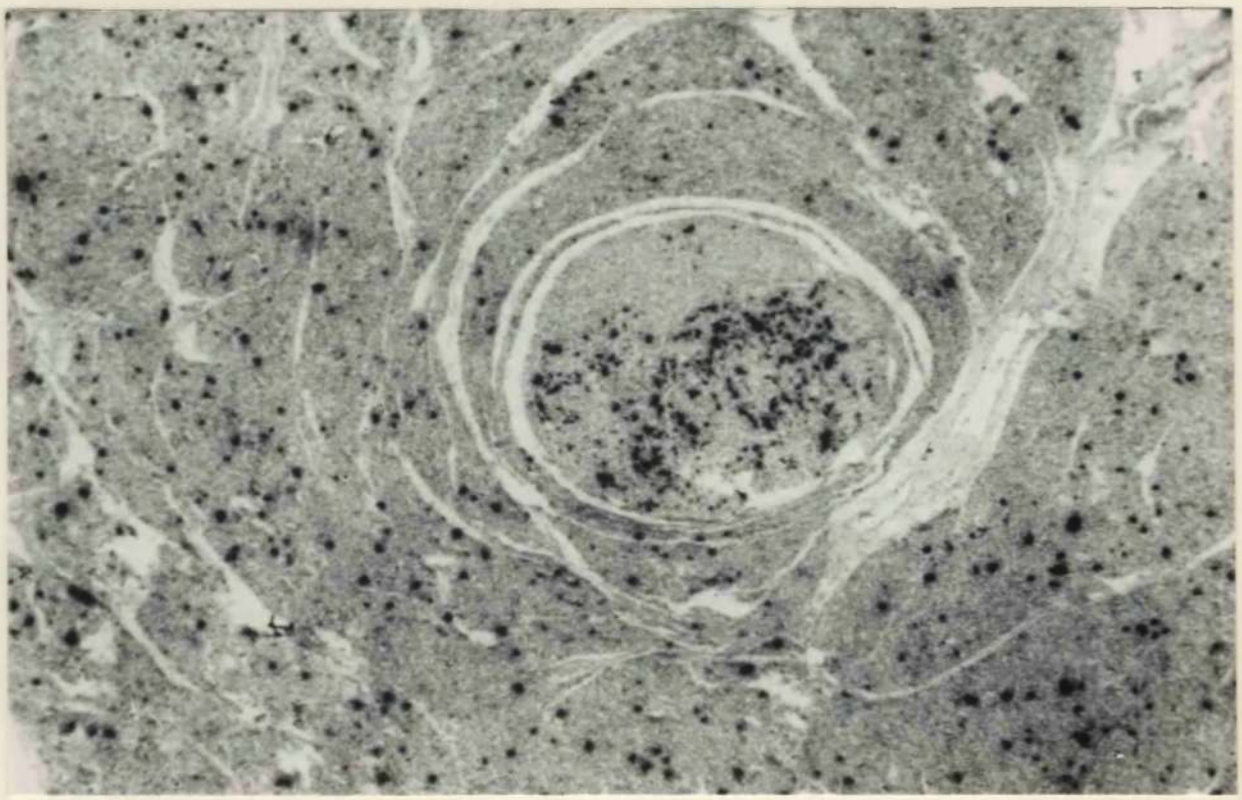

FIG. 4,39

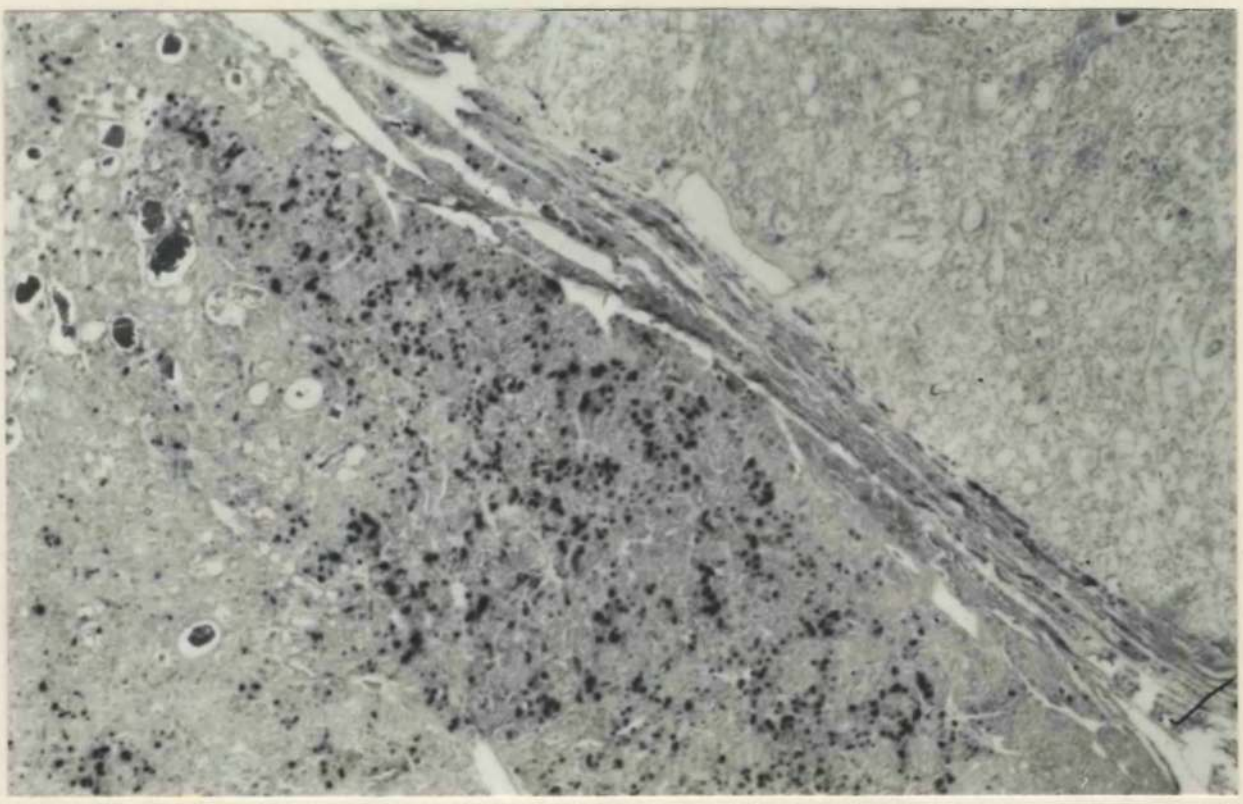

FIG. 4,40

Fig. 4,41. - Case 4114/55. Dyshormonogenetic goitre - abnormal iodo-protein. There is marked variation in uptake-pattern of iodine-131 in the nodule (top). Part of an inactive nodule is present (bottom). The internodular tissue shows epithelial utilisation of radio-iodine. Autoradiograph, Kodak AR10. X 19.

Fig. 4,42. - Case 1337/58. Dyshormonogenetic goitre - abnormal iodo-protein. Parts of 4 nodules shown. Iodine-131 distribution-patterns vary from nodule to nodule and within a nodule. There is a light diffuse epithelial image in the internodular tissue and only occasional small follicles showing more intense colloid-image. Autoradiograph, Kodak AR10. X 13.

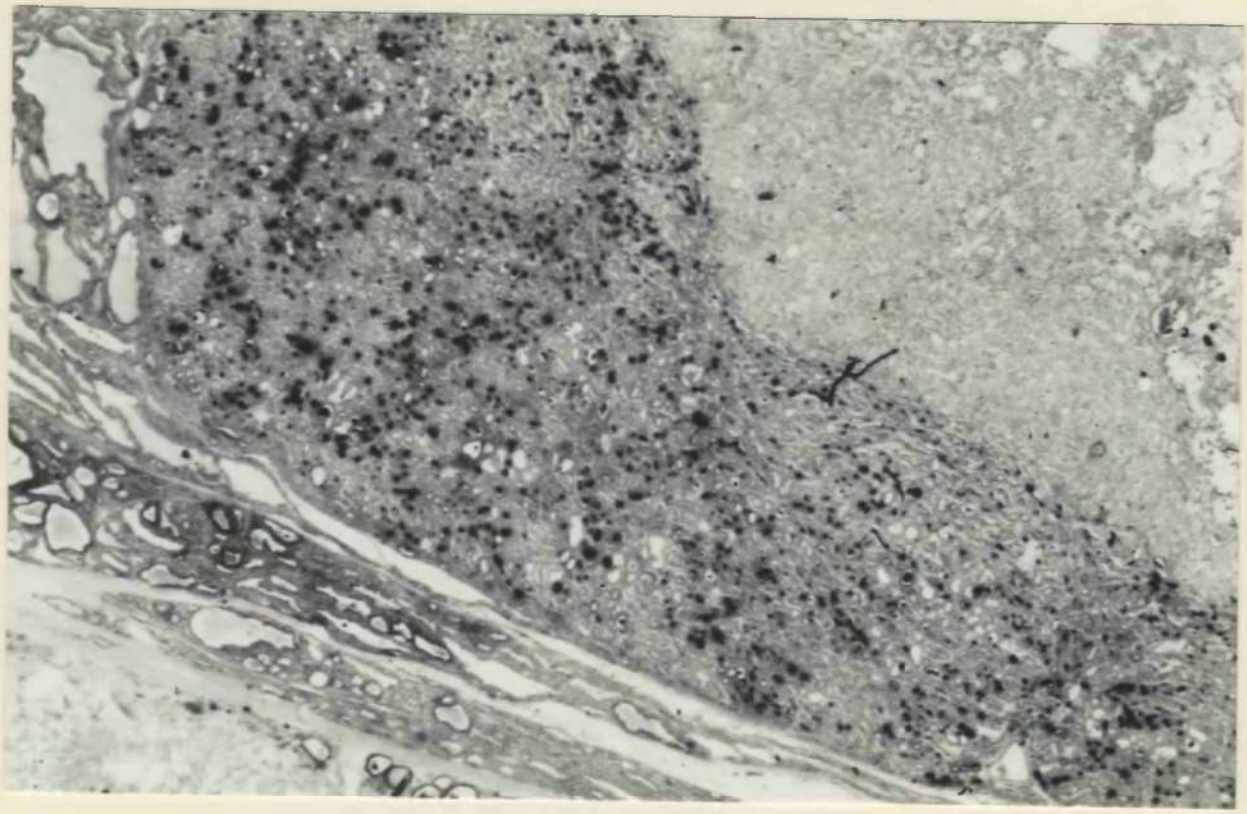

FIG.4.41

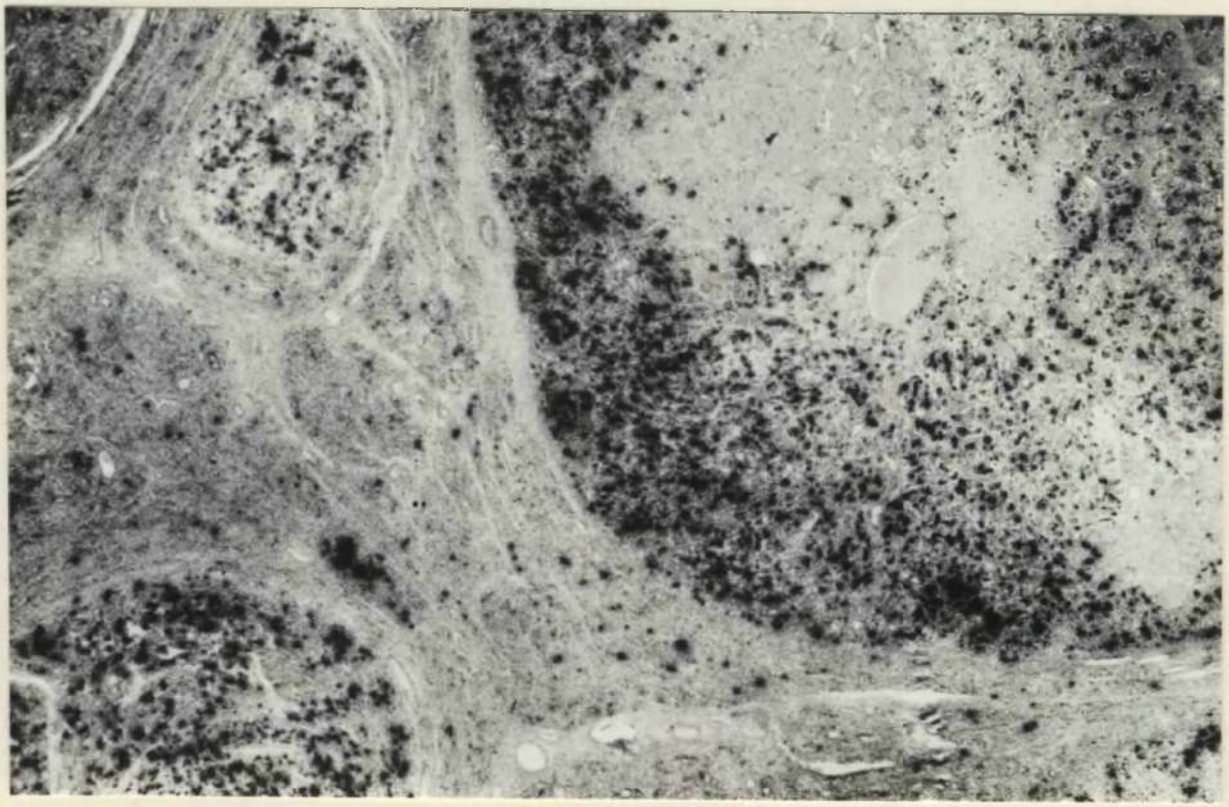

FIG.4.42

Fig. 4,43. - Case 4114/55. Dyshormonogenetic goitre - abnormal iodoprotein. Autoradiograph shows epithelial utilisation of iodine-131 in medium size follicles containing labelled colloid. Kodak AR10. X 230.

Fig. 4,44. - Case 2727/55. Dyshormonogenetic goitre - abnormal iodoprotein. Autoradiograph shows iodine-131 in epithelium, a ring-image, and a more diffuse image over colloid in the follicle. Kodak AR10. X 230.

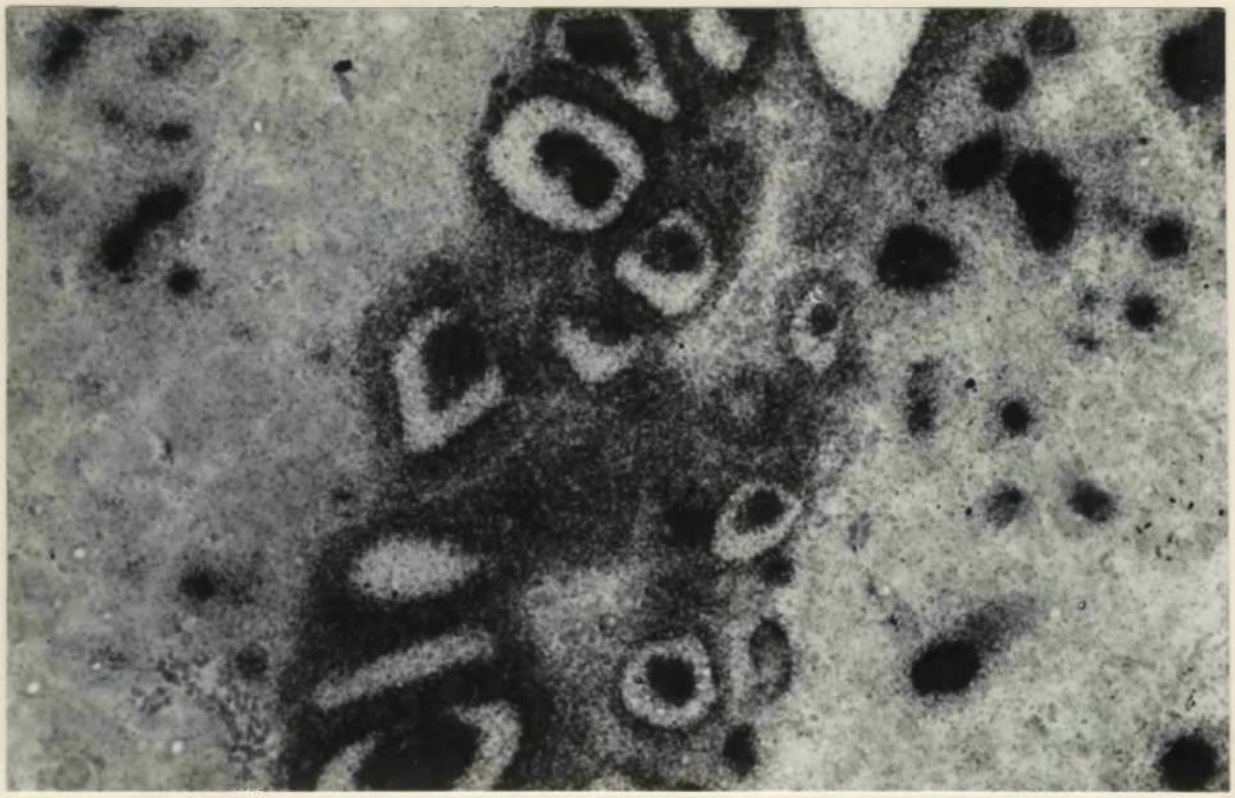

FIG.4,43

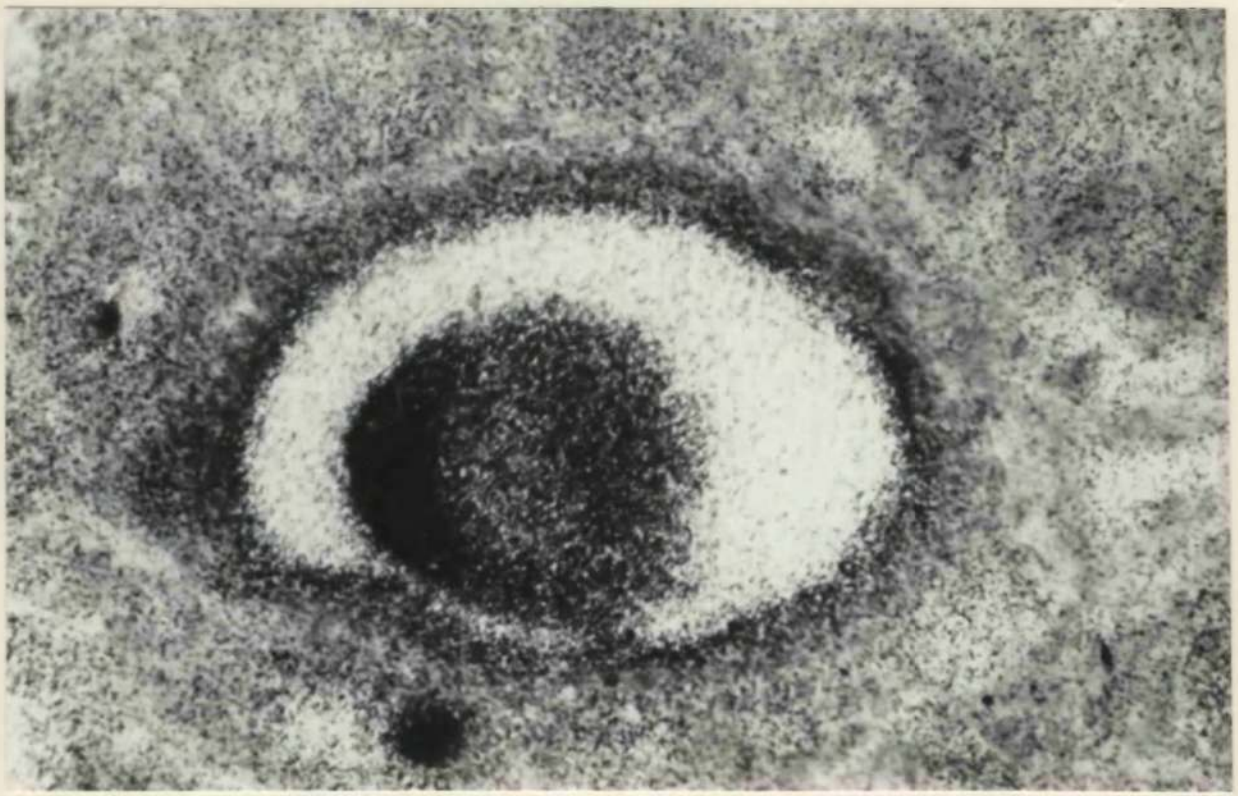

FIG.4,44

Fig. 4,45. - Case 3001/57. Dyshormonogenetic goitre - abnormal iodoprotein. Epithelial cells and a small amount of colloid are present in the lumen of a vessel. H. and E. X 250.

Fig. 4,46. - Case 3001/57. Corresponding autoradiograph shows that the intravascular colloid contains iodine-131. Kodak AR50. X 250.

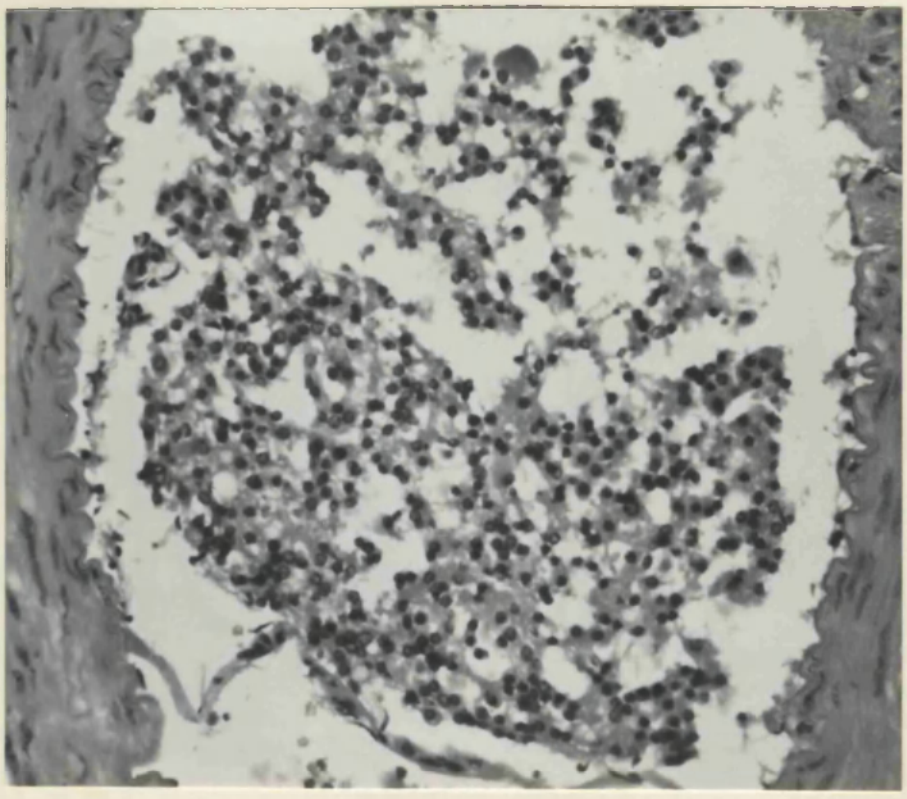

FIG. 4,45

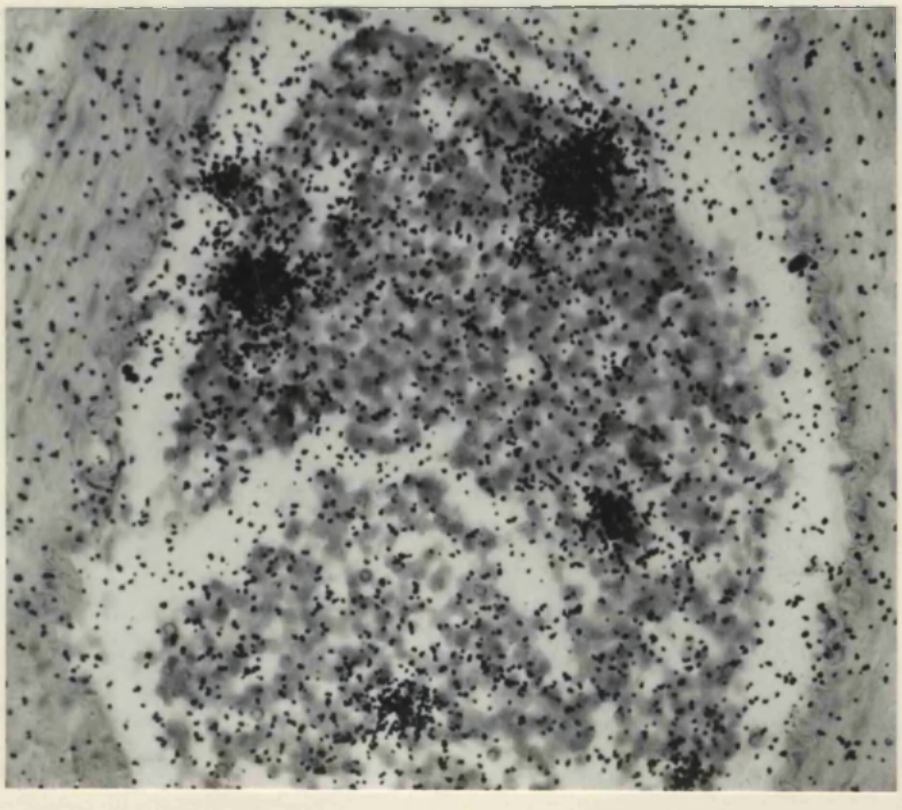

FIG. 4,46

Fig. 4,47. - Case 3467/58. Dyshormonogenetic goitre - impaired dehalogenase activity. In the malignant areas of the gland there is only scanty evidence of utilisation of iodine-131 in colloid. Autoradiograph, Kodak AR50. X 140.

Fig. 4,48. - Case 4505/59. Graves' disease. All colloid present contains iodine-131. Autoradiograph, Kodak AR50. X 95.

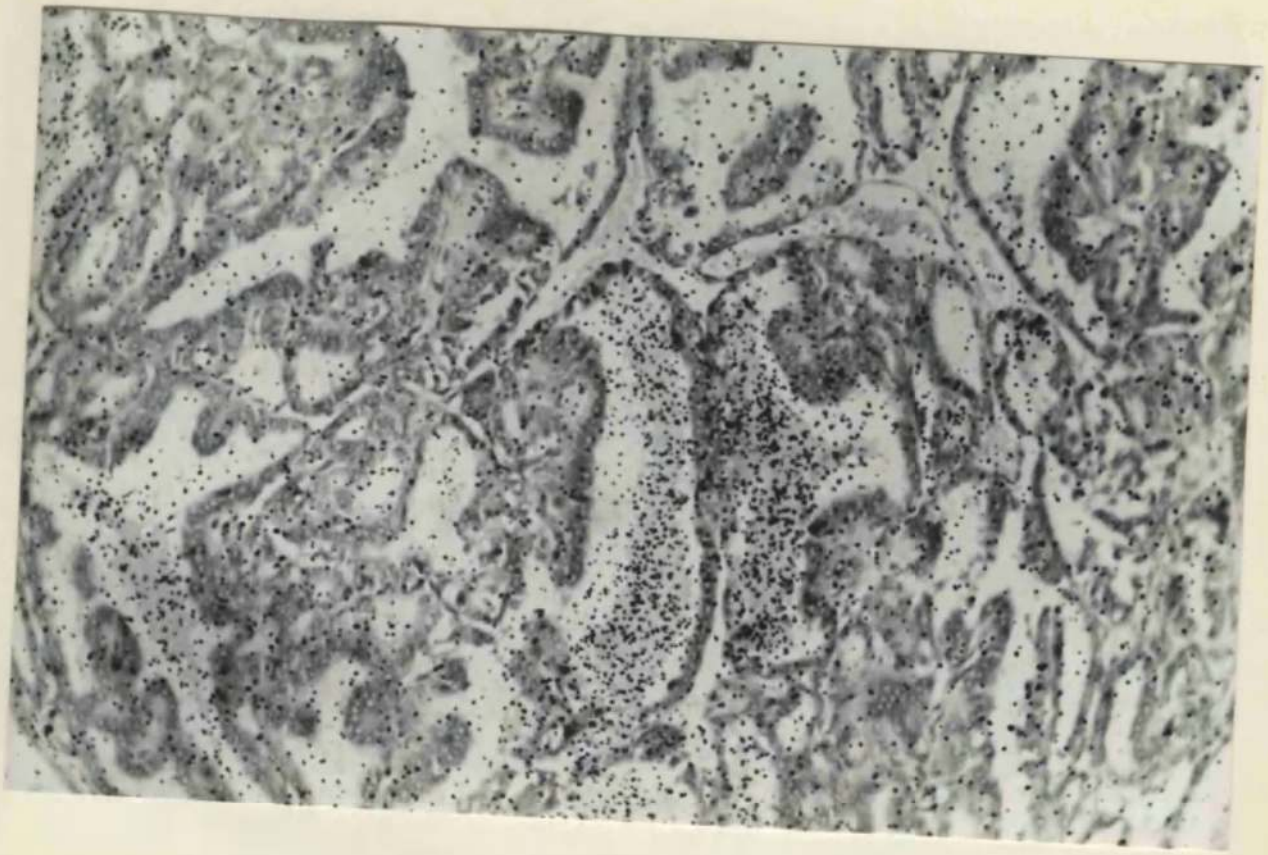

FIG.4,47

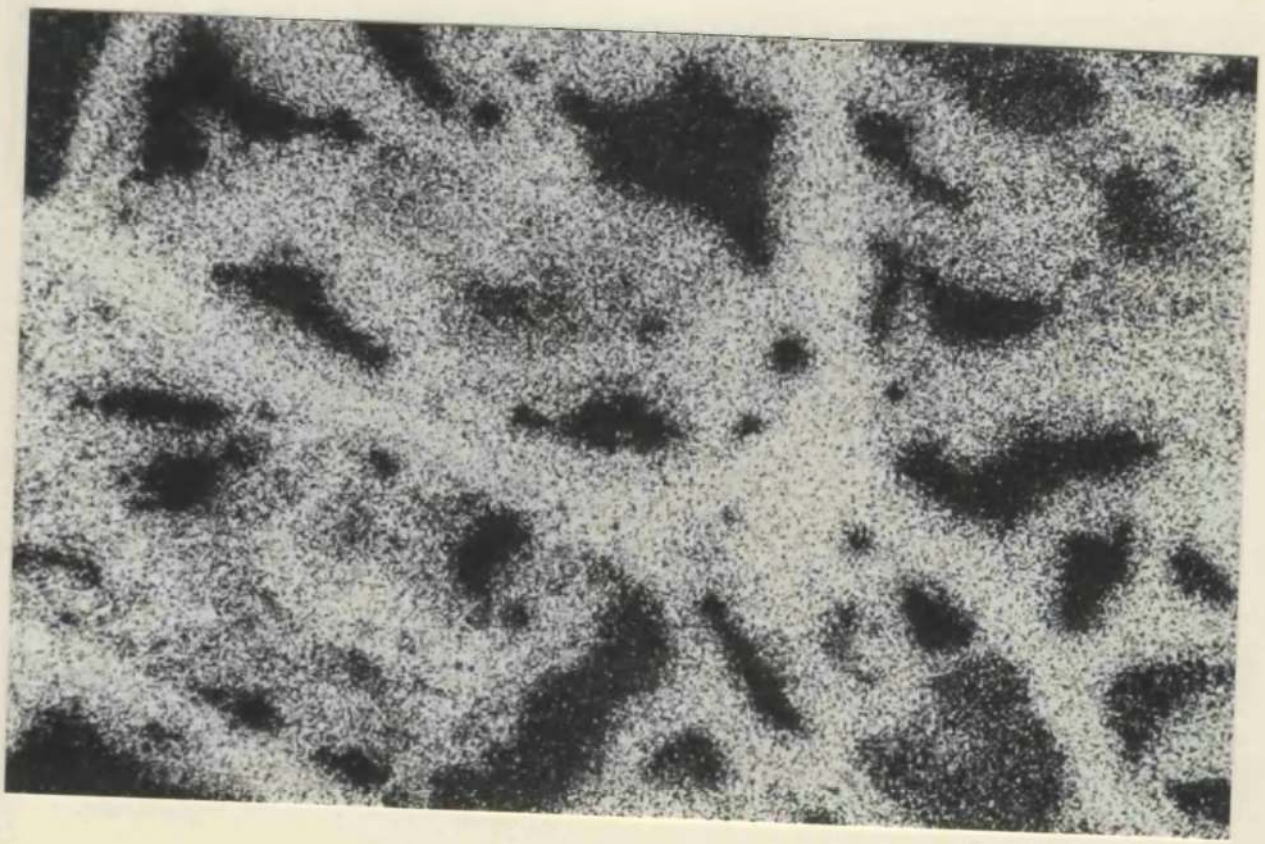

FIG.4,48

Fig. 4,49. - Case P.M. 67/59. Graves' disease. Five days after a therapeutic dose of iodine-131 there is evidence of epithelial utilisation of the isotope. Colloid present shows a more intense image. Autoradiograph, Kodak AR10. X 230.

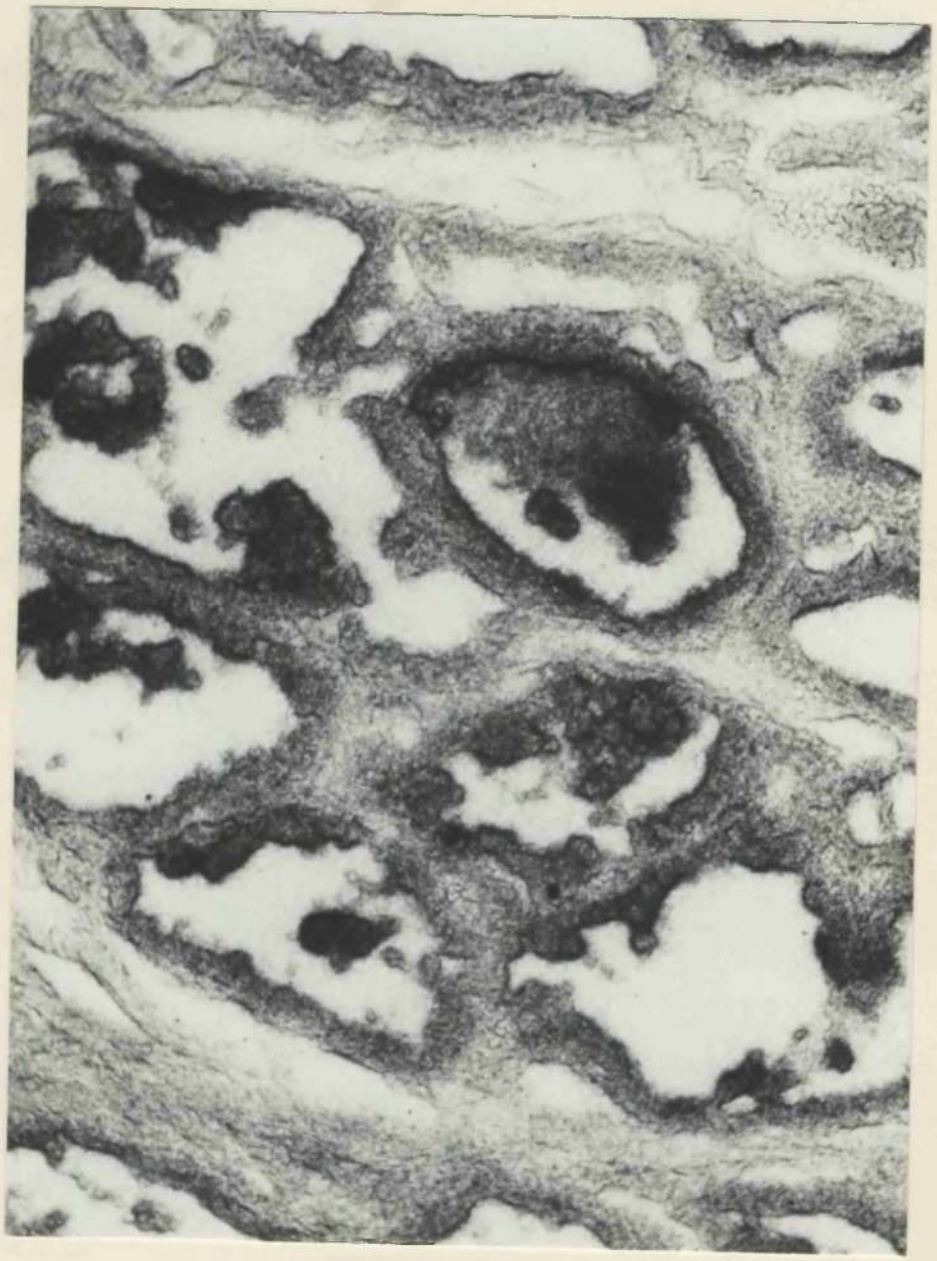

FIG.4,49

Fig. 4,50. - Case 1778/56. Hashimoto's thyroiditis. Groups of follicles in which the epithelium does not show Askanazy-change. H. and E. X 95.

Fig. 4,51. - Case 1778/56. Corresponding autoradiograph shows intense images over colloid in the follicles. Kodak AR10. X 95.

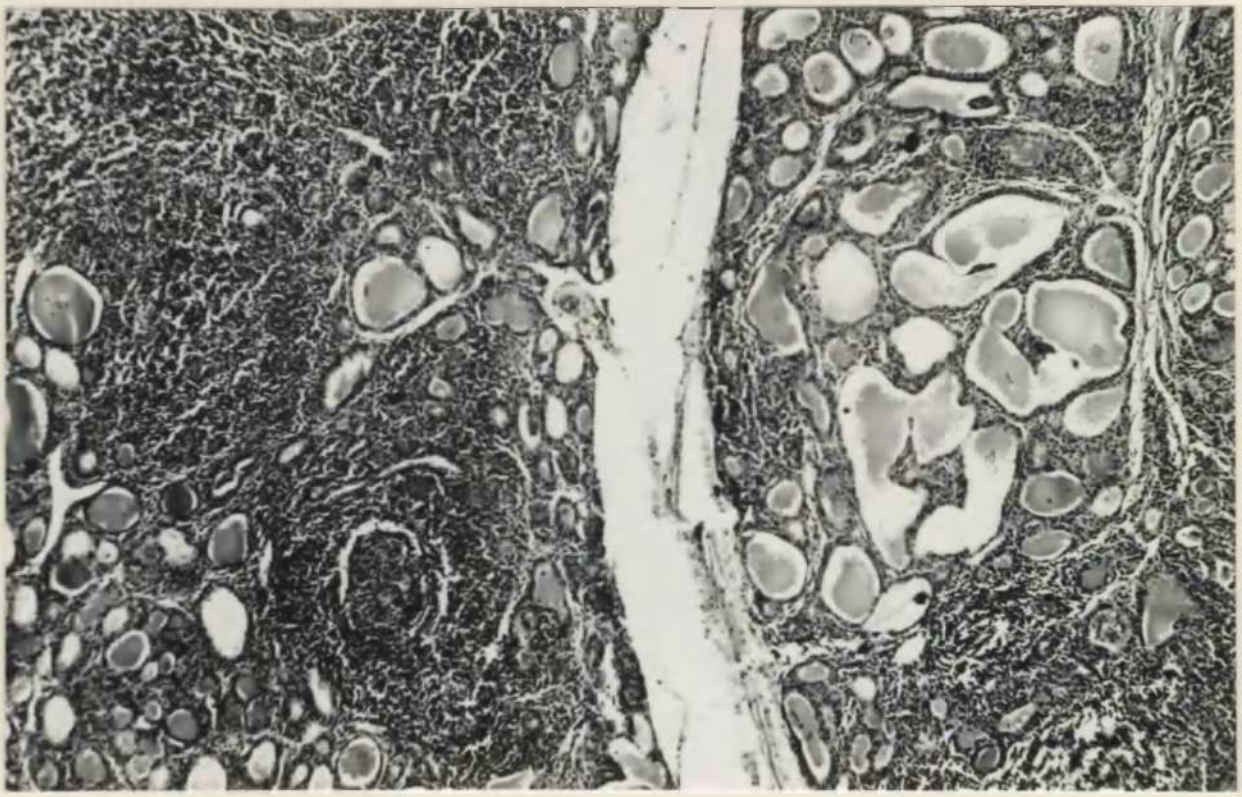

FIG.4,50

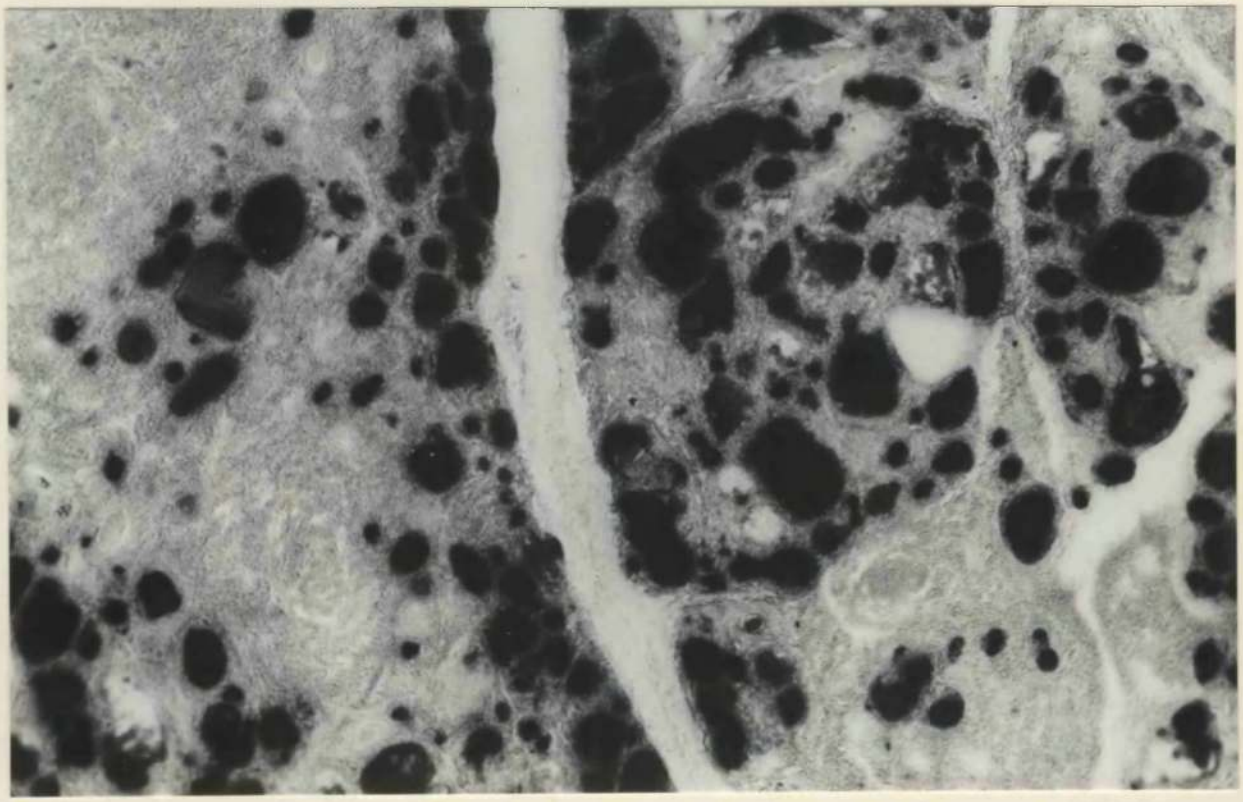

FIG.4,51

Fig. 4,52. - Case 1778/56. Hashimoto's thyroiditis. Askanazy-epithelium is present to the right, more normal epithelium to the left. H. and E. X 95.

Fig. 4,53. - Case 1778/56. Corresponding autoradiograph shows image over follicles lined by normal epithelium (left) is greater than image over follicles lined by Askanazy epithelium (right). Kodak AR10. X 95.

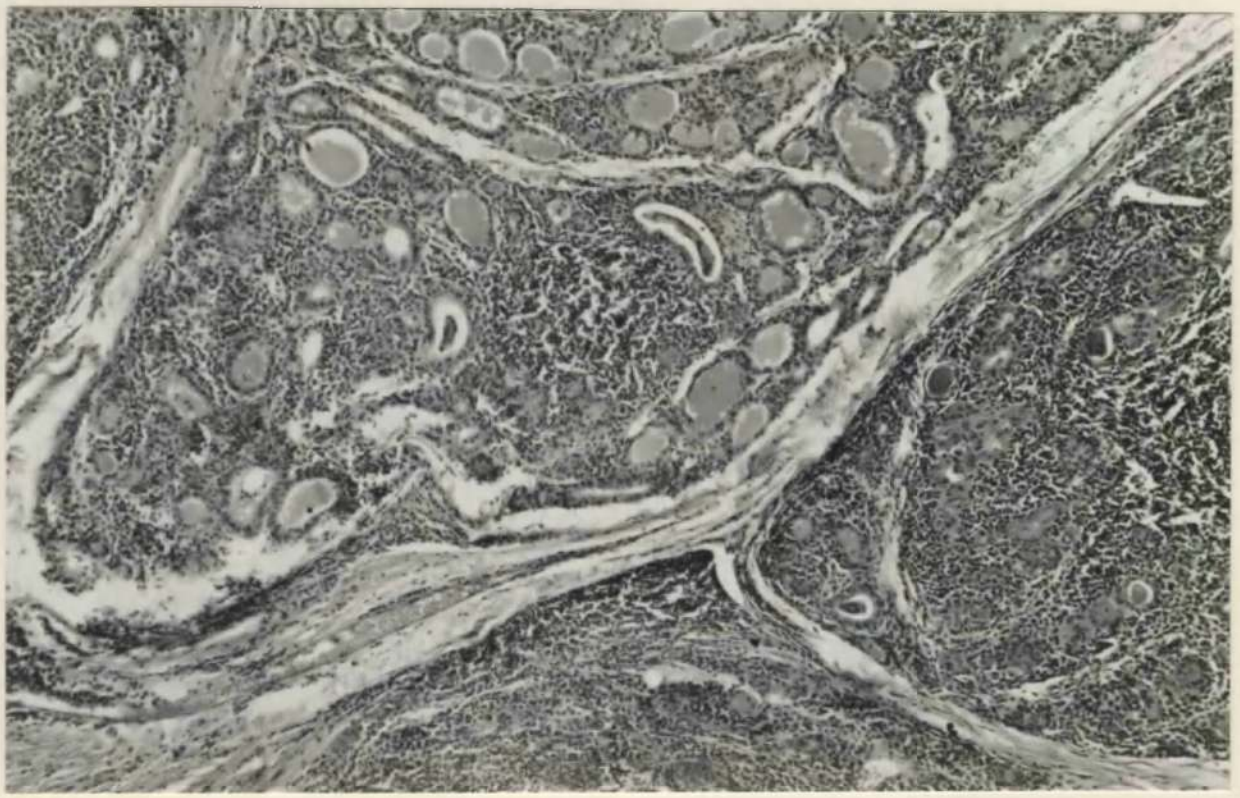

FIG.4,52

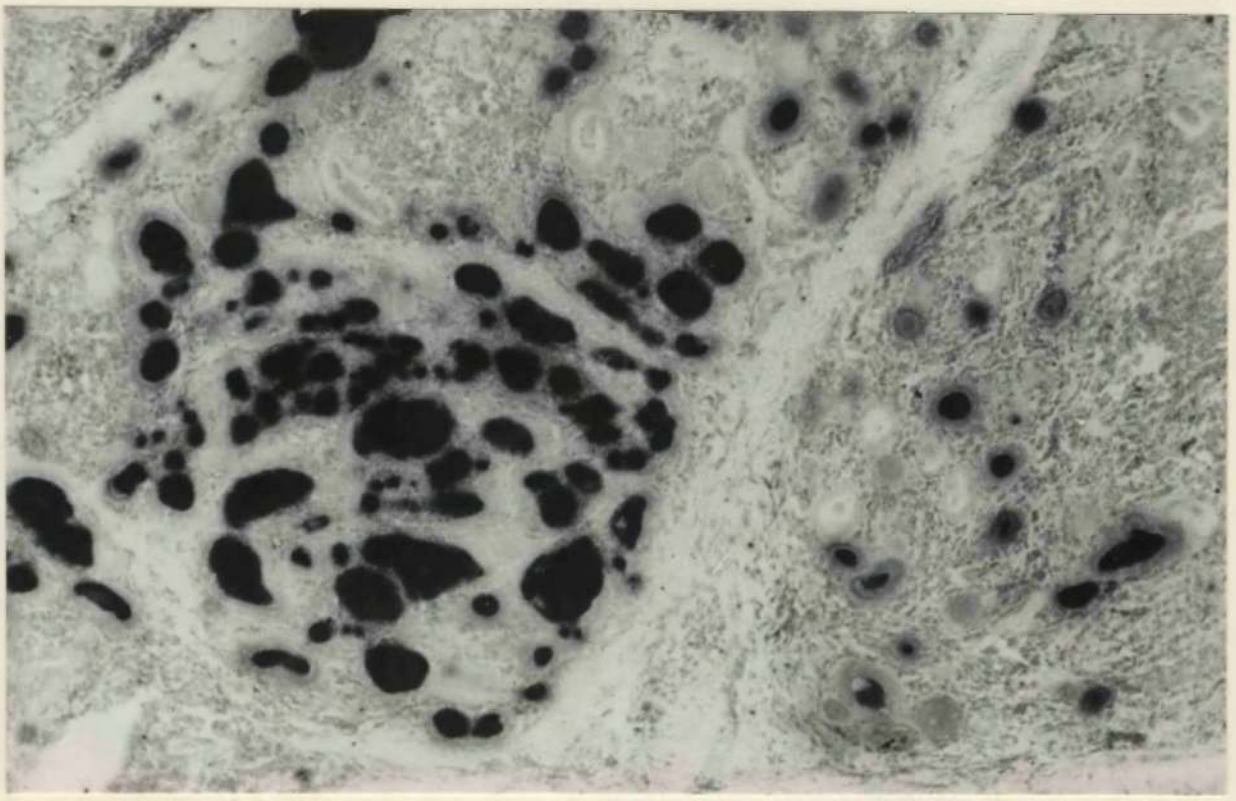

FIG.4,53

Fig. 4,54. - Case 2262/57. Hashimoto's thyroiditis. Lobule showing degenerate follicles, a light plasma cell infiltrate, and, on the periphery extrafollicular colloid. H. and E. X 75.

Fig. 4,55. - Case 2262/57. Corresponding autoradiograph shows iodine-131 labelled extrafollicular colloid on the periphery of the lobule. Kodak AR50. X 75.

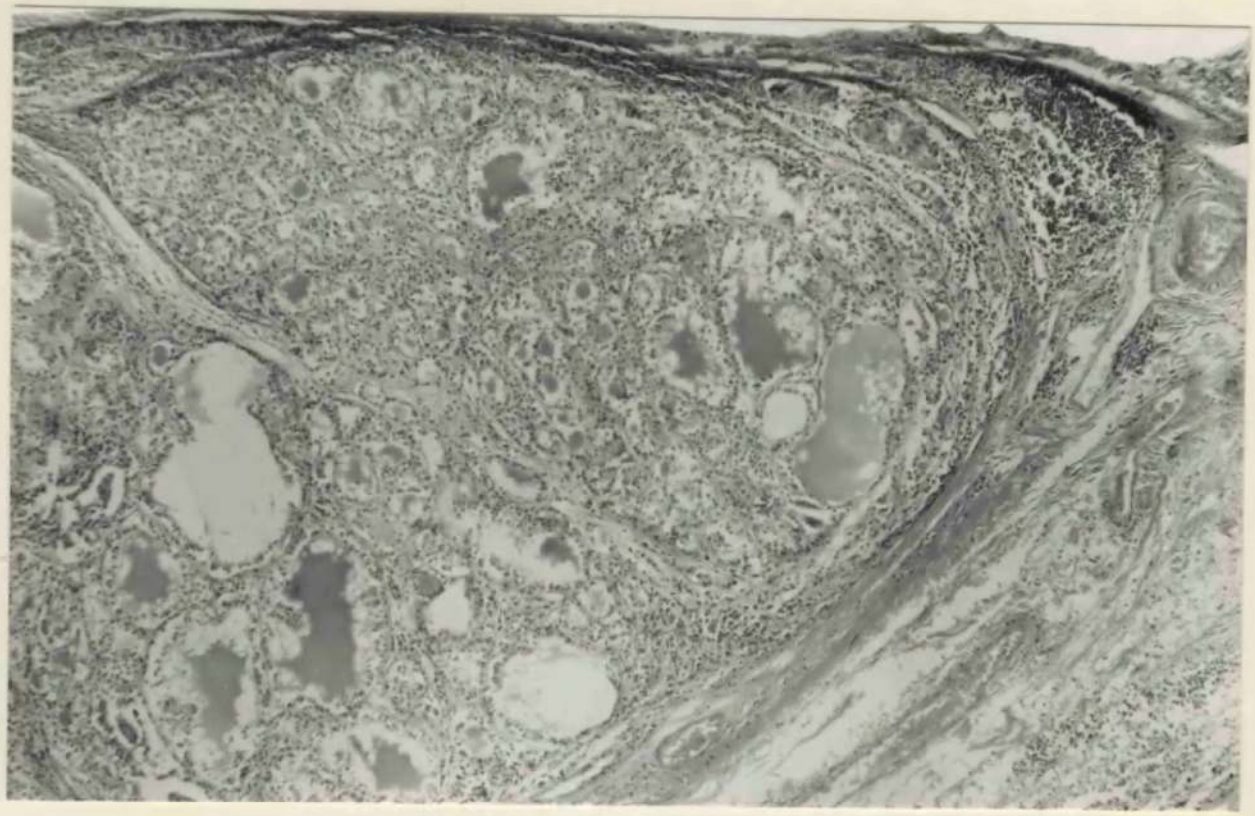

FIG.4,54

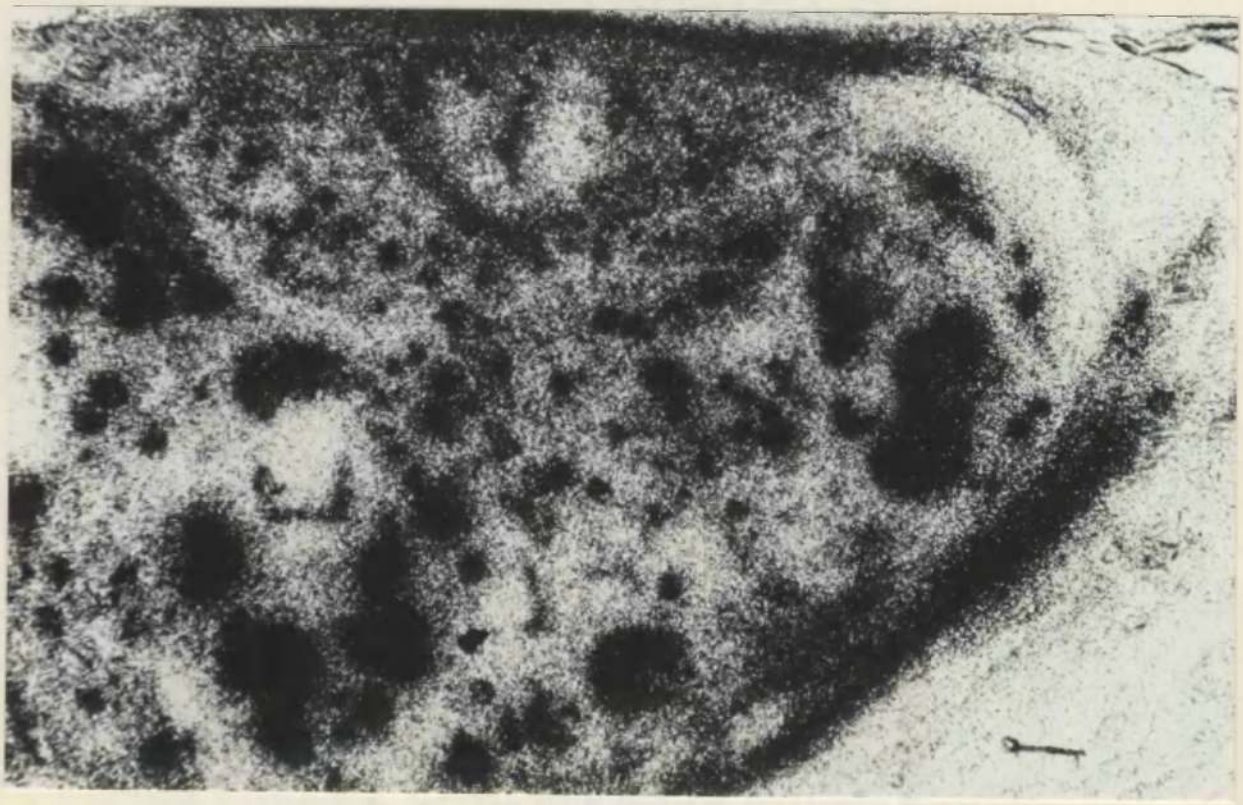

FIG.4,55

Fig. 4,56. - Case 586/57. Hashimoto's thyroiditis. Lobule showing extensive Askanazy-change in epithelium lining small follicles. H. and E. X 75.

Fig. 4,57. - Case 586/57. Corresponding autoradiograph shows the small follicles lined by Askanazy-cells contain bound iodine-131, indicative of epithelial metabolic activity. Kodak AR10. X 75.

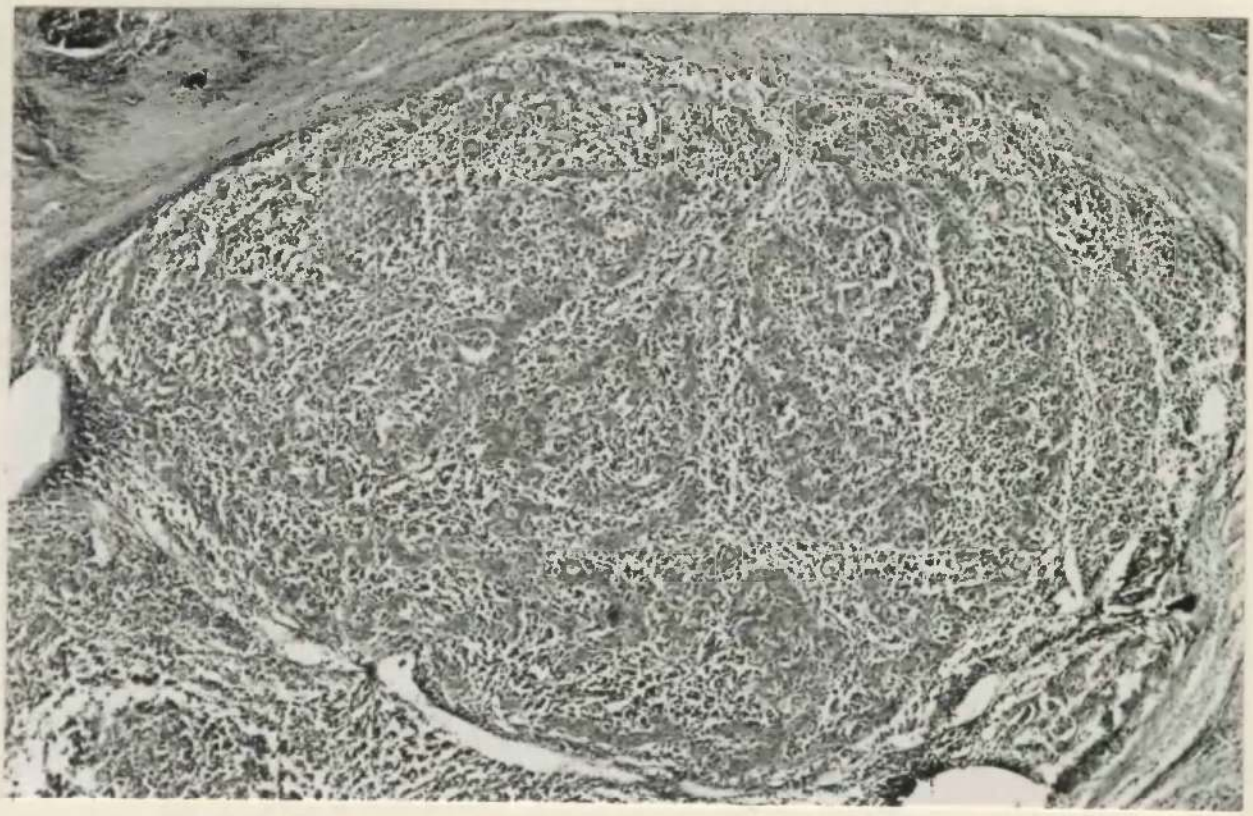

FIG. 4,56

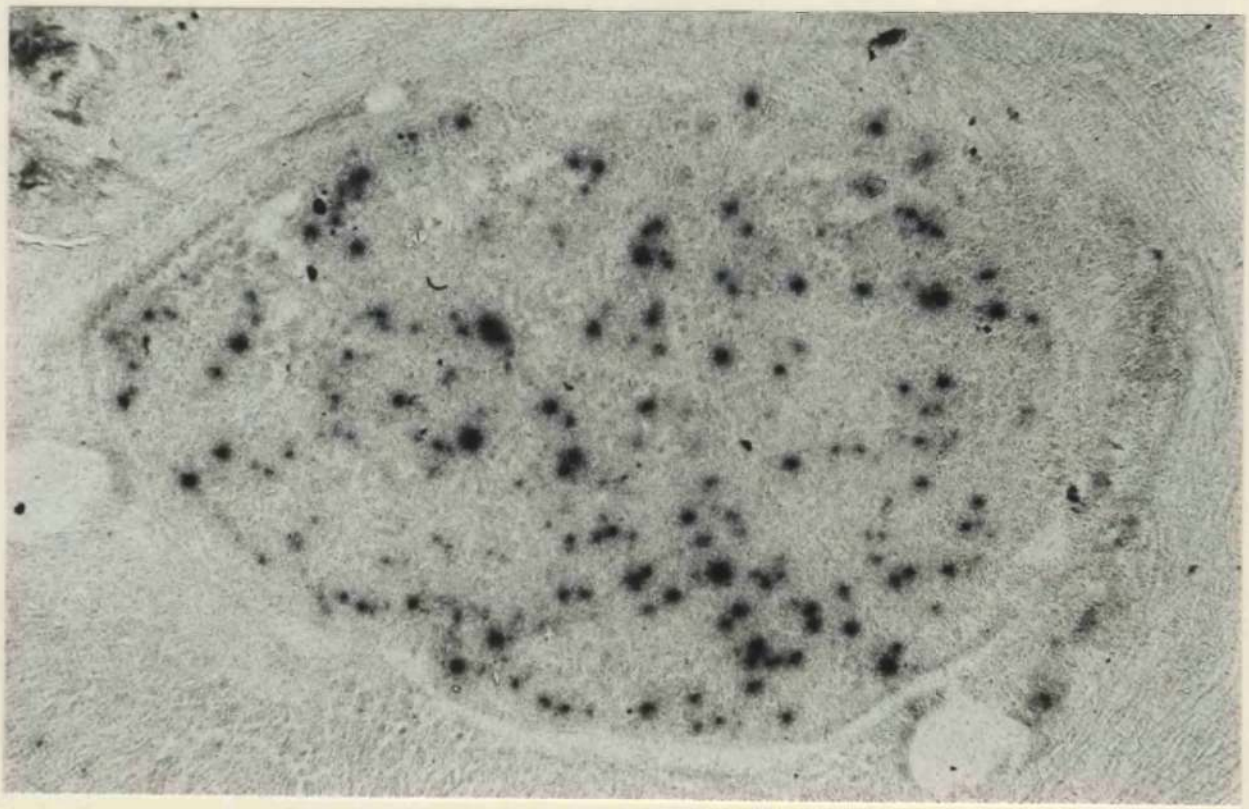

FIG. 4,57

Fig. 4,58. - Case 3849/55. Hashimoto's thyroiditis. The Askanazy-type epithelium occurs in follicular and non-follicular forms. H. and E. X 380.

Fig. 4,59. - Case 3849/55. Corresponding autoradiograph. Iodine-131 is present in follicles lined by Askanazy-cells. Clumps of Askanazy-cells not in follicular form show no evidence of utilisation of radio-iodine. Kodak AR10. X 380.

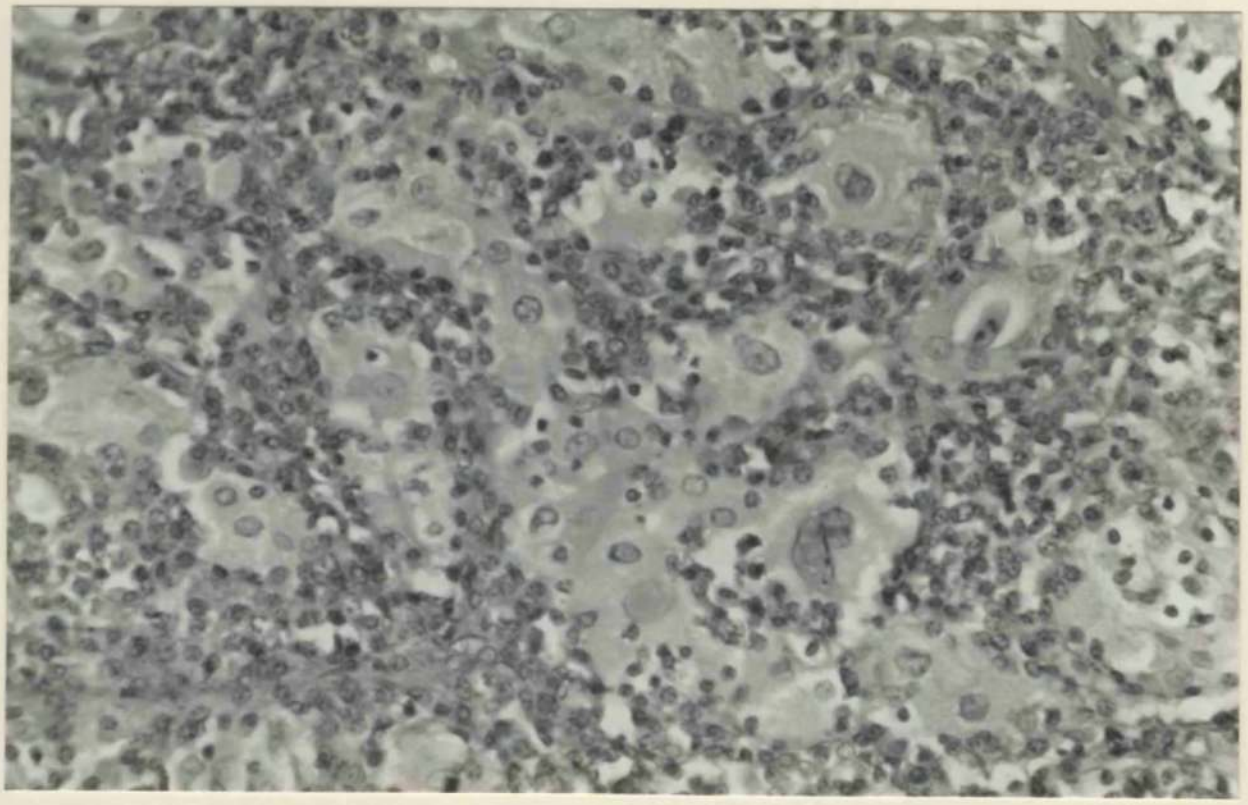

FIG.4,58

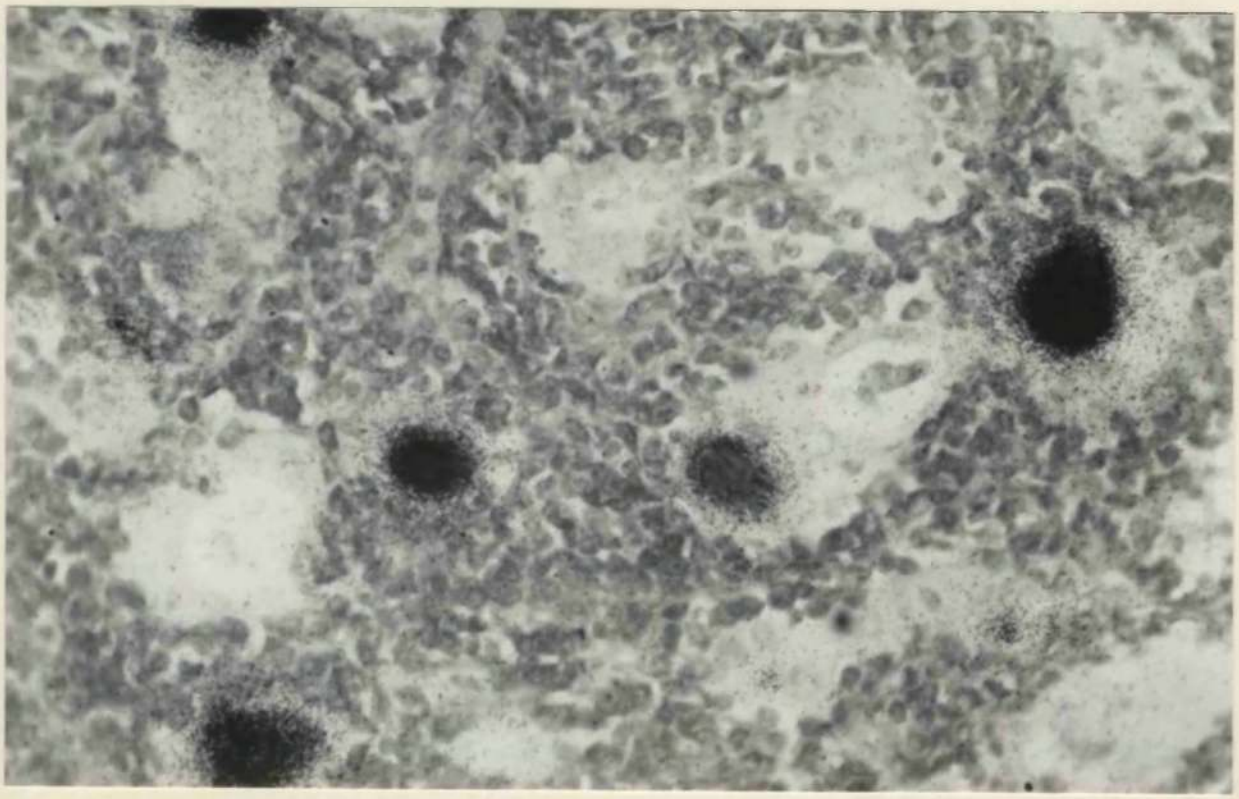

FIG.4,59

P A R T V

Tables 5,1 - 5,4

TABLE 5,1

PHAEOCHROMOCYTOMATA CLINICAL

Case	Age	Sex	Clinical*	Total urinary catechol amines (µg. per 24 hr)	Size	Classification
1 Necropsy	56	F	Sustained hypertension Glycosuria Rogitine±	—	12x10x10cm. 388 g.	Malignant secondaries in lymph glands, liver and bone
2 Necropsy	80	M	Not suspected	—	1.25 cm. in diameter	Benign
3 Necropsy	37	F	Not suspected	—	8 cm. in diameter	Benign
4 Operation	56	F	Paroxysmal hypertension Sweating Rogitine+	1560	8 cm. in diameter	Benign
5 Operation	32	F	Paroxysmal hypertension Glycosuria Rogitine+	1200-4500	5 cm. in diameter	Benign
6 Necropsy	56	F	Sustained hypertension Glycosuria	—	7 x 5 x 4 cm. 120 g.	Benign

*Rogitine+ indicates a significant decrease in blood pressure; Rogitine± indicates an equivocal result.

TABLE 5.2

CATECHOL AMINE ASSAYS ON TUMOUR TISSUE

Case	Adrenaline mg. per g.	Noradrenaline mg. per g.	Ratio adrenaline/ noradrenaline
1 Malignant	0.19	1.97	0.1
2* Benign	0.32	0.2	1.6
3 Benign	1.88	0.07	27.0
4 Benign	1.73	0.33	5.2
5 Benign	1.71	1.64	1.0
6 Benign	Nil	3.75	0.0

*Assay by Professor U.S. von Euler

TABLE 5.3

CATECHOL AMINE ASSAYS ON CASE 1 TUMOUR TISSUE

Catechol amines	Primary tumour	Liver metastases	Primary* after 90 days	Primary* after 300 days
Adrenaline (mg. per g.)	0.19	Nil	0.01	Nil
Noradrenaline (mg. per g.)	1.97	2.1	0.7	0.55

* 10 g. minced tumour tissue kept in 0.01 N HCl at -10°C.

TABLE 5.4

PHAEOCHROMOCYTOMATA: HISTOCHEMISTRY

Case	Control of pH	Histochemical methods			
		Chromaffin	Radio-chromate	Iodate	Radio-iodate
1	No	-ve	—	—	—
2	No	-ve	—	—	—
3	No	-ve	—	-ve	-ve
4	No	Weak +ve	—	-ve	?+ve*
5	Yes	+ve	+ve	+ve	?+ve*
6	Yes	+ve	—	—	—

*See discussion on iodate reaction

P A R T V

Figs. 5,1 - 5,7

Fig. 5,1. - Case 1. The malignant phaeochromocytoma (kidney to right, aorta below).

Fig. 5,2. - Case 1. The primary tumour is composed of mature phaeochromocytes grouped in an irregular alveolar pattern. Masson. X 135.

FIG. 5,1

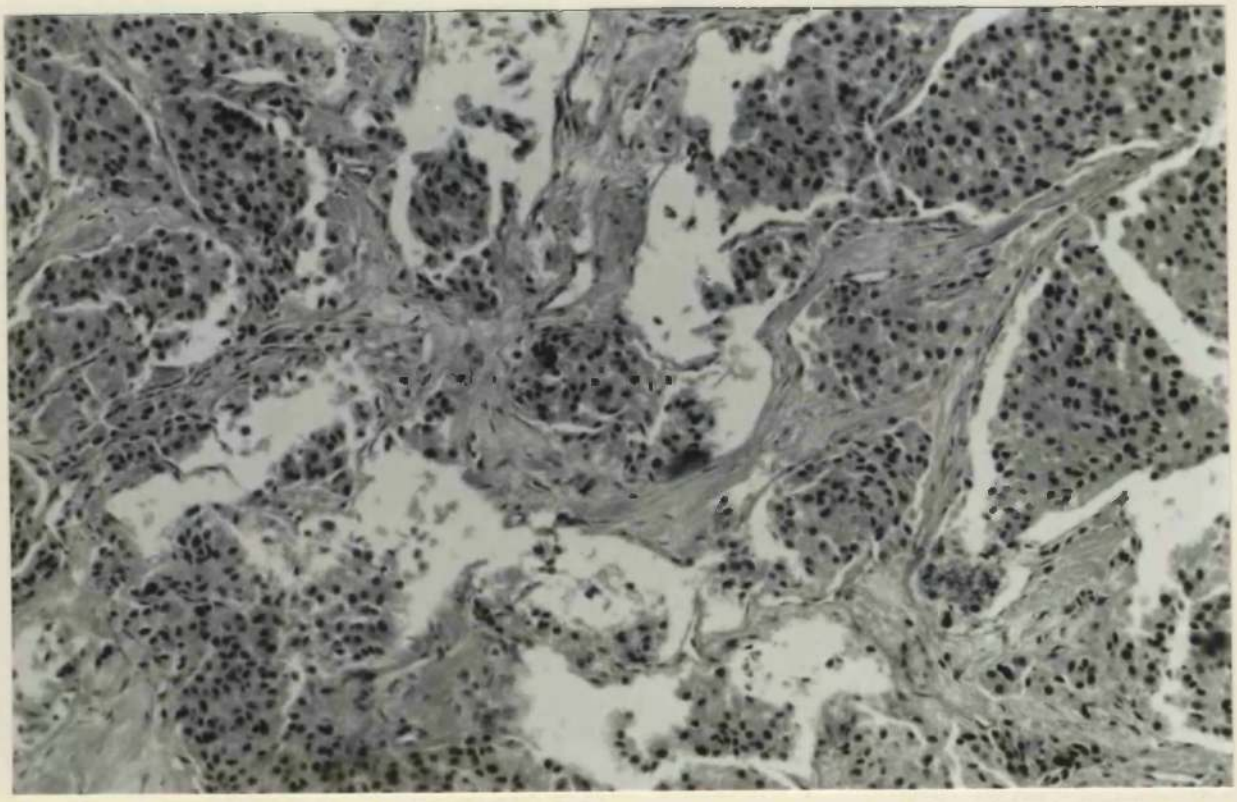

FIG. 5,2

Fig. 5,3. - Case 1. There is slight nuclear and cellular pleomorphism in the primary tumour. Mitotic figures are scanty. Masson. X 350.

Fig. 5,4. - Case 1. Clumps of tumour cells are present in hepatic vessels. Masson. X 400.

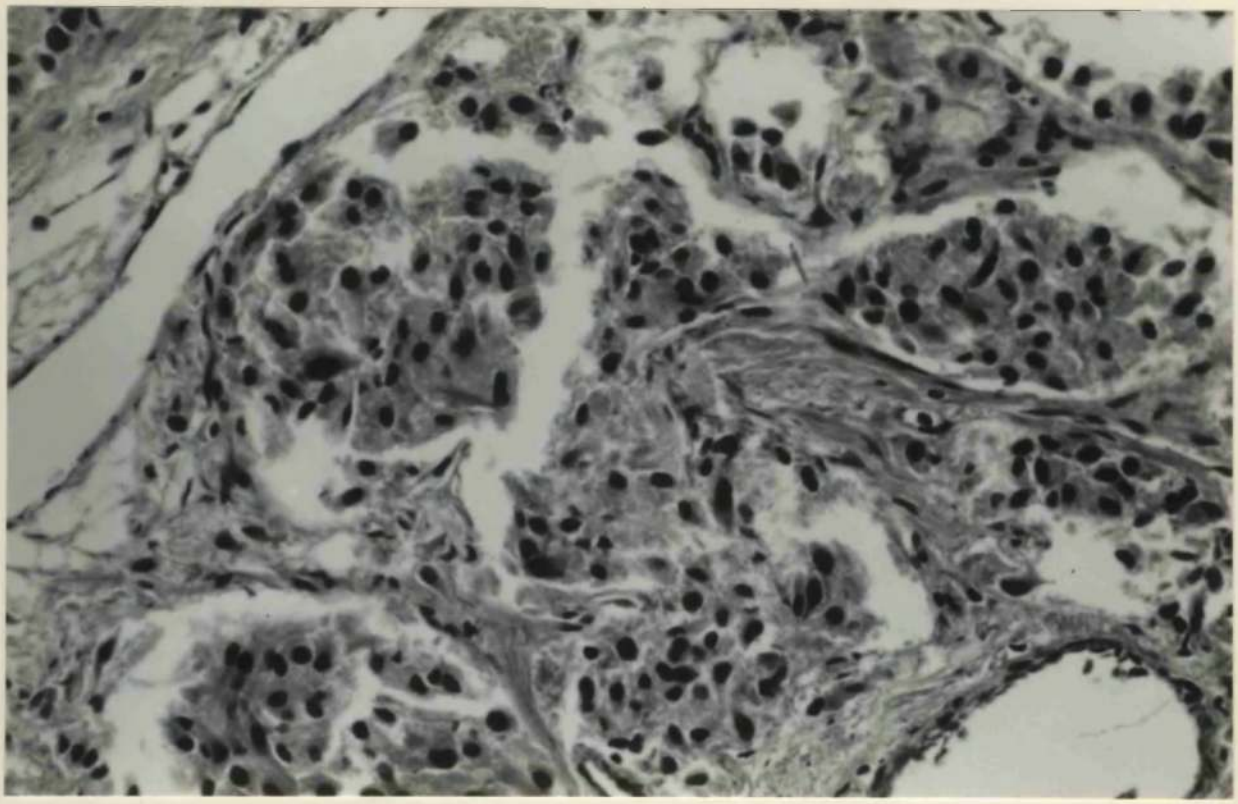

FIG. 5,3

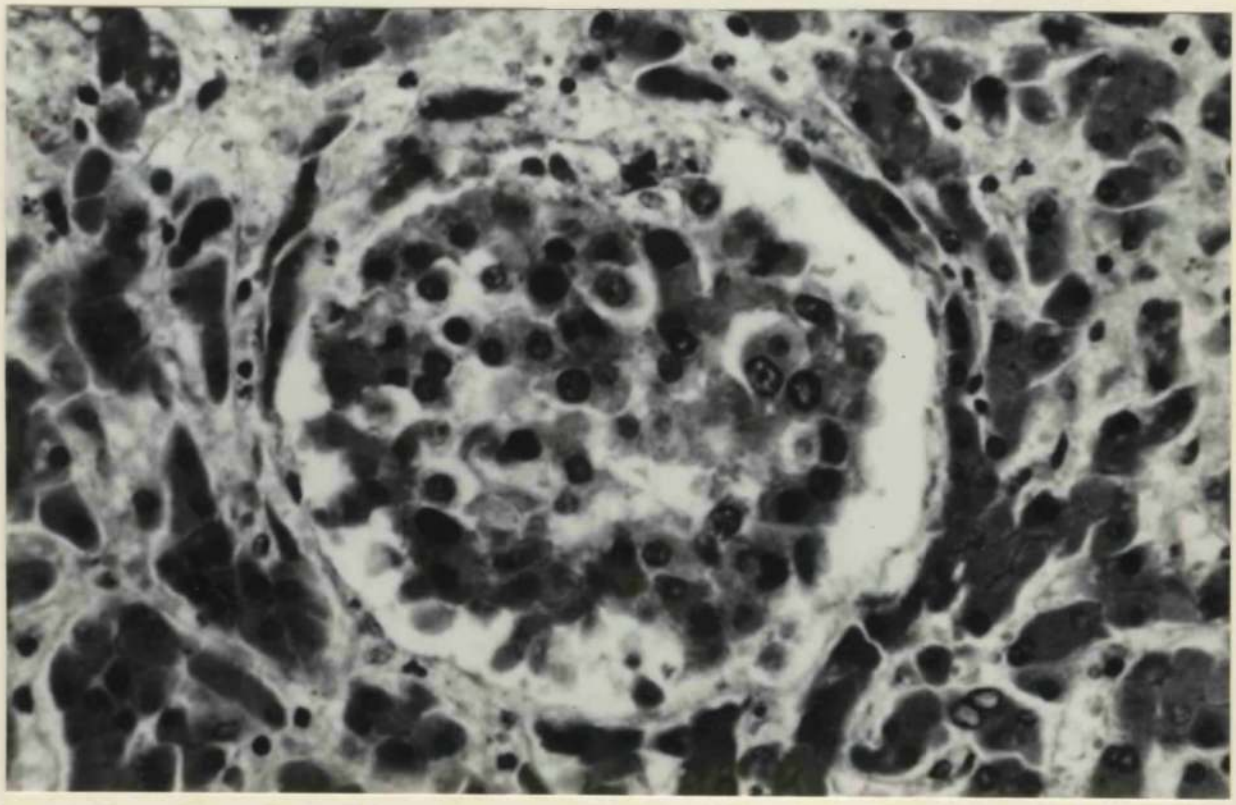

FIG. 5,4

Fig. 5,5. - Case 1. Secondary deposit in ilium. The structure is similar to that of the primary tumour and of the hepatic metastases. Haematoxylin and eosin. X 260.

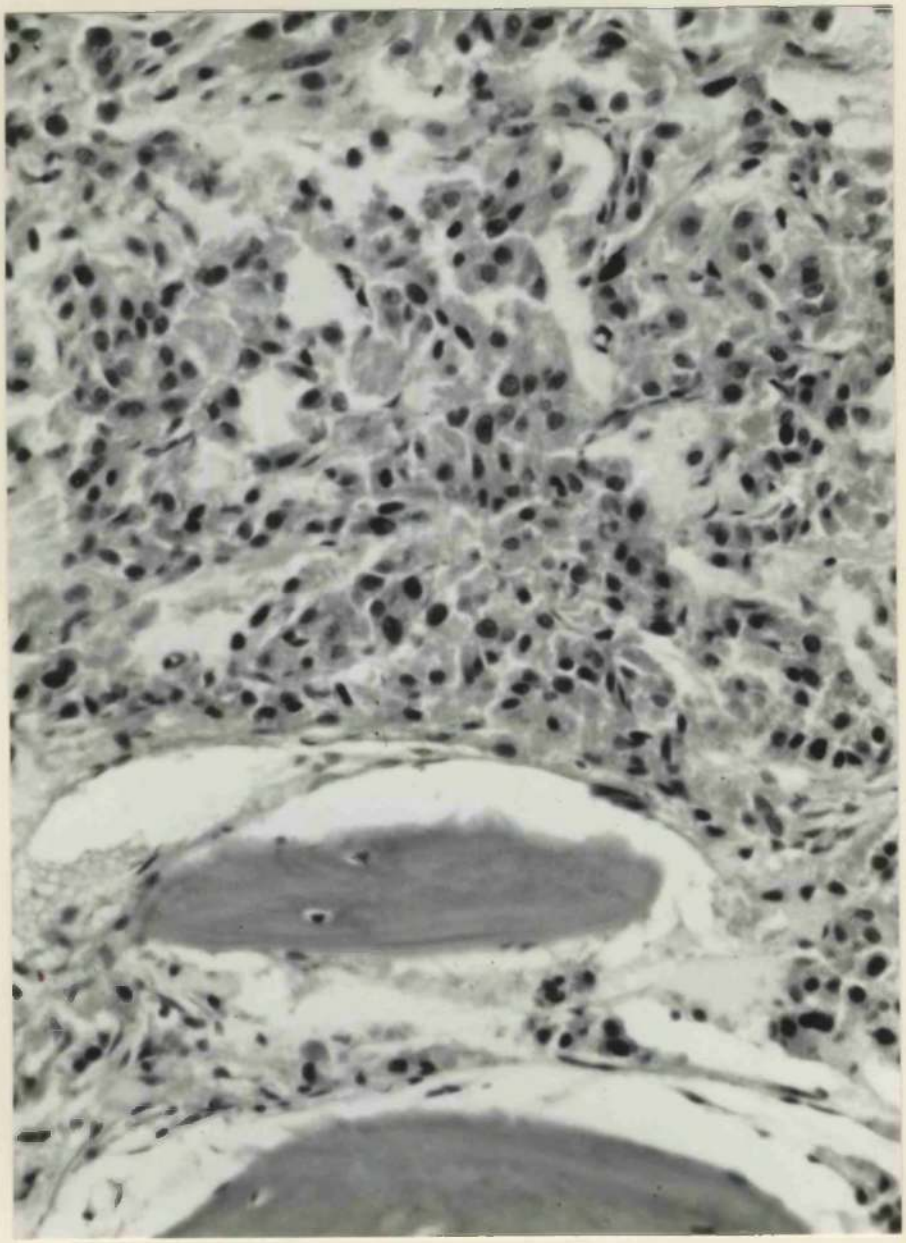

FIG.5,5

Fig. 5,6. - Ox adrenal. The cortex (left) shows only background activity. In the medulla (right) chromium-51 is associated with the brown pigment formed in the chromaffin reaction. Autoradiograph, Kodak AR50. X 310.

Fig. 5,7. - Mouse adrenal. Cortex to left. Medullary cells stained for noradrenaline by the iodate method form clearly delineated dark groups. Counterstained H. and E. X 340.

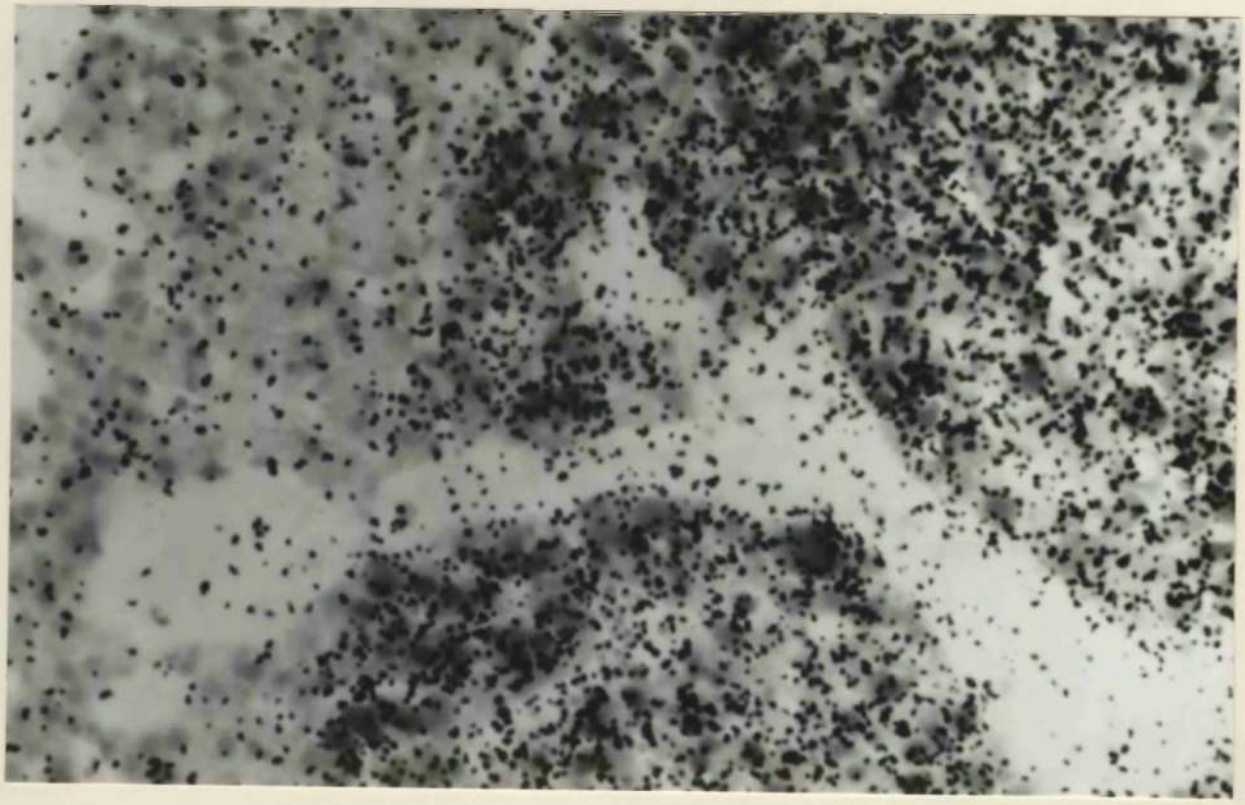

FIG. 5,6

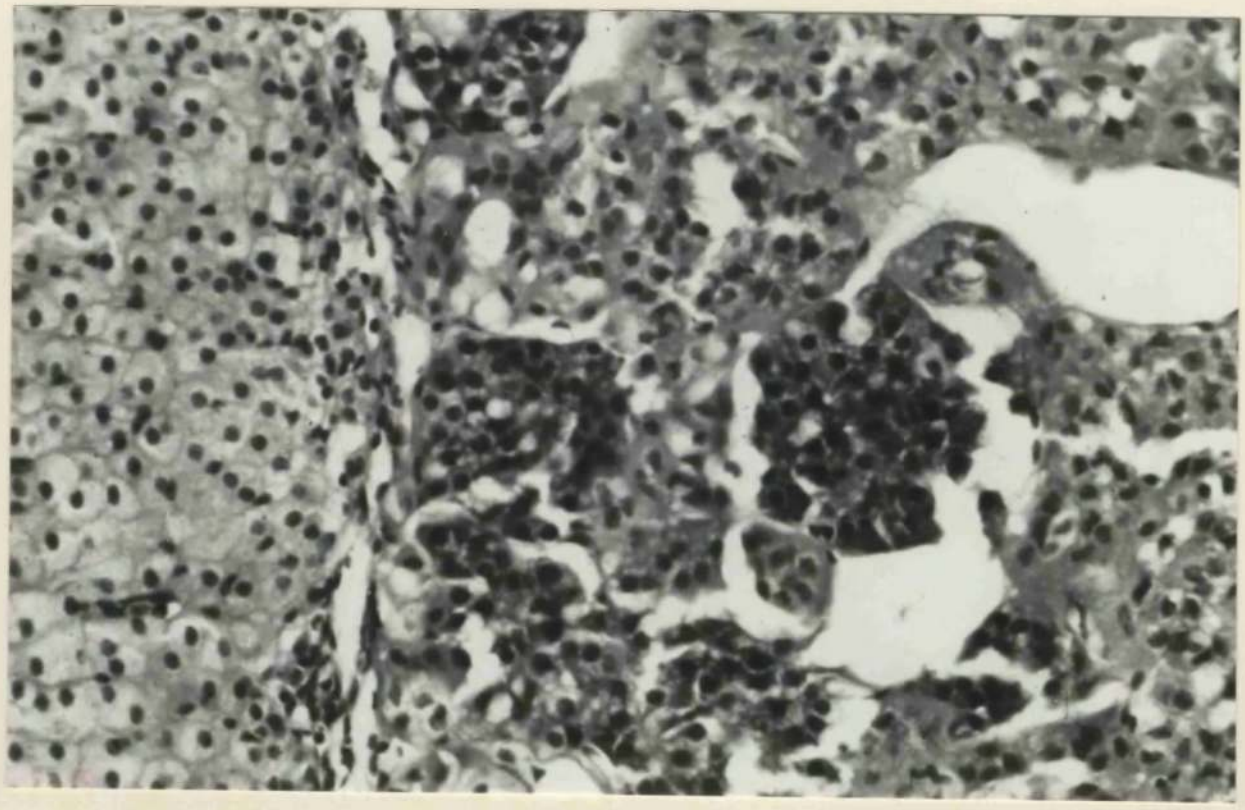

FIG. 5,7

P A R T VI

Tables 6,1 - 6,2

TABLE 6,1

ANALYSIS OF 259 LYMPH NODES EXAMINED FOR TUMOUR METASTASES

Disease	Cases	Total No. of nodes examined	Tumour-free nodes	No. of nodes partially replaced by tumour	No. of nodes wholly replaced by tumour
Carcinoma of breast	6	73	11(15%)	18 (25%)	44 (60 %)
Carcinoma of rectum	8	64	47(73%)	7 (11%)	10 (16%)
Carcinoma of cervix	3	69	63(91%)	4 (6%)	2 (3%)
Carcinoma of bronchus	6	41	27(66%)	6 (14%)	8 (20%)
Non-neoplastic	2	12	12	-	-
Totals	25	259	160(62%)	35 (13%)	64 (25%)

TABLE 6,2

ANALYSIS OF 73 LYMPH NODES FOUND TO CONTAIN COLLOIDAL RADIOACTIVE GOLD

Disease	Tumour-free nodes with uptake of Au 198	Partially replaced nodes with uptake of Au 198	Tumour-free or partially replaced nodes with uptake of Au 198
Carcinoma of breast	4 (36%)	2 (11%)	6 (21%)
Carcinoma of rectum	0 (0%)	0 (0%)	0 (0%)
Carcinoma of cervix	44 (70%)	4 (100%)	48 (72%)
Carcinoma of bronchus	9 (33%)	6 (100%)	15 (45%)
Non-neoplastic	4 (33%)	-	4 (33%)
Totals	61 (38%)	12 (34%)	73 (37%)

P A R T VI

Figs. 6,1 - 6,6

Fig. 6,1. - Tumour-free lymph node from the internal mammary group. A considerable deposit of colloidal radio-gold is present throughout the node and is present in the peripheral sinus. Autoradiograph, Kodak AR50. X 15.

Fig. 6,2. - Tumour-free lymph node from the internal mammary group. The amount of radio-gold present is much less than in the lymph node in fig. 6,1. Autoradiograph, Kodak AR50. X 18.

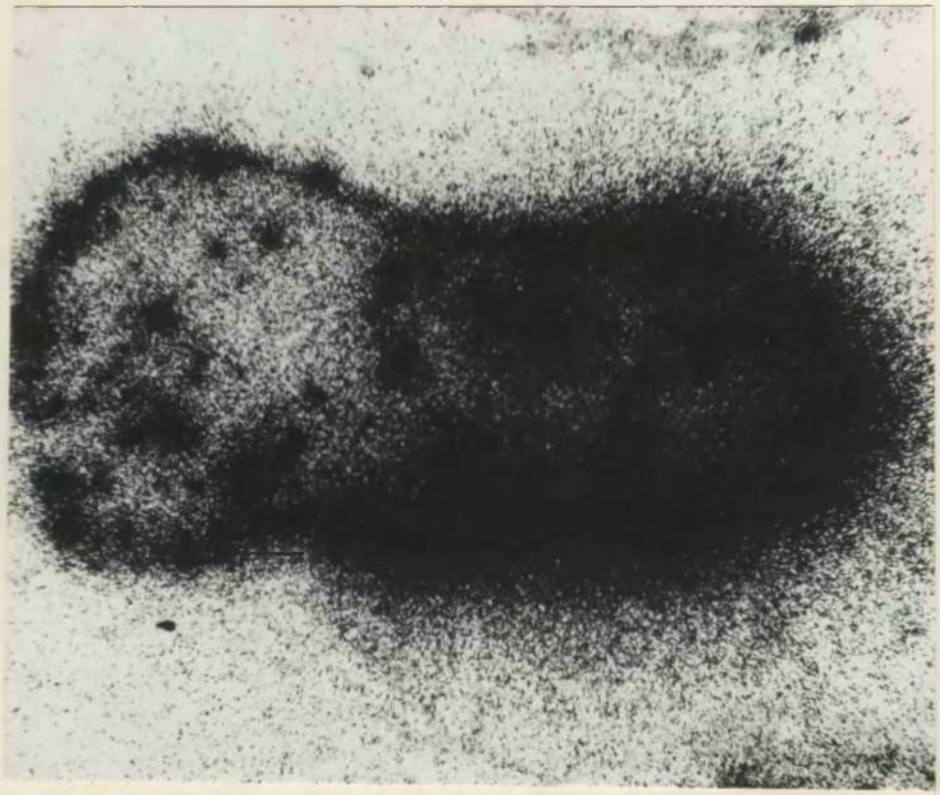

FIG. 6,1

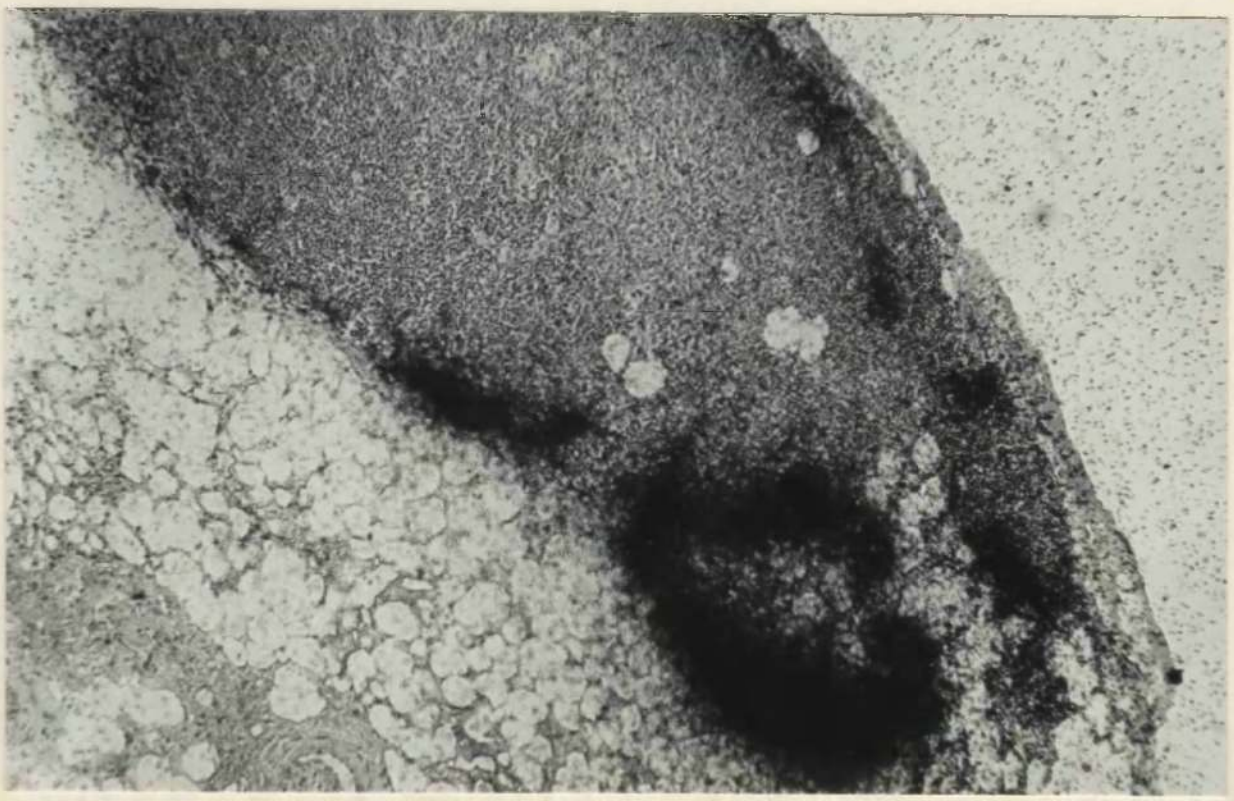

FIG. 6,2

Fig. 6,3. - Mammary carcinoma. Colloidal gold-198 is present at the injection site in macrophages of the stroma. No colloid is seen in the tumour cells. Autoradiograph, Kodak AR10. X 120.

Fig. 6,4. - Tumour-free pelvic lymph node. The colloidal radiogold is present in sinuses but the distribution-pattern is not uniform. Autoradiograph, Kodak AR50. X 35.

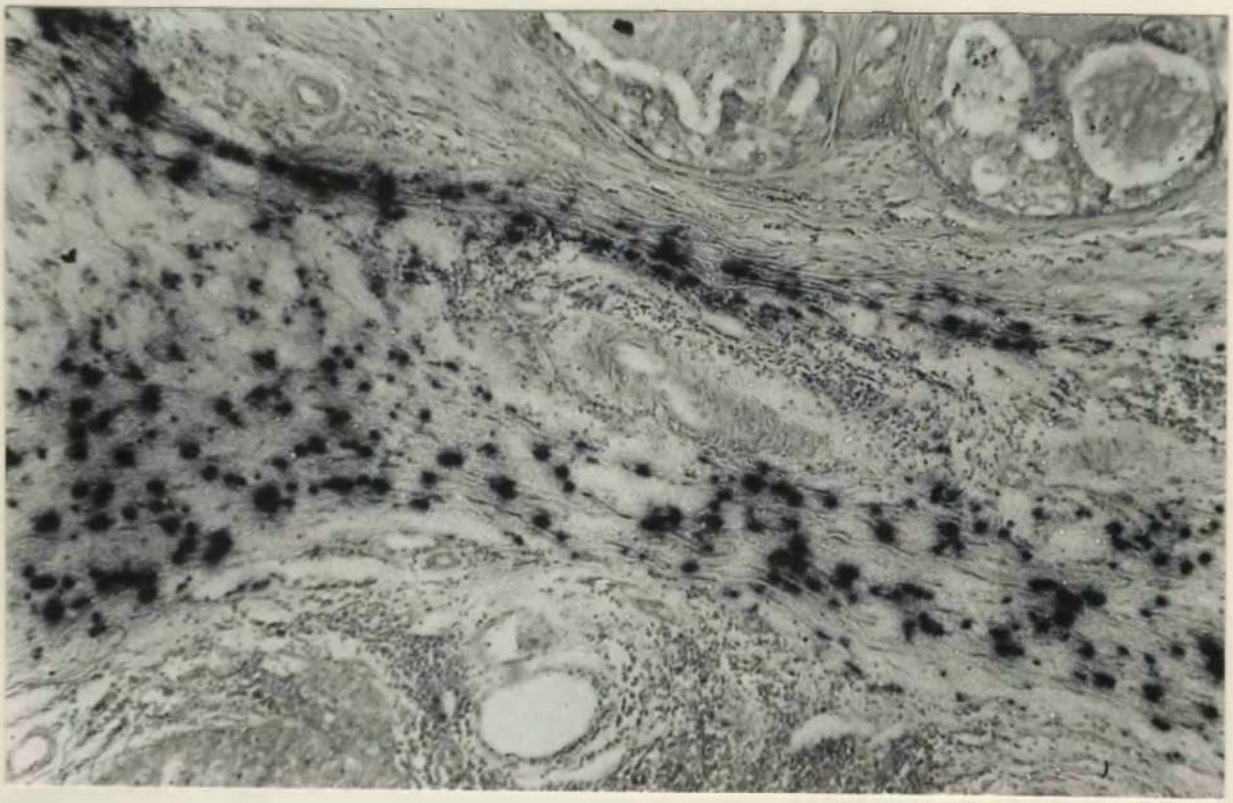

FIG. 6, 3

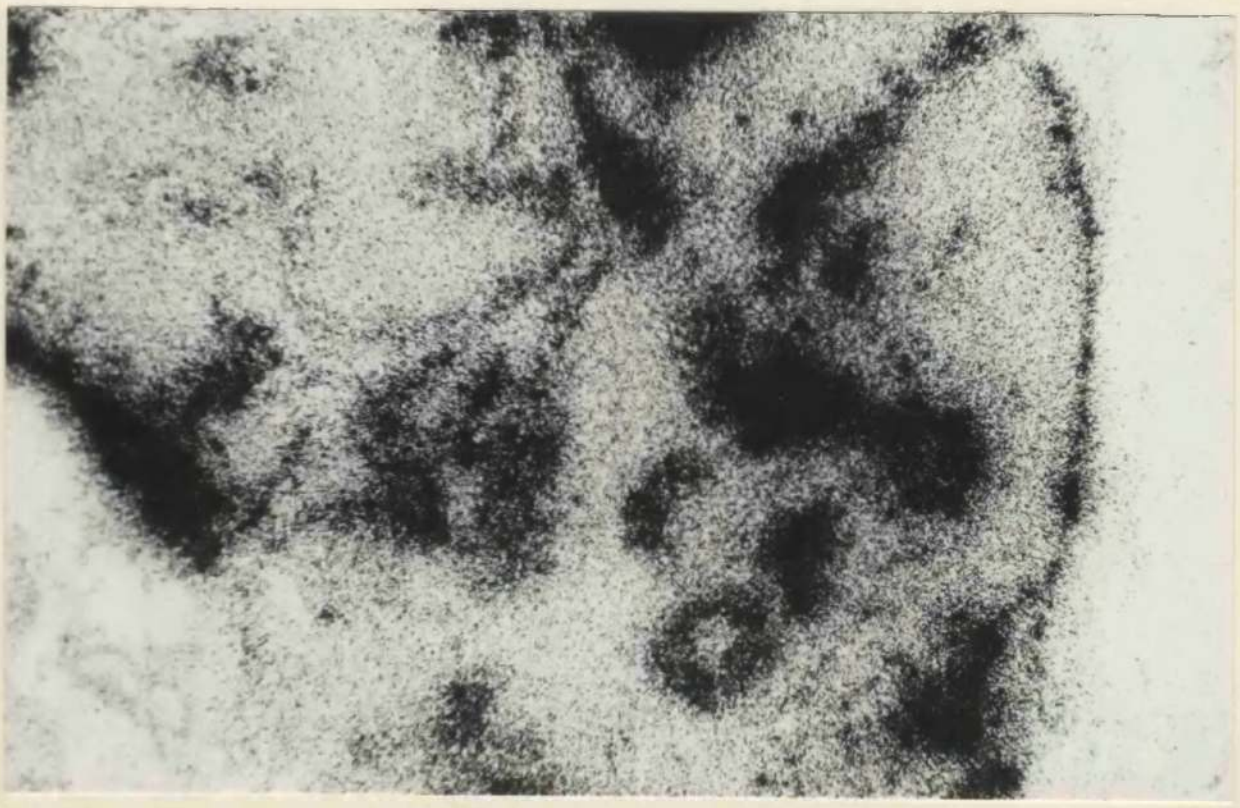

FIG. 6, 4

Fig. 6,5. - Tracheobronchial lymph node with secondary carcinoma (bottom). Deposits of colloidal radio-gold are present in the surviving lymphoid tissue (top). Autoradiograph, Kodak AR50. X 35.

Fig. 6,6. - Axillary lymph node with secondary carcinoma (bottom). Colloidal gold-198 is present in the surviving lymphoid tissue (top) and extends to the tumour edge. Autoradiograph, Kodak AR50. X 90.

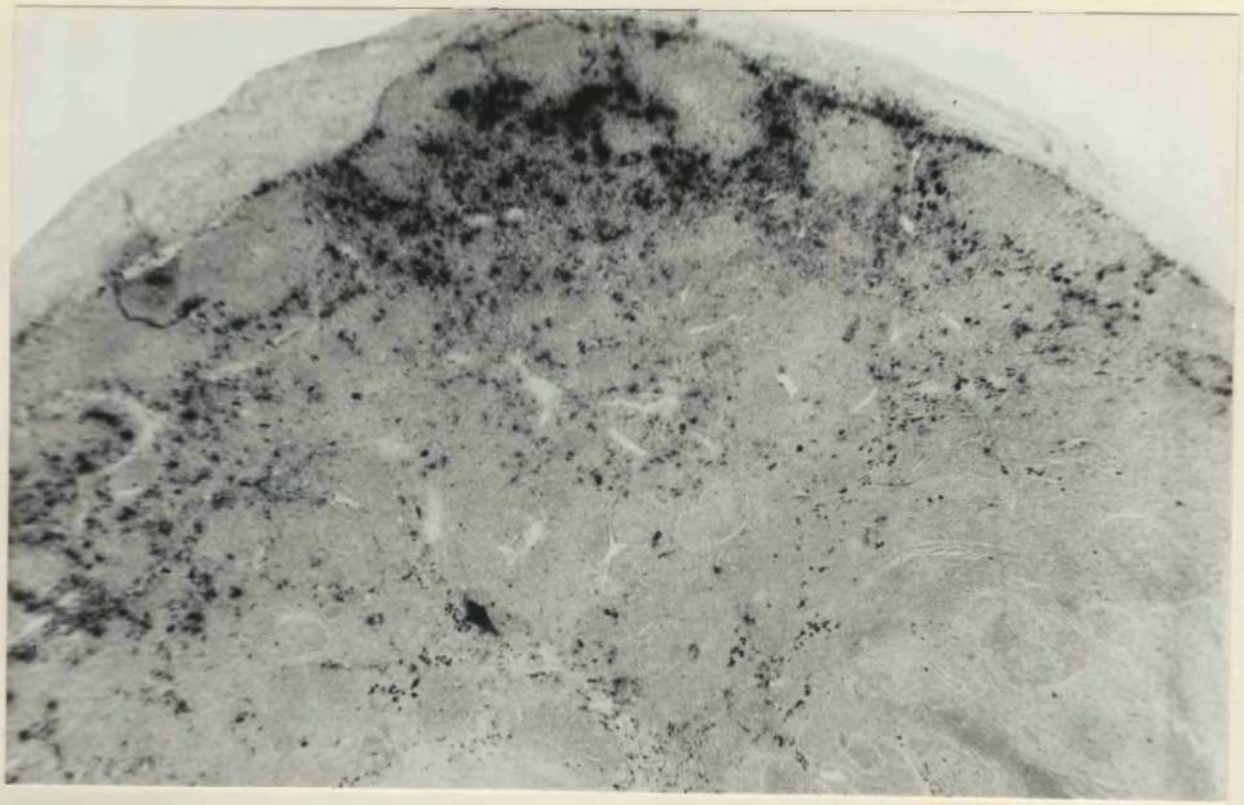

FIG. 6,5

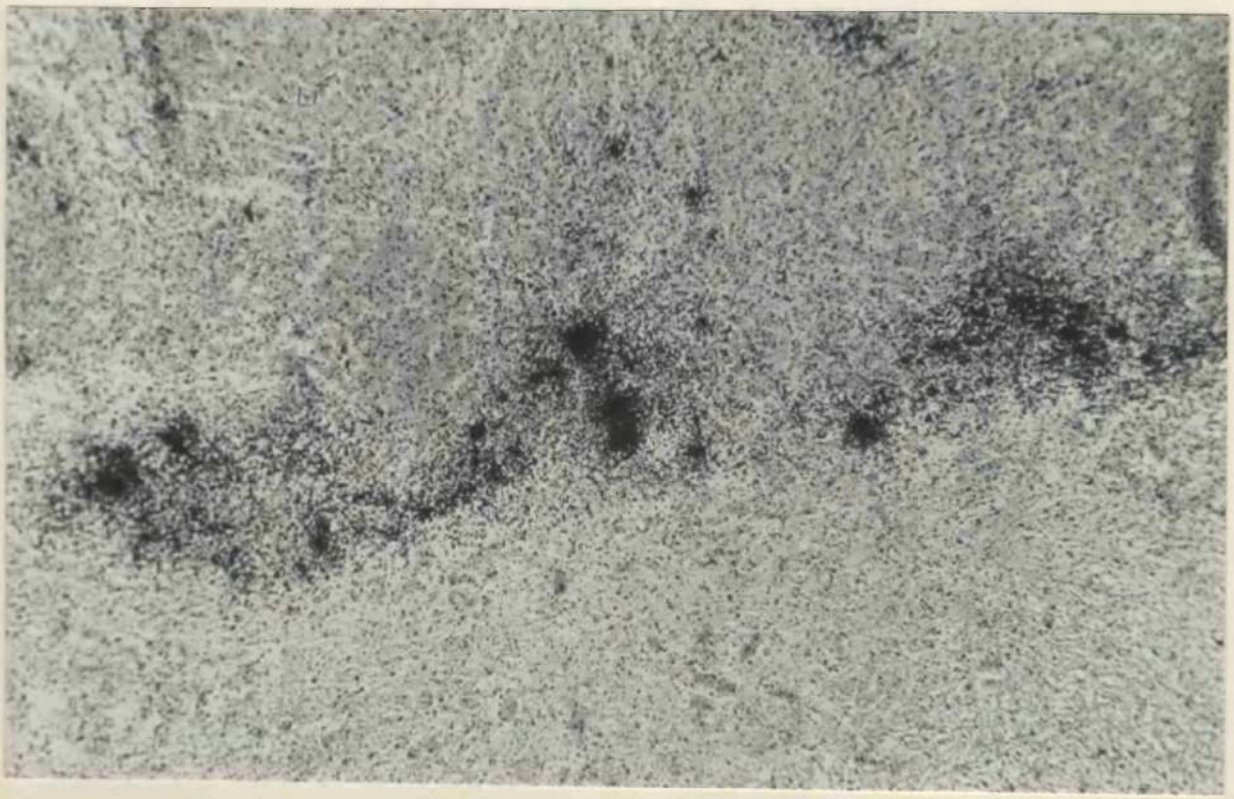

FIG. 6,6