THE ORIGIN AND FUNCTION OF THE MUCOPOLYSACCHARIDES OF THE CONNECTIVE TISSUES

THESIS SUBMITTED FOR THE DEGREE OF

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BY

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INTRODUCTION

This thesis is divided into five parts.

Part I is a histological study of the development of the experimental silicotic focus. Histochemical methods are employed to demonstrate the presence of mucopolysaccharide, which is apparently elaborated by fibroblasts.

Part II is concerned with the effects of cortisone and adrenocorticotrophic hormone (ACTH) on the tissue response to quartz and particularly on the formation of mucopolysaccharide.

Part III shows the presence of sulphated mucopolysaccharide in the quartz focus, by means of radioactive sulphate. It also confirms the fibroblast as the source of this material.

Part IV describes the autoradiographic distribution of "labelled" sulphate in the normal tissues of the mouse, and so determines the specificity of the method for sulphated mucopolysaccharides and the cells which form them in these tissues.

Part V applies the method to human tissues, to elucidate a number of problems with regard to cartilage behaviour in grafts and in storage. The results reinforce strongly the claim of the technique to demonstrate cells which form mucopolysaccharides.

An Appendix gives details of some of the technical methods employed but not described fully in the text.

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PART I.

OBSERVATIONS ON THE DEVELOPMENT OF THE SILICOTIC FOCUS IN THE MOUSE

THE SOLUBILITY THEORY OF SILICOSIS

The nature of the pathological lesions in the lungs of workers exposed regularly to inhalation of stone dust has been recognised for almost a century (Peacock, 1861; Greenhow , 1865), but the mechanism by which the dust exerts its harmful effect is still unknown. For many years it was believed that the fine particles lacerated the tissues in their neighbourhood. However, in 1922, Gye and his co-workers (Gye and Purdy, 1922; Gye and Kettle, 1922; also Gye and Purdy, 1924) produced inflammatory and necrotic lesions in tissues with amorphous silica and colloidal silicic acid (silica gel), and shortly afterwards Gardner (1923) demonstrated the inability of silicon carbide (Carborundum) to cause the intense fibrous reaction which quartz evoked; silicon carbide is extremely hard and its particles have edges as sharp as those of quartz. It was postulated, therefore, that quartz might exert its fibrogenic powers by producing a soluble substance which was toxic to the tissues, and this chemical or solubility theory suggested that the toxic substance was silicic acid. A very significant experiment supporting this idea and helping greatly to disprove the "mechanical" theory was

performed by Kettle (1932a) when he showed that quartz particles coated with a very thin layer of iron oxide were relatively ineffective in causing a reaction. The coating of iron oxide did not affect the sharpness of the particles but lessened greatly the solubility of the quartz.

It seemed, therefore, that a reasonably satisfactory explanation was available for the differing fibrogenic powers of the quartz and silicates; the former was much more soluble than any of the latter. However, it was soon evident that, though, in general, the pathogenicity of a stone-dust bore some relation to the rate at which it released silicic acid into solution, the correlation was not exact. For example, extremely finely-divided silica dust ("20 Angstrom" silica) caused no fibrosis in the lungs of animals, and with silicates the least active in the tissues often had the highest solubility in saline (Gardner, 1938). In 1937 this author said "The solution hypothesis cannot comprehend all of the observed facts: purely mechanical injury does not produce such reaction, for the hard, sharp, particles of diamond, aluminium oxide and silicon carbide cause no fibrosis..... Obviously it still remains for someone to make the crucial experiment which will prove the mechanisms by which silica exerts its

influence on the tissues".

King, whose extensive studies on the solubilities of siliceous materials have been largely responsible for the wide acceptance of the solubility theory, listed its shortcomings but did not advocate abandoning immediately a theory which has served well in many respects (1947). Wright (1950) has more recently taken a similar view. Demolishing the "fresh-fractured surface" theory of silicosis, of which Policard (1947) is still a strong advocate, he outlined some gaps in knowledge still to be filled by experimental investigation, to confirm previous workers' results, and to clarify questions still obscure. At present, the solubility theory holds that particulate silica is fibrogenic because it dissolves at a rate sufficient to cause colloidal silica to form in the tissue fluids, and apparently the particles must be less than 5μ in diameter (Scheel, Fleisher and Klemperer, 1953).

In the last few years, Holt has studied the effect of silicic acid on connective tissue and the polymerisation of silicic acid *in vivo* and *in vitro* (Holt and Osborne, 1953; Holt and Yates, 1954). He concludes that, contrary to previous evidence (Scheel *et al.*, 1953), polymers of silicic acid do form in the

tissues around quartz particles. This form of silicic acid may exert, it is thought, an activity in the tissues analogous to that of the indigenous polysaccharides in forming normal fibrous tissue. This extension of the solubility theory has a considerable bearing on the results to be presented in this thesis and it will be considered later at some length.

PRELIMINARY INVESTIGATIONS

Two years were spent initially in examining the reaction of animal tissues to a large number of different dusts. The materials tested included quartz, silica-free graphite, diamond, talc, silicon carbide, fused quartz, calamine, aluminium phosphate, tridymite, cristobalite, starch, calcium phosphate, dibenzyl pteridine, and a variety of dusts obtained from collieries. A total of 650 animals, mice, guinea-pigs, rats and rabbits, was employed for this purpose. The sites of injection of the materials included the intratracheal (Kettle and Hilton, 1932), intravenous (Gardner, 1938), intrapleural (Bradley and First, 1938; Cooray, 1949), subcutaneous (Kettle, 1932a; Irwin and Gibson, 1938) and the intraperitoneal (Miller and Sayers, 1934). Dry dusts

were also introduced intratracheally, by means of a capsule and catheter (Naeslund, 1939).

From this early experience several conclusions were drawn:-

(a) Technical difficulties in the preparation and sizing of dusts are considerable and beyond the scope of all except a special group. That at the Royal Technical College, under Professors Hibberd and Ritchie, provided some of the earlier substances tested, but they were, unfortunately, unable to prepare materials to order. Insufficient data on the factors of particle size and mode of preparation vitiated results.

(b) No lesion resembling the human silicotic focus could be produced by quartz in the lungs of any of the animals used; reticulin formed readily, but only scanty collagen appeared, even when the dust was introduced dry directly into the trachea. Others have experienced similar difficulties (Carleton, 1923-1924; Belt and King, 1945). However, in the mouse, the peritoneal cavity, and to a lesser extent the subcutaneous tissues, yielded in periods ranging from a few weeks to several months collagenous silicotic foci which closely resembled the human type of lesion. The guinea-pig and rat tissues

reacted similarly, particularly the former, but gave no lesions equal to those in the mouse. It was fortunate that the smallest animal was the most suitable, for many could be used for each experiment. It was even possible to show that the pathogenic properties of dusts could be estimated in the mouse peritoneal cavity by the macroscopic technique evolved by Miller and Sayers (1934) for guinea-pigs. It is noteworthy that Gardner (1938), after many years of work on dusts, abandoned the lung completely; he advocated the intravenous route in rabbits, with the liver as the site in which to study the dusts' fibrogenic powers. Though the value of this method was confirmed, it was considered unsuitable because of the size of the animal.

(c) Finally, the use of one dust preparation would eliminate the variables introduced by mode of preparation and particle size.

On the basis of these conclusions, it was proposed to study collagen formation in the silicotic focus in the peritoneal cavity and subcutaneous tissues of the mouse.

In the last twenty years there has been a renewed interest in collagen formation and break-down (Glynn and Loewi, 1952), and the amorphous ground-substance (mucopolysaccharide) of the connective tissues has received

particular attention (Meyer, 1947). Mostly, healing cutaneous wounds have been employed for histological studies. Though many years have elapsed since it was first clearly shown that the ground-substance plays an essential role in collagen formation (Wolbach and Howe, 1926; also Kolliker, 1861), and newer staining techniques for mucopolysaccharide have been available recently (McManus, 1946; Hale, 1946), its origin is still not precisely known (Klemperer, 1950 and 1952). It was reasonable to surmise that examination of the developing quartz lesion, where much collagen forms in a comparatively short time, would show the presence of mucopolysaccharide and perhaps provide information as to its source.

MATERIALS AND METHODS

The material was D.R.C. (rock crystal) quartz with a mean particle diameter of approximately 1μ. This was suspended in isotonic saline in a concentration of 4g./100 ml. The suspension was made immediately before use and sterilised for one hour at 100°C. Each mouse received 0.5 ml. (20 mg. quartz) intraperitoneally and 0.12 ml. (5 mg.) subcutaneously on the ventral surface.

Series A. A total of 60 mice was used and killed at 6 hrs., 1, 2, 3, 4, 7, 10, 14, 21, 30, 40,

55, 75, 90, 120, 145, 170 days, and 12 and 15 months after the injections of quartz.

Series B. For this experiment of short duration, 24 mice were used: 4 were killed immediately and the others at intervals of 5, 15, 20, 30 and 90 minutes, 6 hours and 20 hours after the injections of quartz.

The animals were examined immediately after death and the tissues placed at once in fixative. Bouin's solution was used routinely, but occasional blocks were fixed in 10 per cent. formol-saline, alcohol-formol-acetic, and Carnoy's fixative. Tissues from several animals were prepared by the freeze-drying technique.

Sections were cut at 6μ and stained with haematoxylin and eosin, iron haematoxylin and Van Gieson, and by Gordon and Sweets's method for reticulin. The following histochemical techniques were used to identify mucopolysaccharides: the periodic acid-Schiff (P.A.S.), colloidal iron (Hale, 1946) and Alcian blue (Steedman, 1950) methods, while metachromasia was demonstrated by thionin, toluidine blue, and cresyl fast violet.

Hyaluronidase from several sources was applied to some sections before staining for mucopolysaccharide.

Most of the observations were made with 0.025 per cent. testicular enzyme (Benger's Hyalase) at pH6. The filtrate of a 24-hour culture of Cl. welchii and 1 in 500 solution of the venom of Bothrops jararaca were also used. The period of incubation at 37° C. with each of these was usually 18 hours, but some sections were incubated for 4 hours and others for 30 minutes. Growth of organisms was controlled by addition of a drop of toluol to the enzyme solution. Control sections were treated with boiled enzyme.

Ribonuclease, 0.1 per cent. solution in distilled water for 1½ hours at 37° C., was used on a number of sections which were then stained by thionin. Sections were treated also in malt diastase, 1 per cent. solution, for one hour at 37° C. The Romieu reaction for tryptophane and the Millon reaction for tyrosine were applied.

RESULTS

Quartz injected into the tissues acts initially as a powerful irritant, evoking an early acute inflammatory reaction. This subsides slowly over several days, to be replaced by the characteristic granulomatous reaction.

MACROSCOPIC APPEARANCES:

For some time the earliest lesions examined were of 6 hours' duration (Series A).

By 6 hours, a considerable proportion of the injected dose is already adherent to the peritoneal surfaces of omentum, mesentery, intestine, abdominal wall, fat, spleen and reproductive organs, on which it collects in a few, large rough masses. Gelatinous, turbid, inflammatory exudate is replacing the aqueous suspending fluid, and the vascular response of acute inflammation is evident throughout the peritoneal cavity.

By 24 hours, much more of the quartz has been taken up, so that most of it is already fixed (fig. 1, p.12). If free dust persists, this also is collected into only one or two clumps. Aggregation into a few masses is characteristic of quartz, and so is the failure of the dust to disperse evenly throughout the peritoneal cavity. It is never found, for example, on the superior aspect of the liver or on the diaphragm.

By 3 days, the free fluid exudate is greatest in quantity, but hyperaemia of the tissues is diminishing. The quartz lesions are larger and have a smoother surface. Free dust is generally absent.

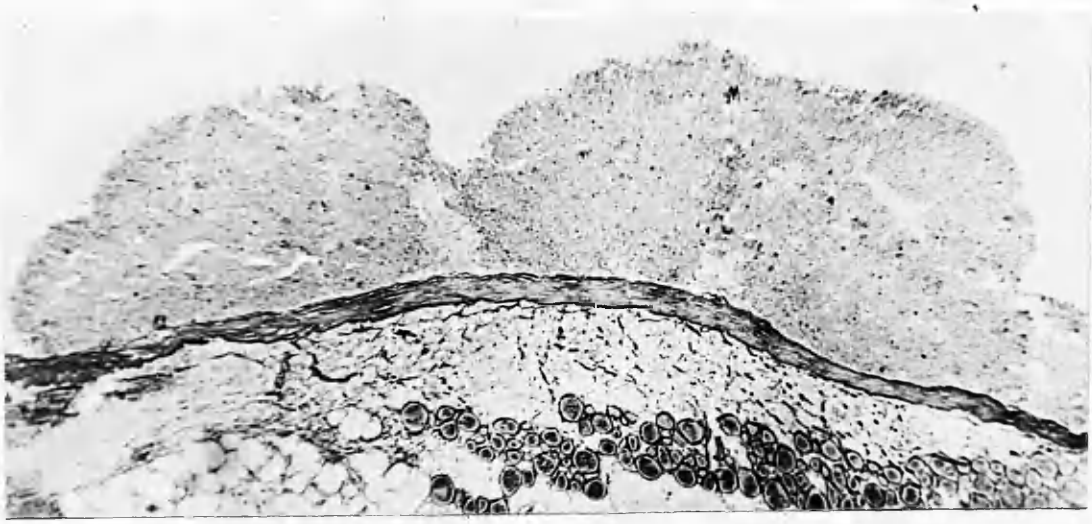


Fig. 1. A mass of dust is firmly adherent to the serosal surface of the abdominal wall 24 hours after the intraperitoneal injection of quartz. Gordon and Sweets's method X 12.

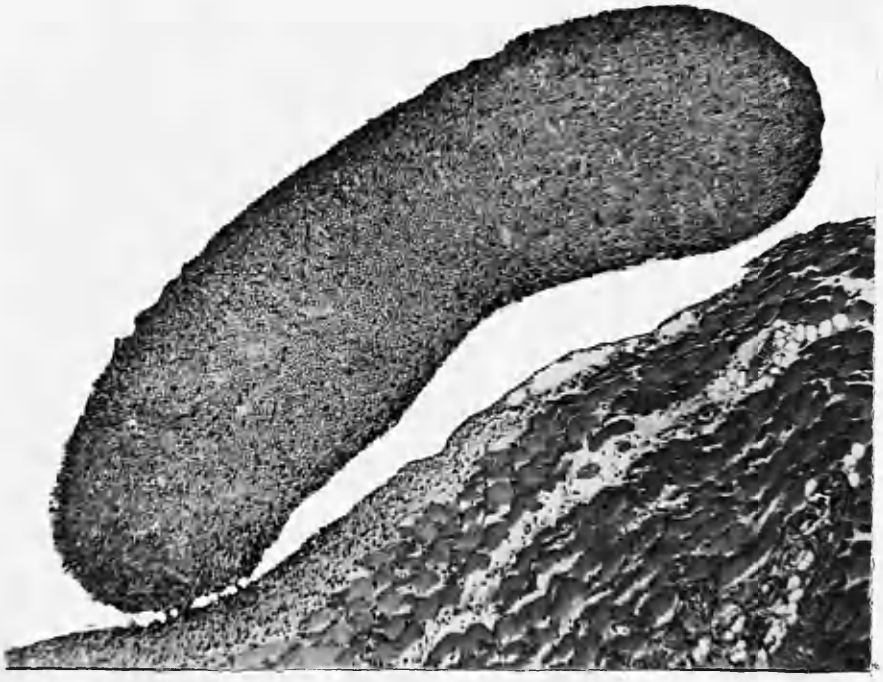


Fig. 2. The mass of quartz forms a large smooth-surfaced lesion adherent at one end to the serosal surface of the abdominal wall. Haematoxylin and eosin X 12.

By 7 days, the lesions have grown and already show evidence of a "proliferative" (Miller and Sayers, 1934) or productive character (fig. 2, p. 12). Only silica lesions exhibit this steady growth. Free fluid is absent and the tissues are no longer congested.

Subsequently, the quartz foci increase slowly in size for several months, by which time they are large, heaped-up and somewhat fleshy (fig. 3, p.14 ; fig. 35, p.106). Thereafter, they become more compact, dense and fibrous.

MICROSCOPIC APPEARANCES:

By 6 hours, all the features of an acute inflammatory reaction are evident in the tissues of the peritoneal cavity, with vasodilatation and abundant exudate containing fibrin and numerous polymorphs and macrophages (fig. 4, p.14; fig. 5, p.15). The polymorph leucocyte usually predominates in the exudate in the tissues and in the free fluid for the first day or two. Large and small mononuclear cells, however, are also present. Scattered throughout the dust masses are fairly numerous cells, including a high proportion of large mononuclear cells (fig. 6, p. 16). A layer of quartz often "gums" the fatty tissues together (fig. 7, p. 17). The particles do not stain with haematoxylin or eosin, but a reliable and sensitive method for determining the presence

Fig. 3. The mass on the abdominal wall surface shows the heaped-up "proliferative" appearance typical of a quartz focus. X 4.

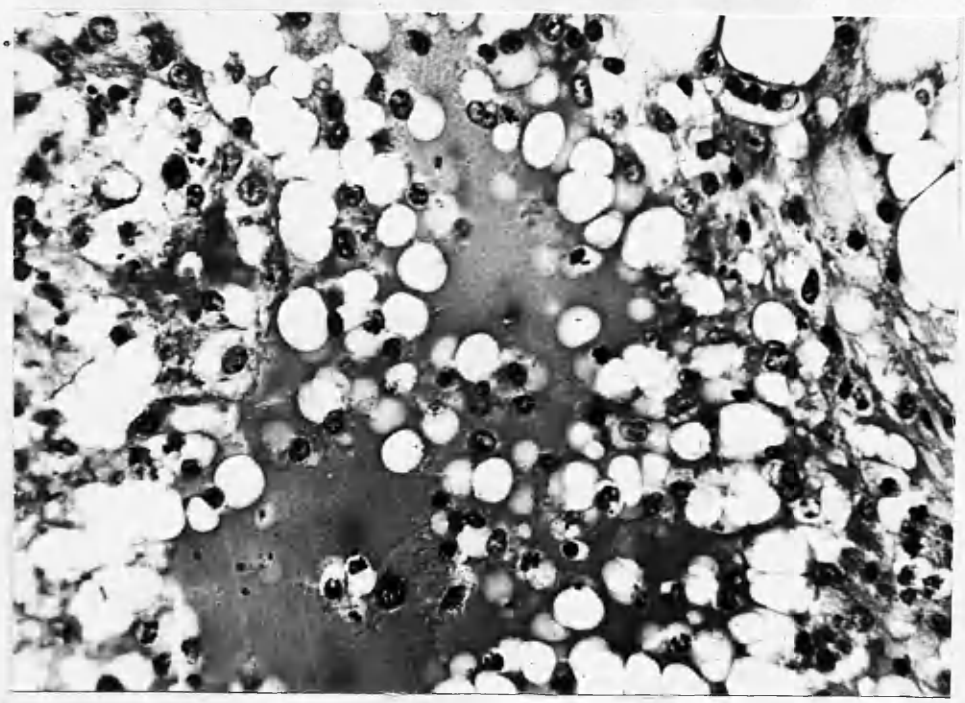


Fig. 4. The inflammatory exudate in the tissues of the peritoneal cavity a few hours after injecting quartz often contains many large mononuclear cells. Haematoxylin and eosin X 500.

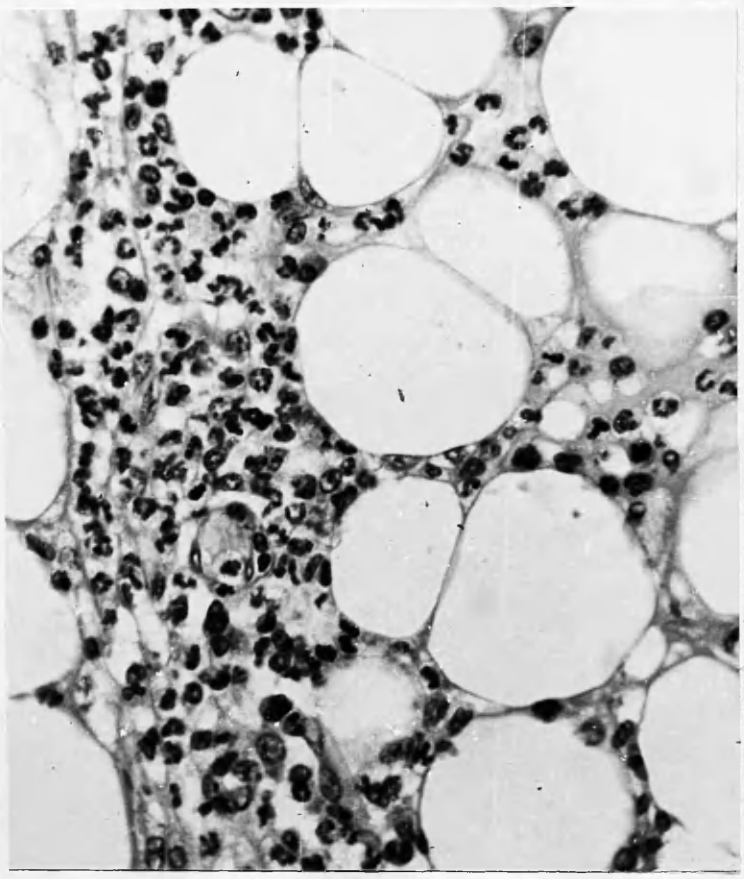


Fig. 5. There are numerous polymorphs in the tissues around a 6-hour quartz lesion in the subcutaneous tissues. Haematoxylin and eosin X 400.

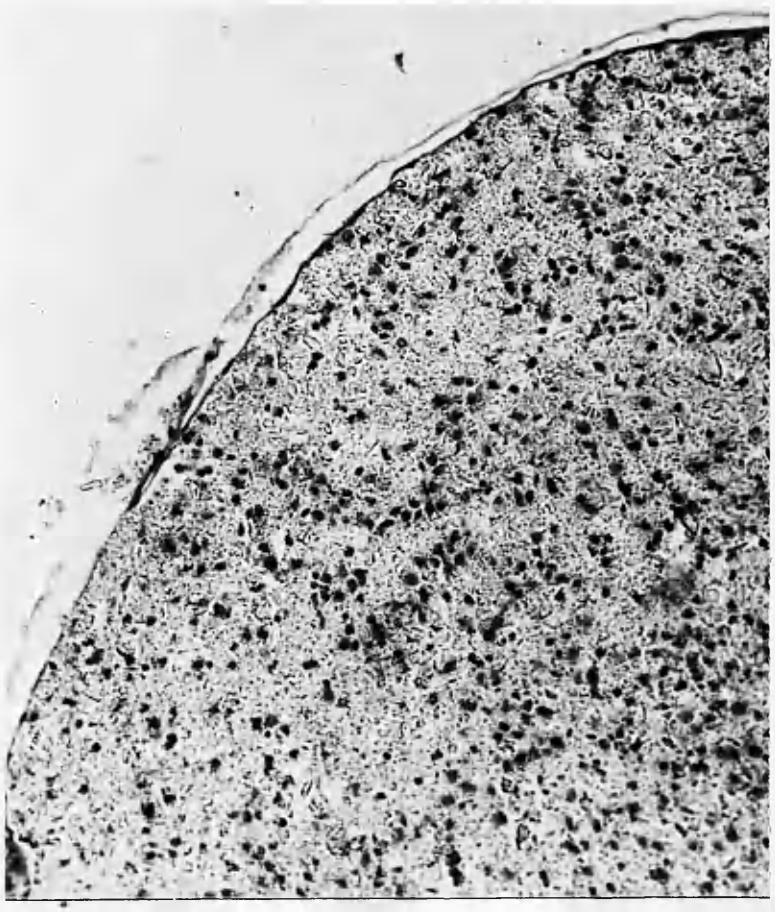


Fig. 6. Many cells, mainly large mononuclear cells, are scattered throughout even the large foci within a few hours. They are killed within a day or two. Haematoxylin and eosin X 150.

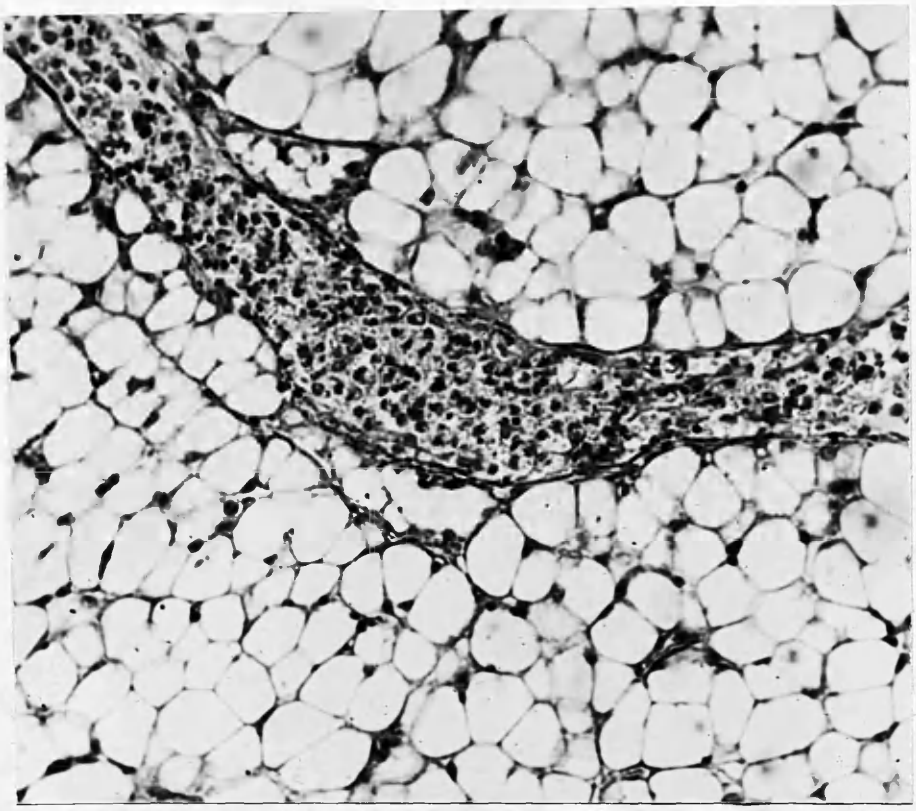


Fig. 7. A layer of quartz infiltrated by cells causes two fatty masses to adhere firmly to each other. Haematoxylin and eosin X 150.

and distribution of the quartz is available, for the dust particles are strongly metachromatic (fig. 8, p.19 ; fig. 9, p. 20). This reaction was studied in detail in a series of experiments reported below which show that it is a property of the quartz dust and not caused by acid mucopolysaccharide. It allows detection of very small quantities of quartz. It should be noted that the anisotropism of quartz is not, contrary to general belief, demonstrable with particles of the diameter used here (of approximately 1μ), and this property is, therefore, of no value in tracing the dust in sections. Only the very few large particles of quartz (5-10μ diameter) in each focus show up on examination by polarised light, but they are also most prominent in thionin-stained sections because of their metachromatic purple colour, as intense as that of the mast cell granule (fig. 9, p. 20). Prior treatment of sections by any of the enzymes fails to affect the metachromasia of the dust.

By 24 hours, the acute inflammatory reaction is more advanced. The abundant, gelatinous free fluid exudate is rich in polymorphs, as is that in the acutely inflamed tissues (fig. 10, p. 21). The dust collections are much larger, and the particles are intensely metachromatic.

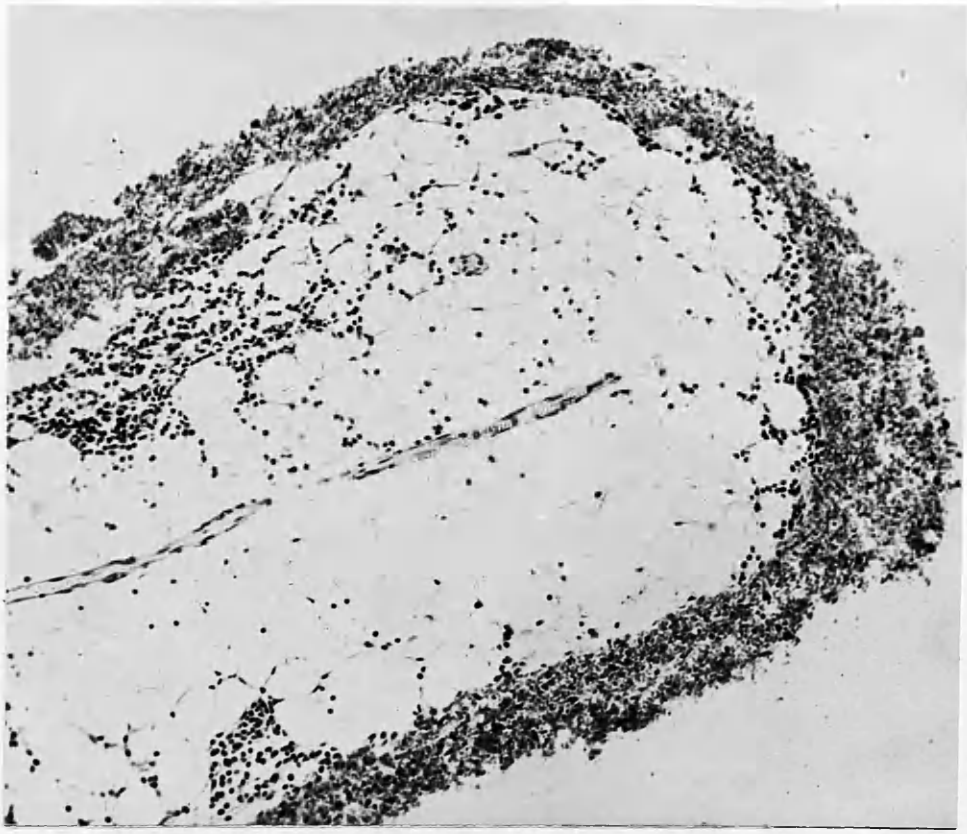


Fig. 8. A layer of quartz, stained metachromatically, adheres to the omental surface immediately after injection. The structures resembling nuclei throughout the dust are nearly all large deeply-stained particles of quartz. Thionin X 200.

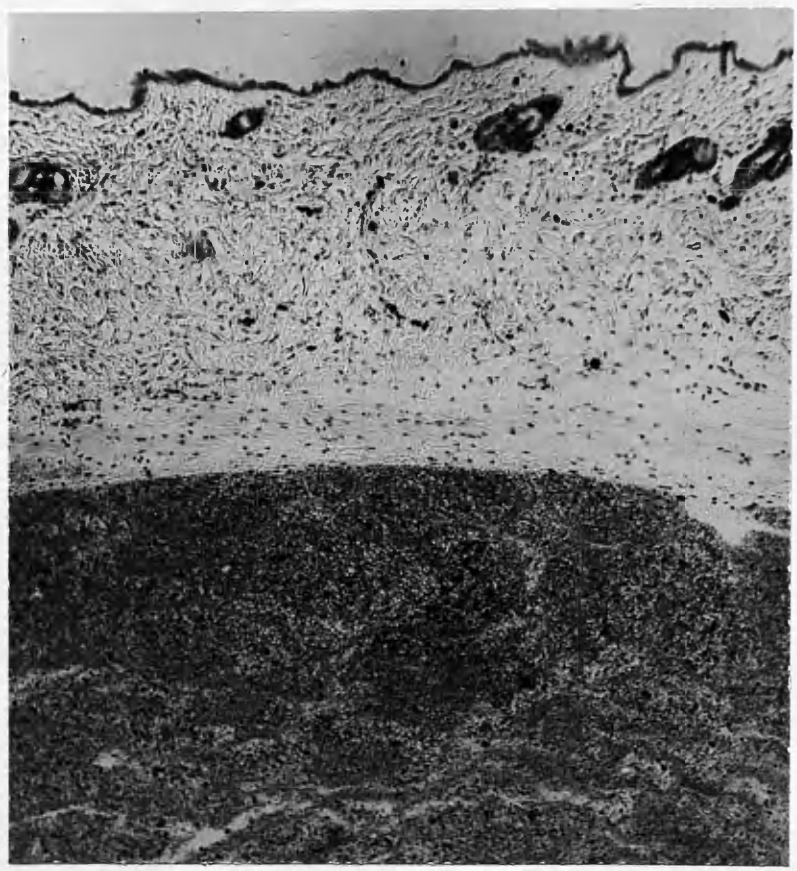


Fig. 9. The subcutaneous quartz mass, immediately after injection, is stained metachromatically. The large particles stain as deeply as the mast cells in the dermis. Thionin X 210.

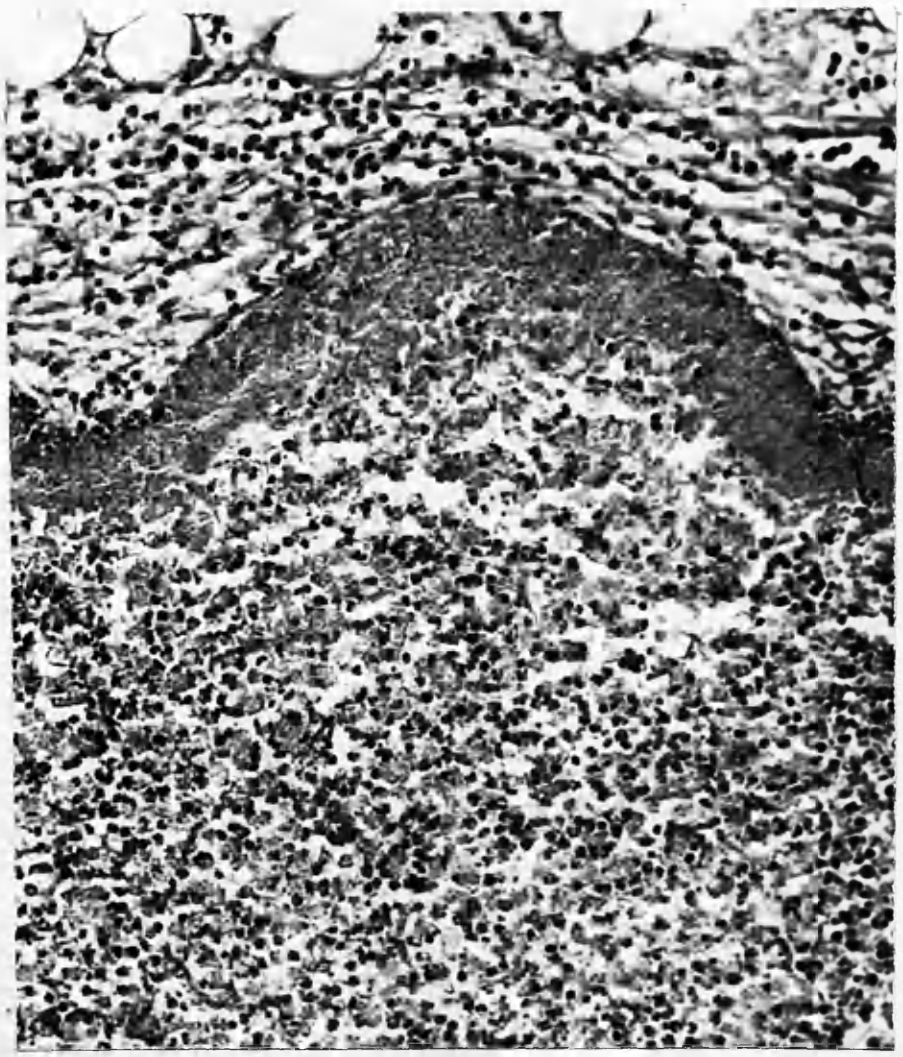


Fig. 10. A large number of cells are present in and around the quartz focus at 24 hours. In this subcutaneous lesion they consist almost entirely of polymorphs. Haematoxylin and eosin X 200.

Many cells surround the foci (fig. 10, p. 21); some penetrate the dust, but are killed and leave chromatin fragments (fig. 11, p.23 ; fig. 12, p. 24). Fat necrosis is absent at all stages of development of the quartz focus, even though in many cases the mass initially forms on the surface of the fat and is later totally enclosed within it.

By 3 days, the first reticulin fibres can be demonstrated at the periphery of each focus (fig. 13, p. 25), in which a rapid increase in the number of large mononuclear cells has occurred. As these cells migrate through the tissues, many are typical elongated fibroblasts (fig. 14, p. 25), but within the focus they usually become polyhedral. Each has a large, pale vesicular nucleus with one or two nucleoli (fig. 12, p. 24). They progressively invade the dust and phagocytose the particles: reticulin forms round each cell (fig. 15, p. 26). It is convenient to refer to them as koniophages. Many are killed and leave fragments of chromatin (fig. 11, p. 23 ; fig. 12, p. 24). Migration is continuous, however. Vascular channels are found only in the superficial layer of the focus. Occasional foci show minimal signs of reaction in their vicinity, and appear relatively inert (fig. 16, p. 27). This delay in onset of the reaction to the dust rarely persists beyond 3 days.

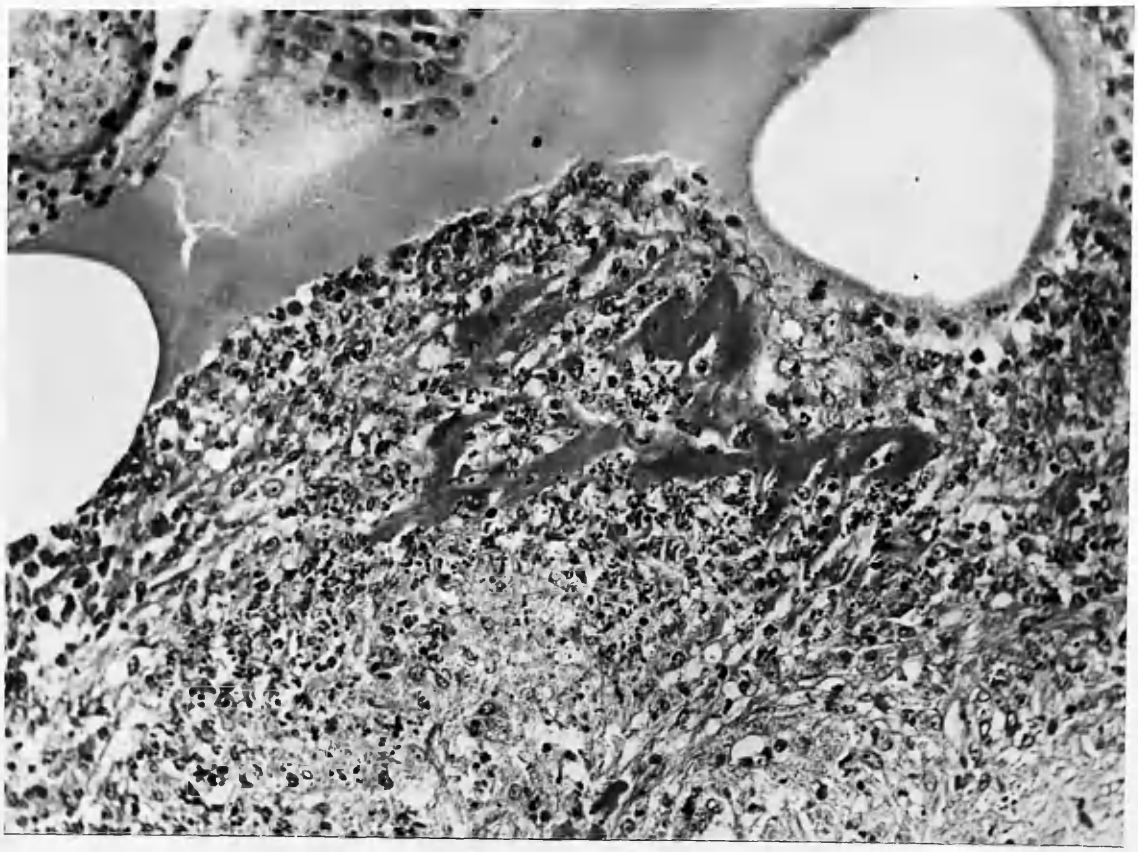


Fig. 11. Abundant lymph and strands of fibrin are present in the vicinity of the lesion at 24 hours. Many cells invade the dust but are killed and leave numerous chromatin fragments. Haematoxylin and eosin X 300.

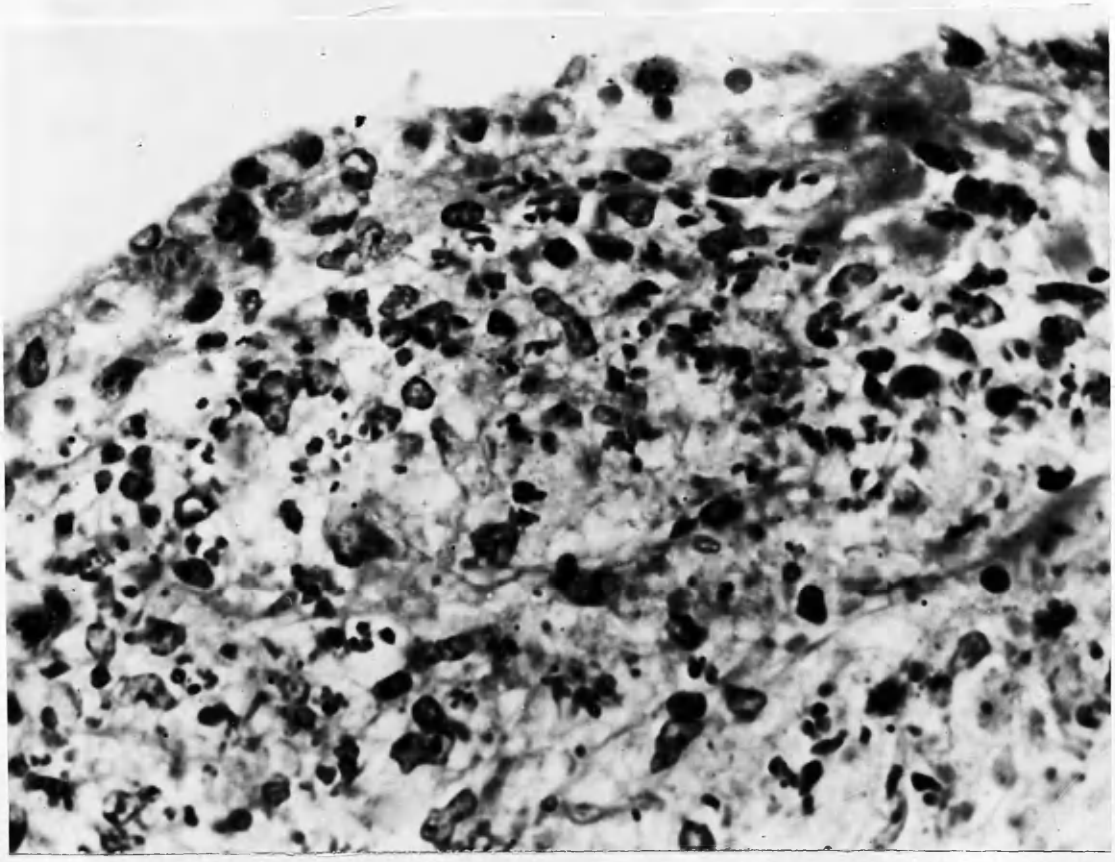


Fig. 12. This shows in more detail the early invasion of the dust by macrophages and polymorphs, many of which are killed leaving many nuclear remnants. Haematoxylin and eosin X 650.

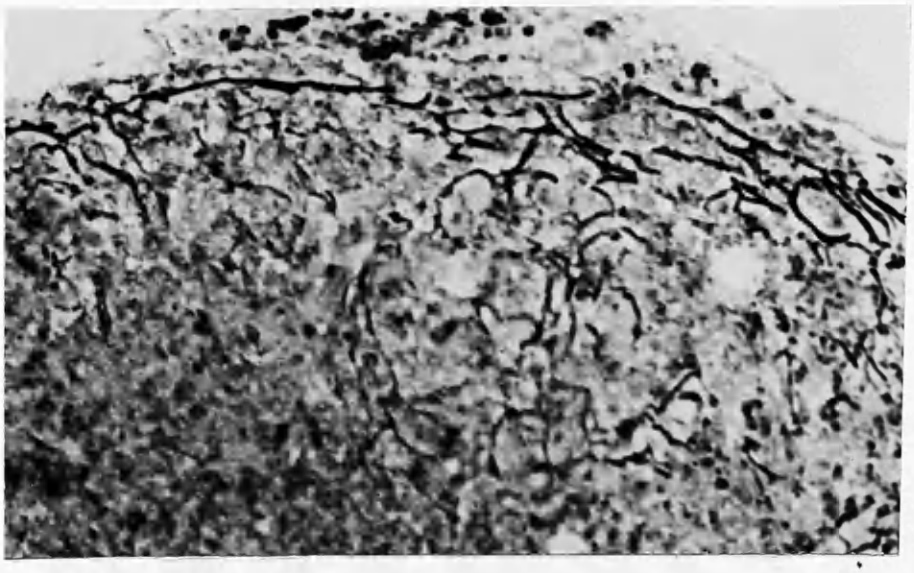


Fig. 13. By 3 days the first reticulin fibres are detected at the periphery of each focus. Gordon and Sweets's method X 400.

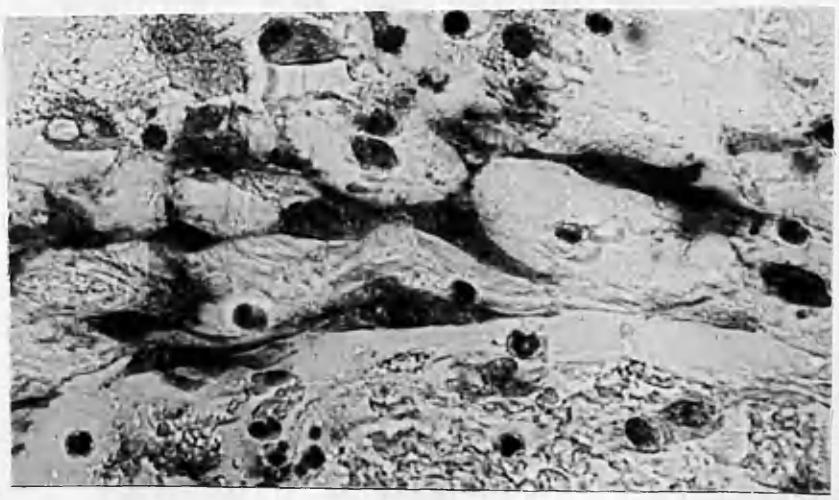


Fig.14. Elongated fibroblasts migrate towards a quartz focus in the peritoneal tissues. Alkaline phosphatase X 800.

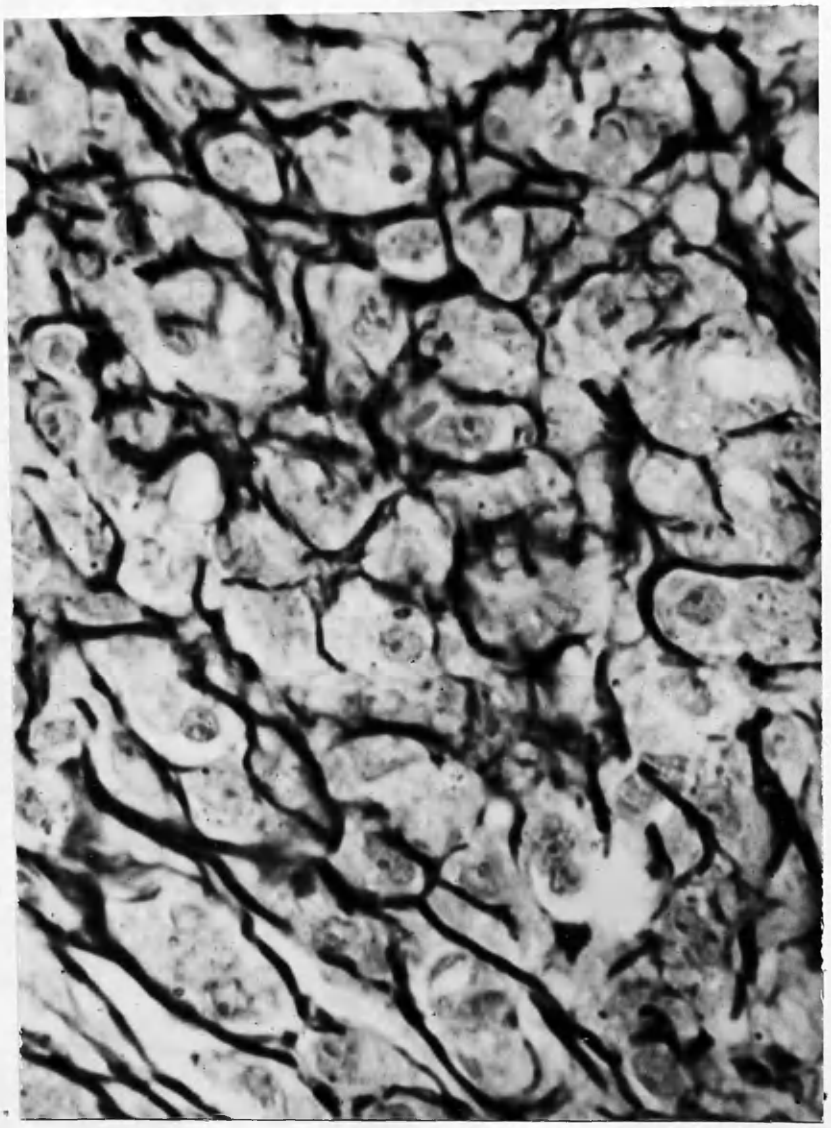


Fig. 15. Reticulin fibres form between the koniophages. Gordon and Sweets's method X 800.

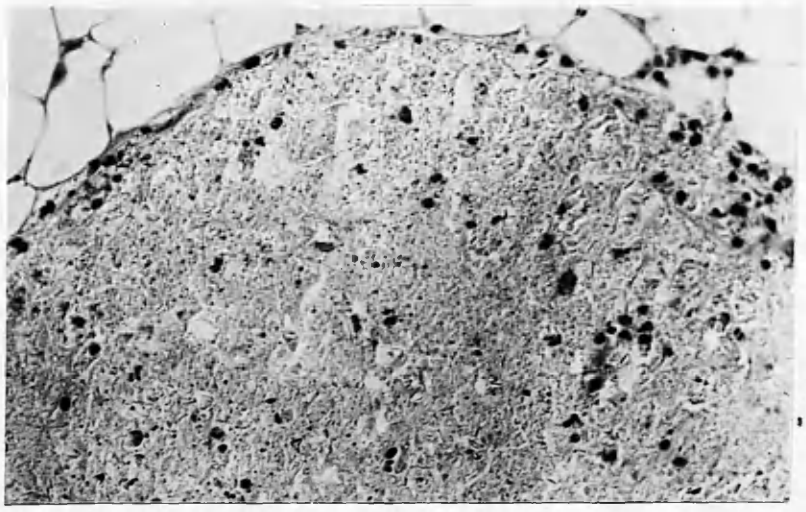


Fig. 16. There is little evidence of an inflammatory reaction to the dust three days after injection. Haematoxylin and eosin X 250.

By 7 days, each focus usually consists of a homogeneous population of koniophages with their well-developed phagocytic and fibroblastic powers (fig. 17, p. 29). Their cytoplasm is strongly metachromatic, and this reaction is unaffected by prior treatment of the sections with ribonuclease, hyaluronidase or diastase; the metachromasia of the central "free" dust is likewise not affected by these enzymes. The koniophage often closely resembles the epithelioid cell of the tubercle follicle, and in fact the smaller silicotic foci are similar in appearance to this structure (fig. 18, p. 30). Many of the koniophages undoubtedly come from the blood stream, but a significant proportion originate in the adventitia of blood vessels and in the taches laiteuses (fig. 19, p. 31).

By this time, a new development has occurred in the koniophage cytoplasm; stainable mucopolysaccharide is present. It stains well by the Alcian blue and Hale methods (fig. 20, p. 32), and feebly by the periodic acid-Schiff routine (fig. 21, p. 33), but any possible metachromasia is obscured by the similar reaction given by the quartz. In a few cells, granules of strongly Schiff-positive material are visible; these are unaffected by diastase or hyaluronidase. Mucopolysaccharide

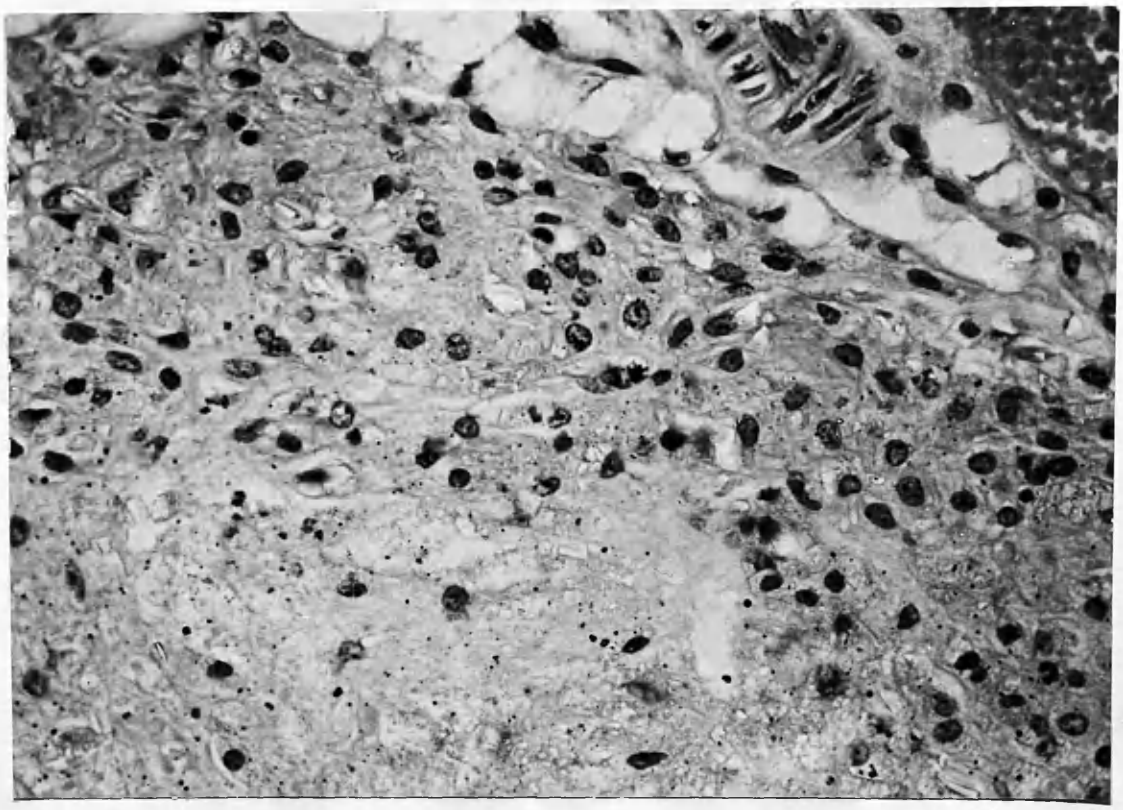


Fig. 17. After a week, the cells invading the dust are almost entirely large mononuclear phagocytes. A few have pyknotic nuclei. Haematoxylin and eosin X 400.

Fig. 18. Within 7 days many of the small quartz collections are entirely intra-cellular and enclosed in a reticulin network. Gordon and Sweets's method X 200.

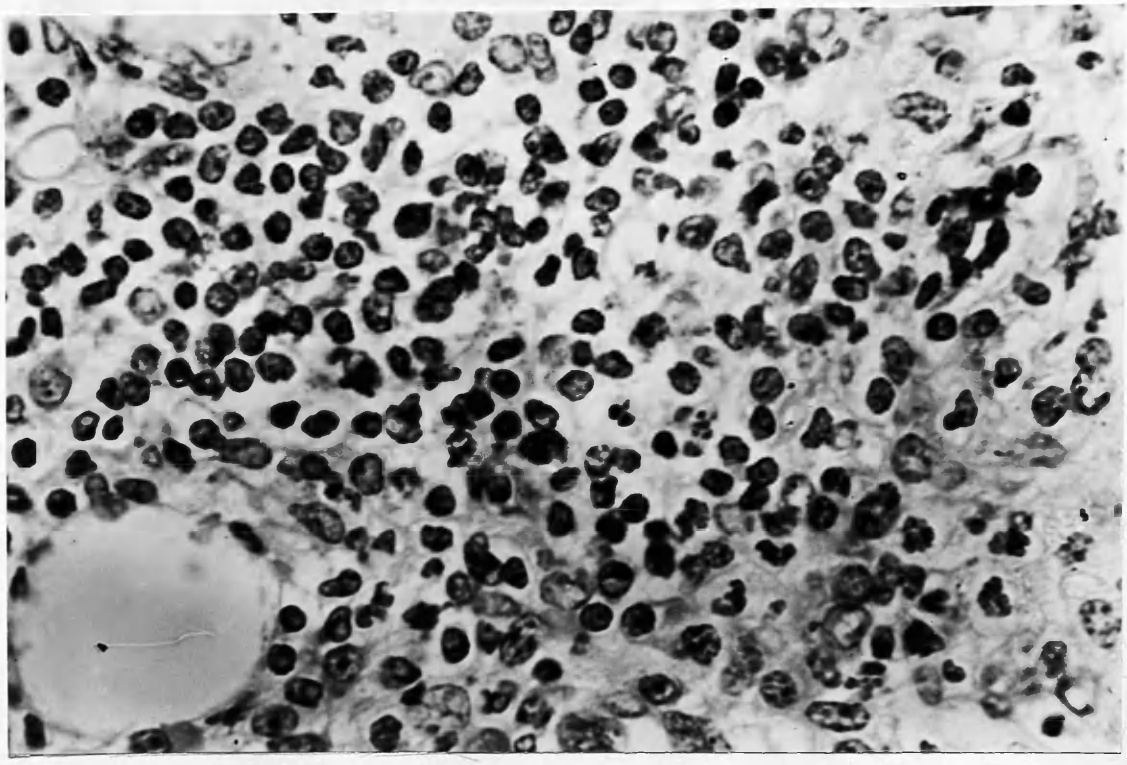


Fig. 19. There are many cells present intermediate in form between the large mononuclear cells of the koniophage type and the small lymphocyte-like undifferentiated cells in the taches laiteuses. Haematoxylin and eosin X 800.

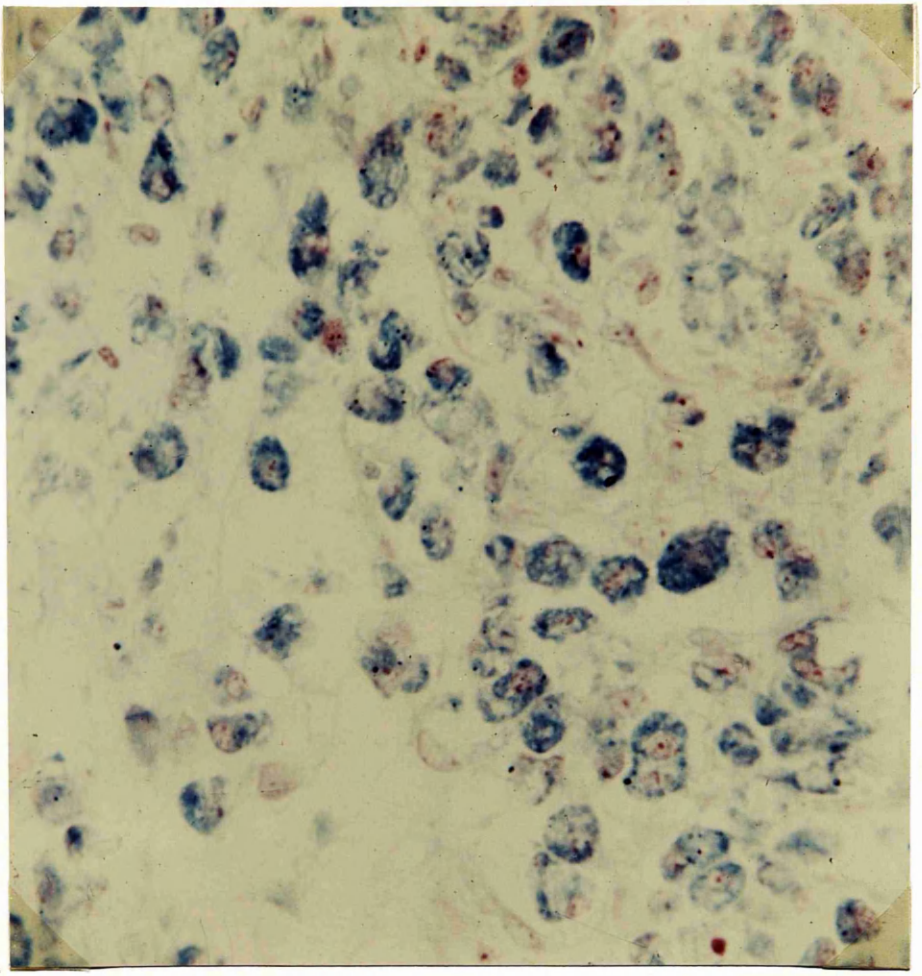


Fig. 20. The cytoplasm of the koniophages contains acid mucopolysaccharide. As a rule, koniophages do not remain discrete like this but congregate in compact masses. Hale's method X 600.

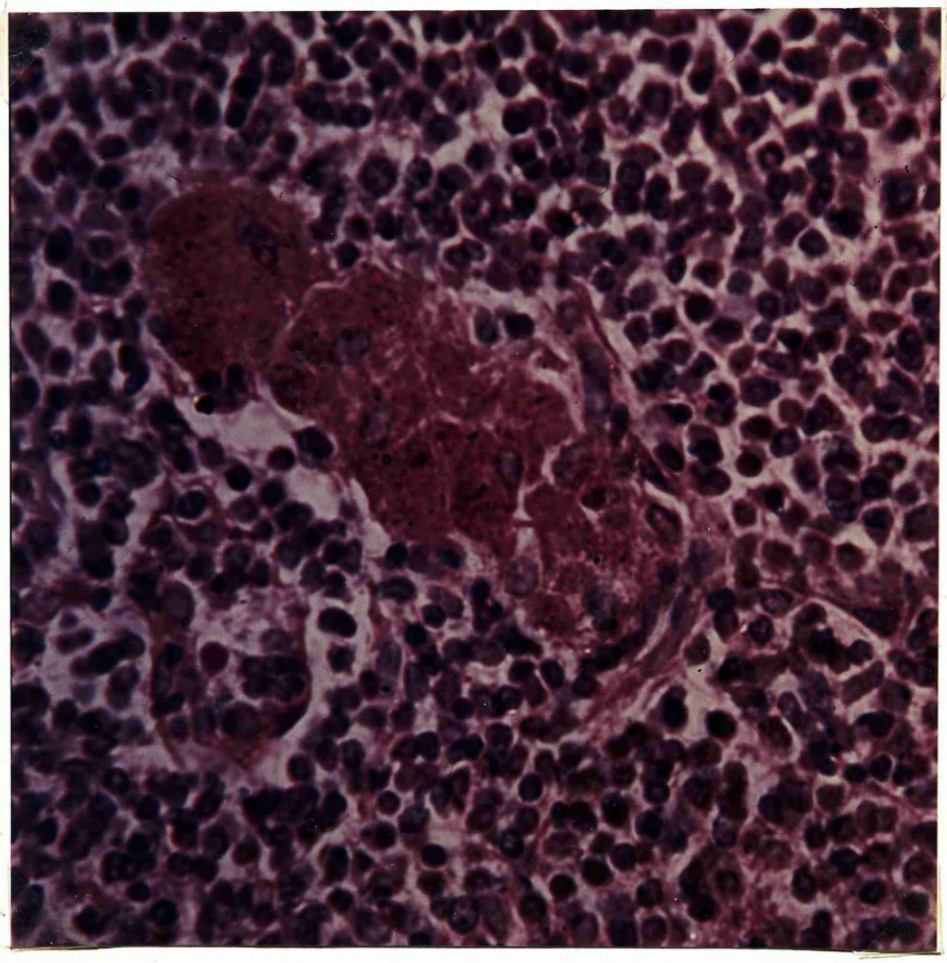


Fig. 21. A small group of koniophages within a sinus of a mesenteric lymph node are identified by the presence of Schiff-positive material in their cytoplasm. Periodic acid-Schiff X 700.

is detectable in some cells before the end of the first week, but by this time all show the reaction. Even after a week some has been liberated, apparently by secretion and from the cytoplasm of dead cells (fig. 22, p. 35), but the amount is usually not considerable at this stage. All types of hyaluronidase abolish the Hale and Alcian blue reaction of the extracellular material, but the intracellular material is virtually unaffected (fig. 23, p. 35). Diastase has no effect. This pattern of reaction to the enzymes persists all through the evolution of the focus.

There is abundant reticulin around each focus, and already the dust in the smaller foci is wholly intracellular and enclosed in a complete reticulin network (fig. 18, p. 30). Some of these fibres begin to stain pale red by Van Gieson's method. This loss of argyrophilia and increase of fuchsinophilia proceeds rapidly as the fibres mature.

Quartz-laden cells only rarely leave the large dust foci, but, exceptionally, isolated koniophages may be fairly numerous in the surrounding tissues. They are readily identified by their cytoplasmic mucopolysaccharide (fig. 20, p. 32) and metachromasia.

Fig. 22

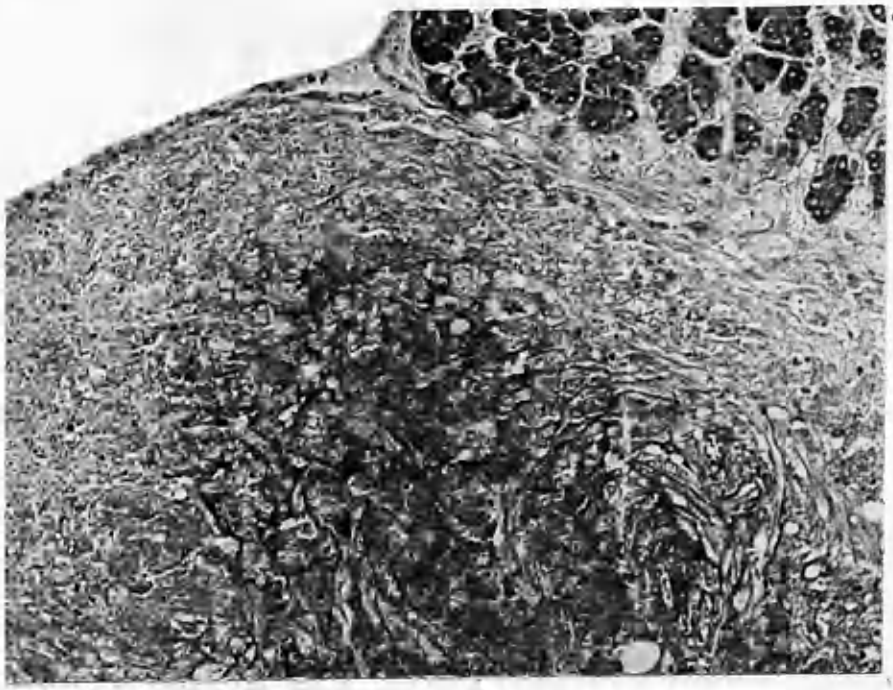
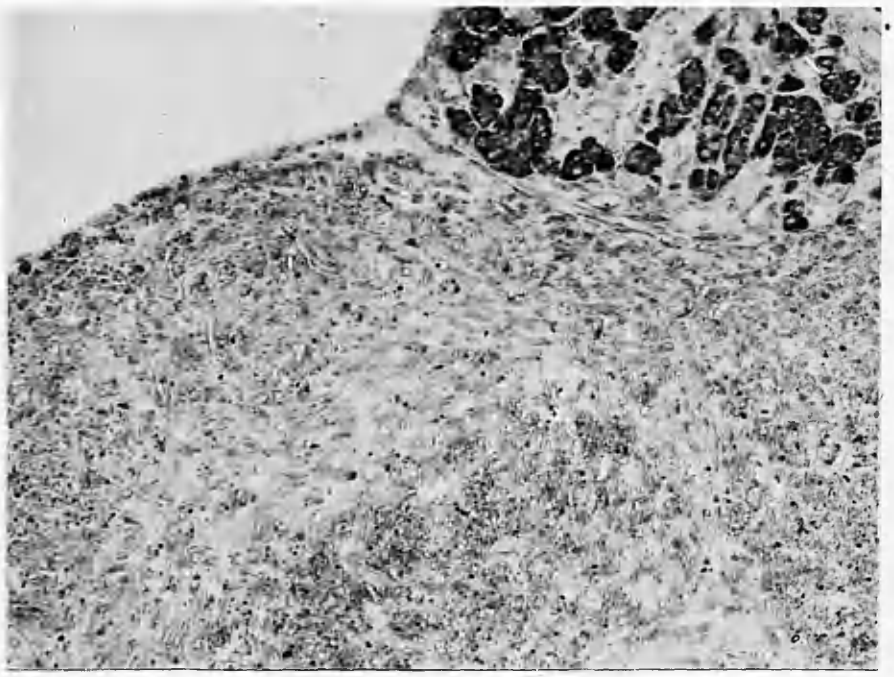


Fig. 23



Figs. 22 and 23. Abundant acid mucopolysaccharide is present in a 4-day omental lesion. In the lower figure, the mucopolysaccharide has been removed by treatment of the section with hyaluronidase. Hale's method X 200.

Polymorphs, plasma cells and lymphocytes are now relatively scanty in the vicinity of the dust foci.

By 2 months, several well-defined zones have formed in the quartz focus. The most superficial consists of dense, concentric bundles of collagen and reticulin fibres and fibrocytes (fig. 24, p. 37). The cells possess only scanty cytoplasm. Little mucopolysaccharide persists. Corresponding with the lack of cytoplasm is the loss of metachromasia in this zone; the dust is bound in collagen and fails to react. Incineration at 700°C. for 1 hour destroys the collagen, however, and restores the metachromasia of the dust. Scanty capillaries are present.

Adjoining the fibrous capsule is a cellular layer, composed of koniophages which are enclosed in a mesh of reticulin and collagen (fig. 15, p. 26 ; fig. 25, p. 38 ; fig. 26, p. 39). The collagen fibres are still haphazard in arrangement, branch like the reticulin fibres, and are not yet orientated into concentric lamellae (fig. 27, p. 40). Each cell still has a large nucleus and its abundant cytoplasm is quartz-laden (fig. 27, p. 40), metachromatic, and contains much stainable mucopolysaccharide (fig. 28, p. 41 ; fig. 29, p. 42). The zone appears to be avascular.

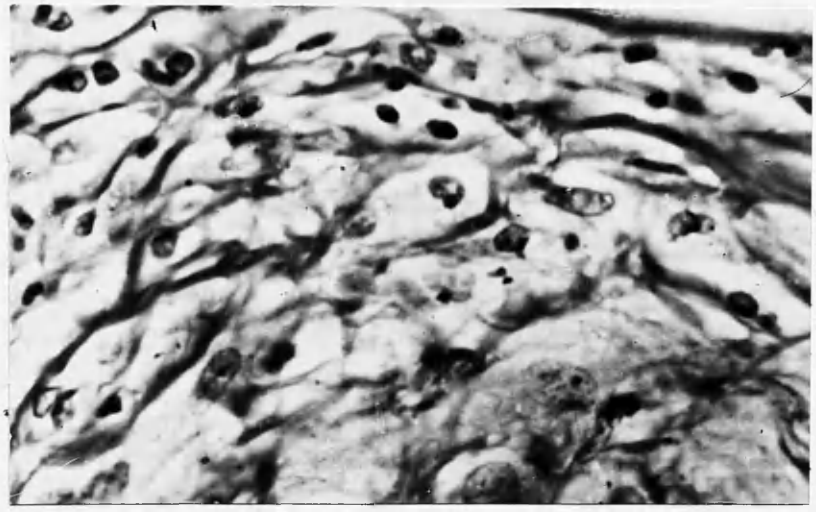


Fig. 24. In this 55-day lesion, the koniophage nuclei in the upper part (zone 1) are smaller, elongated, and fibrocytic in type. Dense collagen fibres enclose these cells. In the lower part of the figure (junction of zones 1 and 2), the cells are much larger and possess large, pale-staining vesicular nuclei. Iron haematoxylin and Van Gieson X 800.

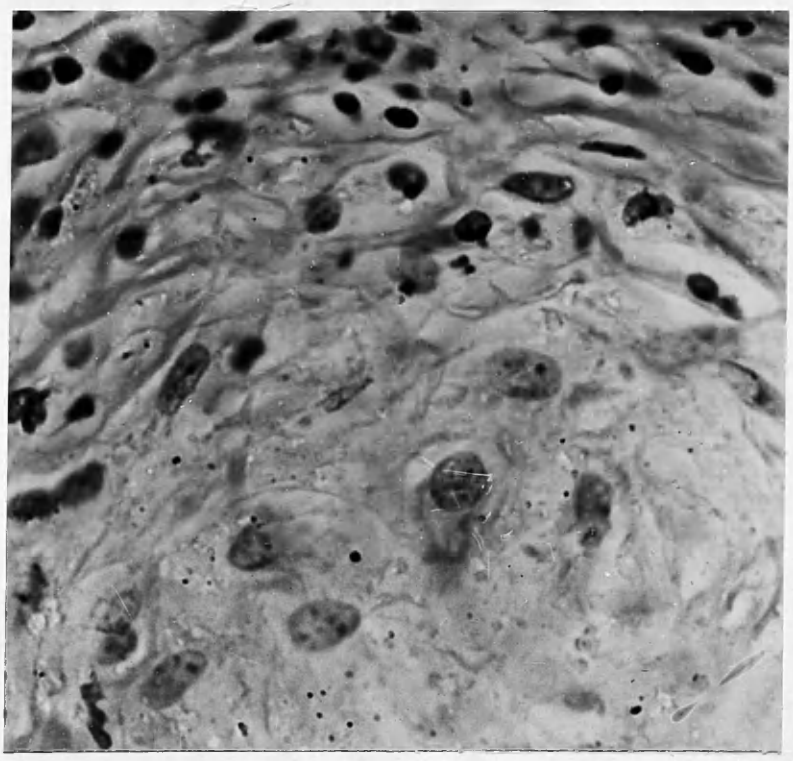


Fig. 25. This covers the same area as fig. 24 and also shows more of the cellular zone 2, which consists of large, actively-fibroblastic, koniophages. Iron haematoxylin and Van Gieson. Red filter X 800.

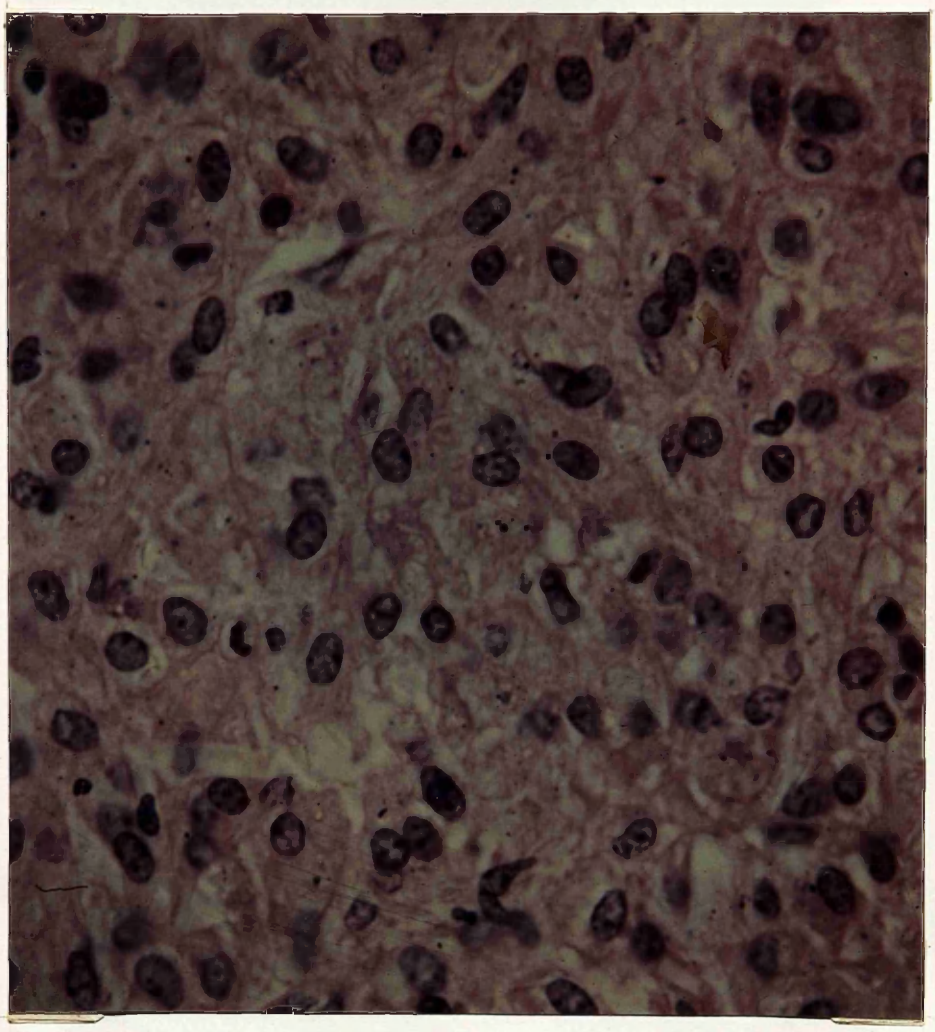


Fig. 26. The cells of the cellular second zone, though functionally fibroblasts (fig. 15, p. 26), have abundant quartz-laden cytoplasm. The small dark structures are nuclear fragments and dust particles. Haematoxylin and eosin X 800.

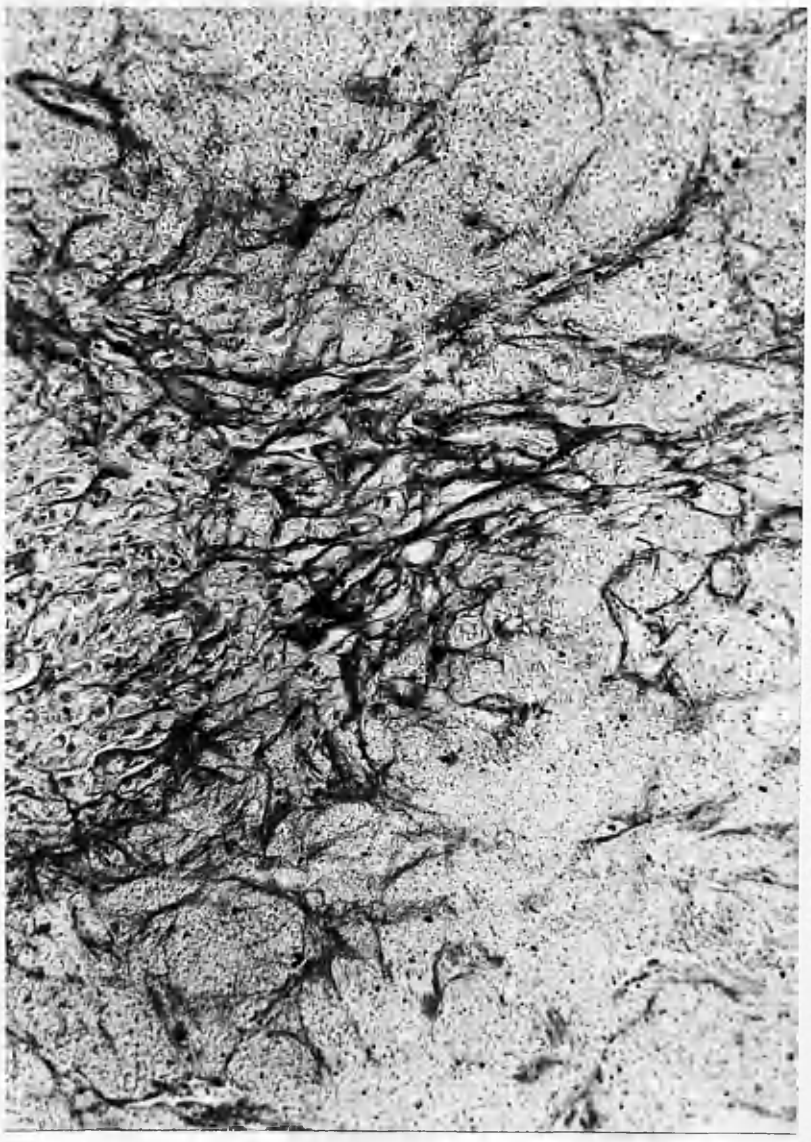


Fig. 27. From the cellular second zone, connective tissue fibres often spread far ahead of the cells into zone 3. Iron haematoxylin and Van Gieson X 150.

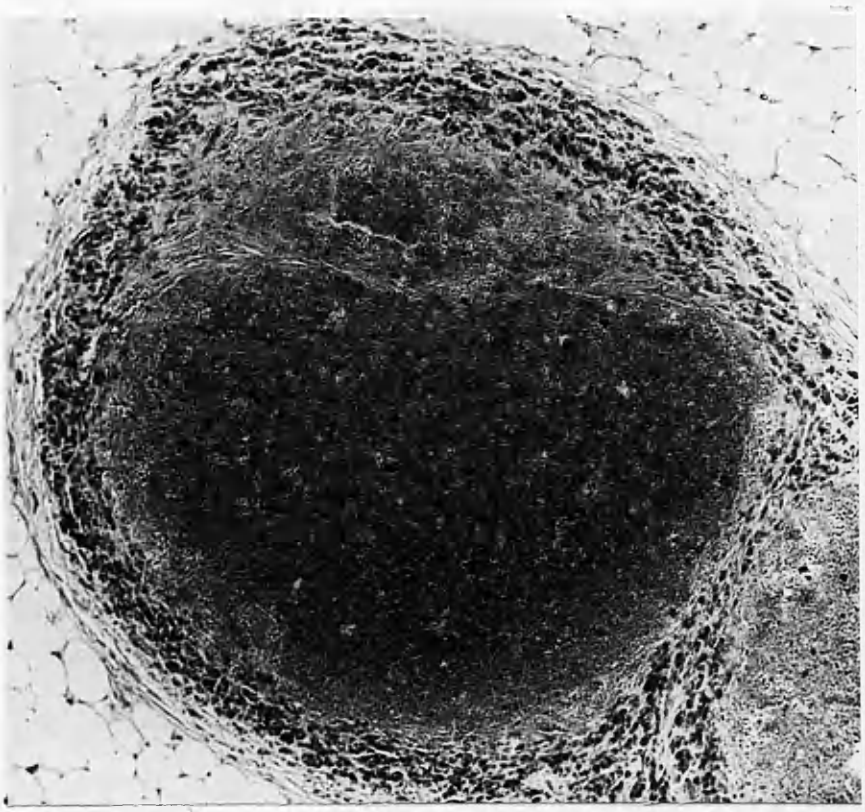


Fig. 28. The koniophages invading the dust (zone 2) contain mucopolysaccharide in their cytoplasm. Similar material is released from these cells and diffuses into the central mass of unphagocytosed dust. A reduced concentration is present round the fibres traversing the upper part of the free dust. Hale's method X 100.

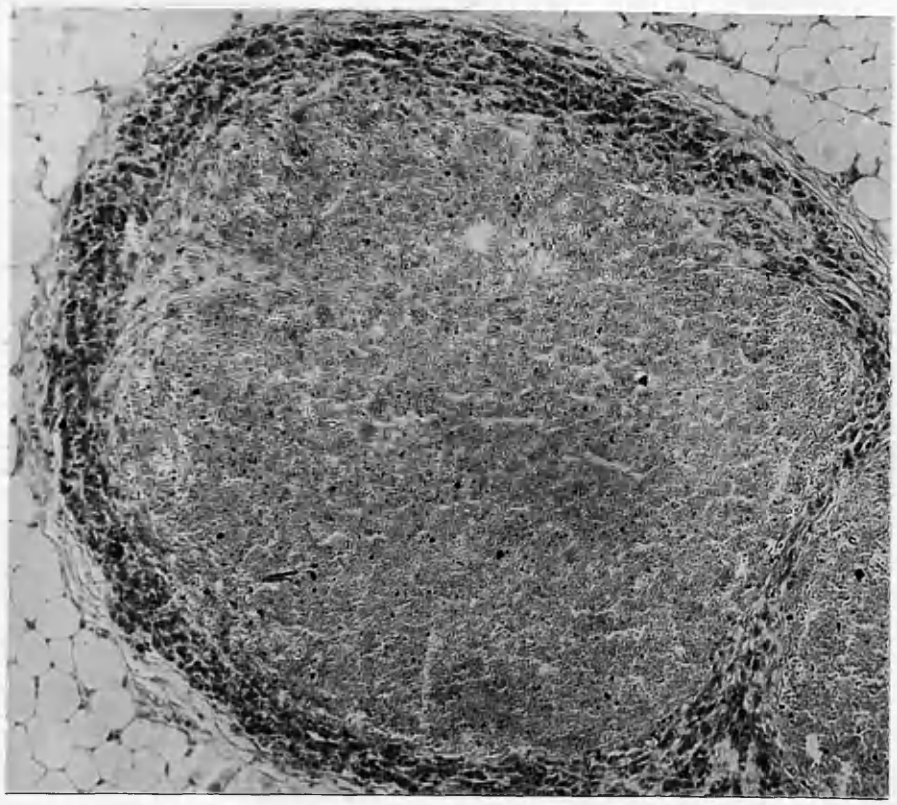


Fig. 29. Treatment with hyaluronidase removes all the extracellular mucopolysaccharide, but that in the cytoplasm is largely unaffected. Hale's method X 100.

The third zone consists almost entirely of reticulin fibres and abundant mucopolysaccharide, spreading in from the cellular second zone (fig. 28, p. 41 ; fig. 29, p. 42). The fibres sometimes completely traverse the central part of the focus (fig. 30, p. 44). The few cells lag so far behind the fibres that these seem to form without cell intervention (fig. 27, p. 40). Much faintly basophilic extracellular mucopolysaccharide is usually present, but immediately around each reticulin fibre the quantity stainable is diminished (fig. 28, p. 41). Blood vessels are not found; they appear to be unnecessary for the formation of the large quantities of fibrous tissue. However, endothelium-lined channels are sometimes detectable, and may originate almost in the centre of the dust mass. These are probably lymph channels, and red cells are not found within them. They contain fluid like lymph and occasional koniophages (fig. 31, p. 45). From their presence it seems that, despite the relatively avascular nature of the focus there must be a considerable fluid exchange, no doubt by diffusion inwards of tissue fluids. In this third zone, most of the quartz is still unphagocytosed, but the extracellular mucopolysaccharide reduces its metachromatic reaction. The reduction is sufficient to delineate the area containing mucopolysaccharide, which

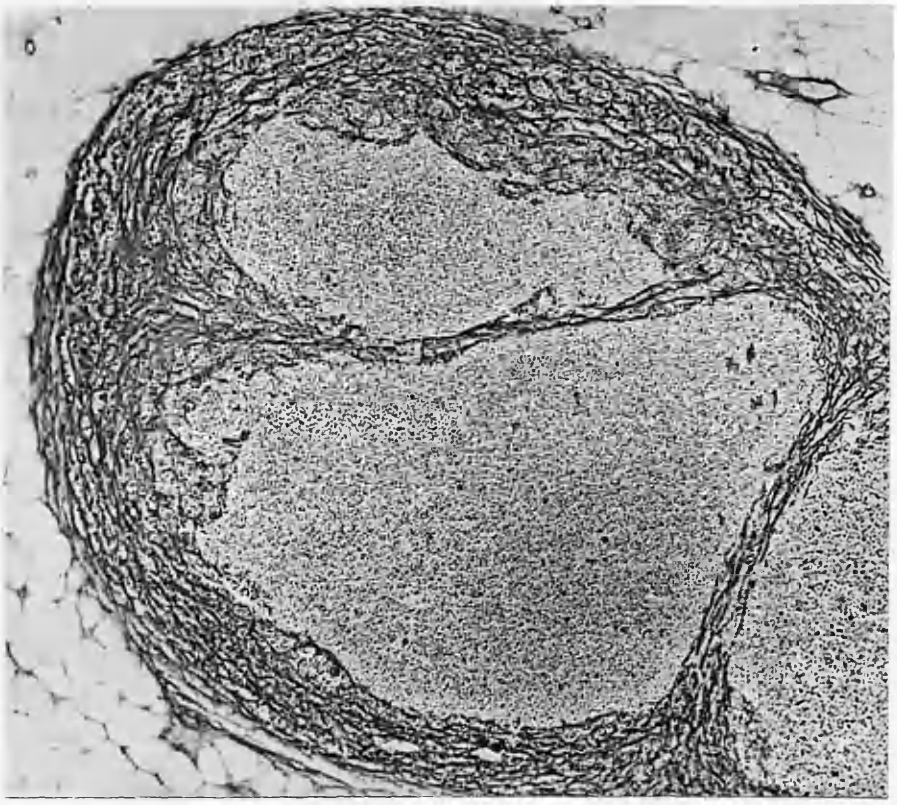


Fig. 30. Young connective-tissue fibres encapsulate the quartz lesion and others split up the main focus into smaller masses. Few cells are detectable in the region of these fibres which traverse the central zone of the free dust. Gordon and Sweets's method X 100.

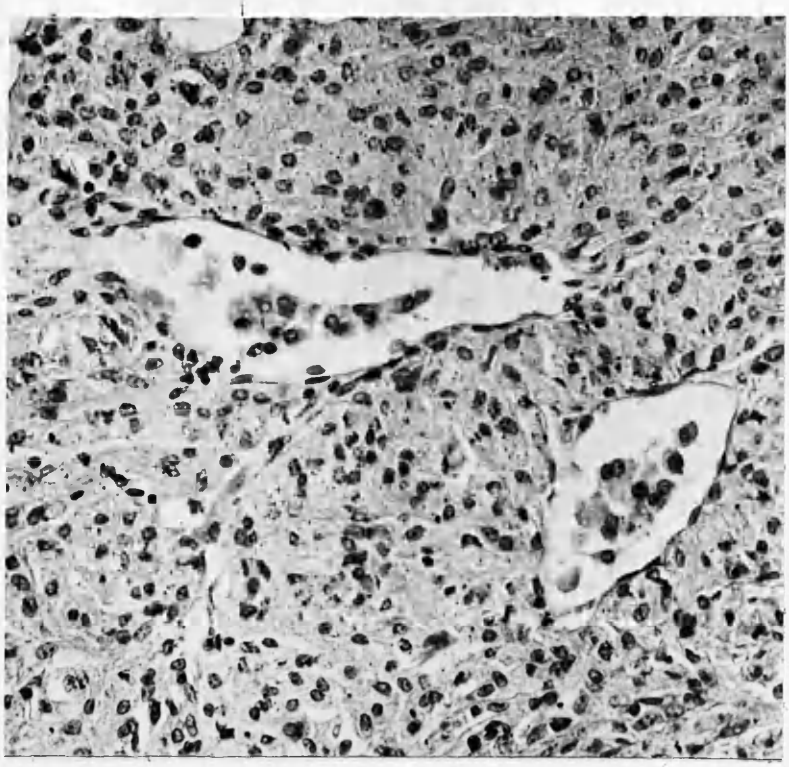


Fig. 31. There are endothelium-lined channels, usually containing lymph and mononuclear cells, in the cellular second zone. Haematoxylin and eosin X 250.

can, of course, be shown directly by the Alcian-blue and Hale methods.

Lastly, the centre of the focus consists of unphagocytosed dust. A few reticulin fibres may traverse it, and some mucopolysaccharide may diffuse in (fig. 28, p. 41 ; fig. 29, p. 42 ; fig. 30, p.44). The dust is strongly metachromatic, but occasionally it completely fails to react with thionin at any pH, even though no mucopolysaccharide can be stained and the Millon and Romieu reactions fail to detect protein in this zone. It is still possible, however, to evoke a metachromatic reaction with cresyl fast violet or toluidine blue. The quartz-laden cytoplasm of the koniophage never fails in this way to stain metachromatically.

Polymorphs are usually few in number around a focus of this age, but collections of lymphocytes and plasma cells are occasionally found (fig. 32, p. 47). However, none of these cells appears to play an essential part in the evolution of the quartz focus.

Subsequently, development of the lesion is relatively straightforward. The quartz, at least in the peritoneal cavity, exerts an unfailing chemotactic effect, and cells arrive until all the dust is intracellular. Eventually,

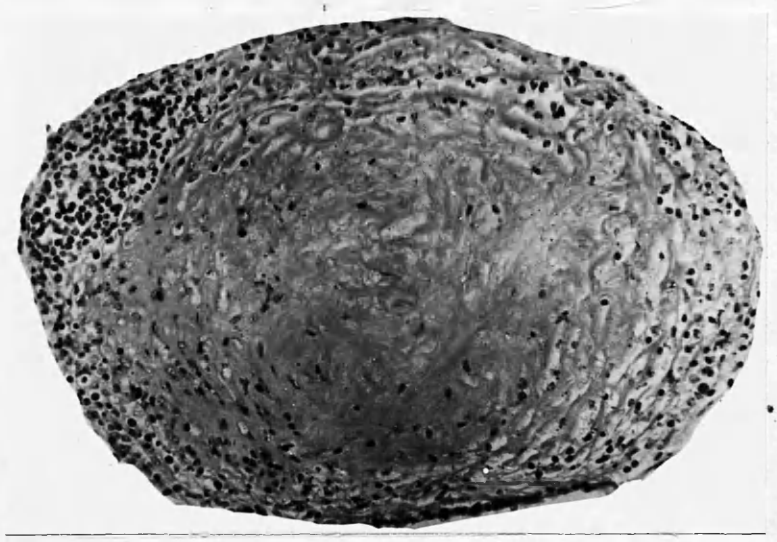


Fig. 32. This 75-day silicotic focus consists in the centre of dense, almost acellular, collagen. Collections of lymphocytes and plasma cells are occasionally found at the periphery. Iron haematoxylin and Van Gieson X 200.

the focus consists of concentric whorls of dense, hyaline collagen (fig. 32, p. 47). The nuclei are few and fibrocytic in type, and stainable mucopolysaccharide eventually disappears. If a focus is exceptionally large, it may eventually fail to evoke a phagocyte response, even in the peritoneal cavity. In consequence, much unphagocytosed dust persists indefinitely, and though this mass in the centre of the focus contains no cells it is ultimately impregnated with calcium salts, which stain very deeply with haematoxylin.

No systematic examination was made of the retroperitoneal lymph nodes, but mesenteric lymph nodes were often removed and sectioned by chance. Quartz is never abundant in these, but small collections of quartz-laden koniophages are found at times in the sinuses (fig. 33, p. 49), detected readily by means of their cytoplasmic metachromasia. It is worth noting too that lymphoid tissue is rarely found in association with the main dust masses, which can develop without the presence of this type of tissue.

The Metachromatic Reaction

This was present in the earliest (6 hour) lesions, and it suggested the presence of mucopolysaccharide on the

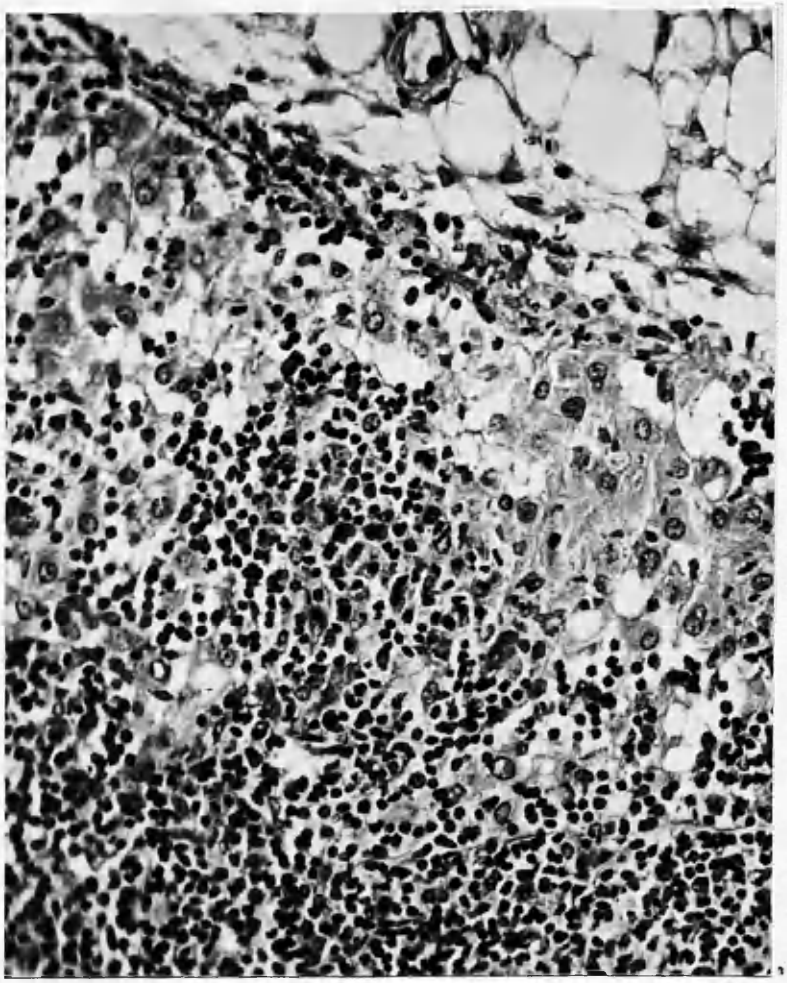


Fig. 33. Quartz-laden koniophages are present in the sub-capsular sinus of a mesenteric lymph node. They form reticulin in this site in the same way as in foci elsewhere. Haematoxylin and eosin X 400.

surface of the dust particles, causing them to aggregate and adhere to the peritoneal tissues and preventing their wide dispersal throughout the peritoneal cavity. Examination of lesions of short duration was undertaken to confirm this possibility. Each time it was found that metachromasia was present and considerable amounts of dust were already adherent. Accordingly, lesions of progressively shorter duration were studied. Even in lesions of 30 minutes' duration, these features were present intra-peritoneally and the dust in the subcutaneous tissues had already aggregated into a firm elastic mass, easily dissected out intact. For this reason the reaction was examined from the time of injection onwards.

When quartz is introduced into the peritoneal cavity, some of it adheres at once to the surface of the omentum and mesentery, so that these tissues appear diffusely and finely "dusted"; this material is bound to the surface sufficiently firmly to resist vigorous shaking during processing to paraffin. On the omentum, it forms a remarkably complete layer which progressively thickens; by 30 minutes the layer is already considerable. Small areas of the mesentery are similarly affected.

Microscopically, the dust layer which is present on the omentum and mesentery of animals killed immediately

after administration of quartz is very thin (fig. 8, p. 19). Its metachromasia is very slight but only because of the small mass of dust involved, for by 5 minutes the reaction shows up the adherent quartz well. Incidentally, the neighbouring tissues are already hyperaemic, the earliest sign of the ensuing acute inflammatory changes. Subcutaneously, metachromasia is marked even in the earliest, immediate, lesion and the presence of much suspending fluid between the aggregates of quartz particles causes only slight patchiness in staining. By 20 or 30 minutes, the quartz stains evenly and strongly (fig. 9, p. 20).

Since the metachromatic reaction was present in the very earliest lesions, it appeared to depend on some intrinsic quality in the quartz, and this was tested for in vitro. A suspension of 20-25 mg. of the dust was made in a solution of thionin, and after a period of time similar to that used for staining sections, the particles were washed twice in isotonic saline. The dust mass was only faintly purple, but examination of a smear by transmitted light, essential for determining the presence of metachromasia, showed that the larger particles were stained intensely purple. This showed that the metachromatic reaction was an intrinsic property of quartz. None of the other methods stains quartz particles in sections.

Special Features in the Subcutaneous Lesion

Quartz injected into the loose subcutaneous tissues of the mouse elicits essentially the same reaction as that in the peritoneal cavity. Several points of difference, however, are worthy of note.

The focus is relatively loose and watery in appearance for the first ten or fifteen minutes, but with care the quartz can be retained during processing. The adhesion of the particles is of remarkable strength and occurs quickly, so that within 20 minutes they form a smooth elastic mass which is readily dissected out intact.

Within a few hours, the amount of gelatinous fluid around the focus is great. Though it coagulates at once on contact with fixatives containing acetic acid, it is not possible to demonstrate mucopolysaccharide by staining methods. By 5 or 6 days, the mass exhibits a lobulated or bosselated surface, like that of the foetal kidney. Sometimes a subcutaneous mass of 6 to 9 months' duration still contains "free" dust inside a fibrous capsule, a consequence of the less vigorous macrophage response in the subcutaneous tissues compared with that in the peritoneum.

Finally, the mast cells which are engulfed within the dust are identified with difficulty because of the equally

intense purple colour of the large quartz particles, but the failure of mast cells to collect around the focus as it develops is noteworthy.

DISCUSSION

A. QUARTZ: PHYSICAL AND CHEMICAL CHARACTERISTICS

1. The Adhesive Properties of Quartz. These are so constant and characteristic that they must play a significant role in determining the nature of the tissue reaction. It was surmised that quartz "liberated" a mucoid substance which then bound the particles, and the metachromatic reaction was accepted as confirmation of this idea till it was shown to be an intrinsic property of the dust: it is considered separately below. There is other evidence, however, that mucopolysaccharide was involved in the adhesive phenomena, particularly in the subcutaneous tissues.

When lamp-black is injected intraperitoneally, the omentum of guinea-pigs becomes intensely blackened almost immediately and the pigment cannot be washed off; Buxton and Torrey (1906) claimed that this was due to a deposit of fibrin entangling the particles, but adduced no direct evidence to support the idea. Fibrin appeared to play no significant part in binding the quartz particles. It has been shown (Cooray, 1949) that mucoid material surrounds the

phagocytes of Kampmeier's foci in the pleura. The taches laiteuses of the omentum and mesentery are the peritoneal counterparts of the Kampmeier's foci, and they undoubtedly help to immobilise the quartz. This idea of the "stickiness" of the fixed tissue macrophages also explains the results with carbon. However, it completely fails to explain the fact that much of the quartz adheres to the serous surfaces in large masses quite unrelated to lymphoid or other cellular tissues, and there becomes incorporated into collagen. Even the quartz particles still free in the peritoneal cavity aggregate into masses. Therefore, though inert dusts like carbon can "dust" the omentum and mesentery in a way similar to that shown by quartz, the latter exhibits unique features.

It was only later, after publication of the earlier results (Curran, 1953), that it was realised that the firm elastic mass which forms in about 20 minutes in the subcutaneous tissues is closely related to Ranvier's "Boule d'oedeme"; in this, combination takes place between water injected subcutaneously and the hydrophilic ground-substance of the connective tissues themselves (Day, 1949), while other fluids like alcohol and ether fail to form a bulla and diffuse away. The mass of clear jelly adherent to the skin at the site of injection of aqueous solutions

appears even when the solution contains much heparin, so that fibrin, on this and other grounds, can be excluded from its composition (Edgerley, 1952). Bensley's (1934) conclusion that the jelly is a connective tissue bulla identifiable as connective-tissue ground-substance or amorphous intercellular substance appears to be correct, and from this it is clear that, in the subcutaneous tissues, indigenous mucopolysaccharides bind the quartz into the firm gelatinous mass. It is not possible to stain these mucopolysaccharides, but the reaction of the bulla on contact with acetic acid is striking. The mucoid material surrounding the macrophages in the taches laiteuses no doubt reacts in similar fashion to aqueous suspensions, but this is not sufficient to explain the adhesive properties exhibited by quartz in the peritoneal cavity, for the reasons listed. In any case, quartz exhibits its powers of agglomeration in other tissues: "The less noxious dusts, producing only the simplest of foreign-body reactions, tend to be dispersed into smaller and smaller aggregations, which become more and more widespread" and "More harmful dusts, on the other hand, tend to retain a nodular distribution, and sometimes coalescence is carried to the point of producing large confluent lesions" (Belt and King, 1945).

It is difficult to postulate a mechanism which causes quartz to behave in this way in these different sites in the tissues. Though the tissue ground-substance probably always plays a part, and indeed its participation is sufficient to explain the fixation of inert particles like carbon, the unique behaviour of quartz can be explained only by some physical or chemical property peculiar to this substance. Later, evidence is presented which suggests that a layer of colloidal silica is present on the surface of quartz. This layer might help to bind together the particles in the tissues. In support, unpublished observations show that silicate particles, which, unlike quartz, exhibit very little metachromasia and presumably have little colloidal silicic acid on their surface, show little tendency to aggregate into masses.

2. The Metachromatic Reaction of Quartz. No evidence was found in published work to suggest that particulate quartz is metachromatic, and it seemed probable that sulphated or strongly acidic mucopolysaccharide diffused throughout the dust mass soon after its introduction into the tissues and gave the purple colour. Mast cells, for example, were one possible source for such a substance.

Eventually, however, quartz particles themselves were found to react <u>in vitro</u> with the dye to produce the purple metachromatic colour (p. 51). Several explanations for this are possible, in view of the different theories of metachromasia. If metachromasia be due to a tautomeric form (imino base) of the dye in labile equilibrium with the orthochromatic form (Lison, 1935), quartz must preferentially adsorb the tautomeric. According to Michaelis (1944), however, the surface of "normally-staining" substrate adsorbs a monomolecular layer of the dye, whereas the surface of "metachromatically-staining" substrate adsorbs a polymolecular layer; on this basis high polymers of the dye molecule must form on the surface of quartz. On the other hand, the reaction might be akin to the potentially misleading colour-reaction described by de Boer and Houben (1951), who showed that adsorption of a pale yellow organic compound (p-nitrophenol), with peripheric dipoles (-OH group) in its molecule, on the surface of an inorganic salt, shifted the absorption spectrum of the molecule towards longer wavelengths, with the development of an intense yellow colour; the colour was not caused by the formation of a salt. In similar fashion, adsorption of phenolphthalein on aluminium oxide

results in a bright red colouration which is not due to an alkaline reaction of the surface.

The question of pH is important; within wide limits metachromasia does not depend on pH and should be carefully distinguished from the pH-effect of indicator dyes. Thionin, for example, was shown to be constant in its colour at least down to pH2, so that though the pH of the surface of quartz particles is now believed to be in this zone (Holt and Yates, 1954), this is no explanation of the metachromatic change in the dye.

Since publication of the earlier results, more information has been gained. Colloidal silica produces a metachromatic reaction *in vitro* with thionin (Kelley and Miller, 1935), while colloidal solutions of various sodium silicates added to a solution of toluidin blue O shift the absorption band maxima of the dye from 620 mμ to about 540 mμ and cause a visual change from blue to purple (Merrill, Spencer and Getty, 1948). It is possible to infer from these results that the surface of the quartz particles consists of a layer of colloidal silica or silicate. Holt (personal communication, 1954), has confirmed the *in vitro* metachromatic reaction of particulate quartz. His other work has a considerable bearing on the present discussion.

Studying the polymerisation of silicic acid *in vitro* and *in vivo* by means of radioactive silicic acid, Holt concludes that formation of polymers of silicic acid is probably possible in the tissues only because quartz particles form large aggregates with consequent exposure of a very large surface area (Holt and Yates, 1954). In this way, a local concentration of silicic acid high enough for the production of polysilicic acid is built up. In perfusion experiments similar to Day's (1952), colloidal silica can act like mucopolysaccharides of high molecular weight; silicic acid sols, so dilute that they have viscosities little different from that of saline, contain particles capable of reducing the permeability of tissue which has been treated with testicular hyaluronidase to remove the indigenous mucopolysaccharides of the collagenous tissue (Holt and Osborne, 1953). On this basis, it is suggested that in lesions where fibrotic tissue is forming, silicic acid may act as a filling material in the collagen network and may organise the fibrils in a manner similar to that shown by the mucopolysaccharides. In the silicotic focus, there must be not only solution of the quartz but also subsequent polymerisation of the silicic acid which is formed. The surface of a silica particle has a pH of about 2 from

formation of orthosilicic acid (Holt and Yates, 1954). In the heavily buffered tissue fluids the pH rises rapidly to 7.4, passing through the critical range of 5.5 to 6 where polymerisation is rapid. In contrast, cement dust, which can also yield a high concentration of silicic acid, is alkaline, and the silicic acid which it releases in the tissues is thus brought to the physiological level of pH 7.4 from a higher value. At no time does the silicic acid enter the pH range in which polymerisation is rapid, and consequently the polymers necessary for the production of collagen fibres are not formed. It is noteworthy that cement dust is innocuous in the tissues (King, 1947). The polysilicic acids formed in the tissues by quartz cannot be removed by any enzymic action, and thus persist to exert their harmful effect.

Two criticisms can be brought against this hypothesis: (a) Scheel et al. (1953) believe that it is unlikely that colloidal silicic acid can form in the tissue fluids, and this has been a defect in the solubility theory. However, they take no account of the unique aggregating powers of quartz particles in the tissues and the fact that quartz exerts its effects not in the tissue fluids but within cytoplasm.

(b) Dextran and starch, macromolecular substances, can act like chondroitin sulphate and hyaluronic acid in perfusion experiments (Day, 1952), but no one has suggested on this account that either substance plays any part in the formation of collagen.

Despite these criticisms, Holt's results merit consideration, for they render the solubility theory more feasible. The importance of the metachromatic reaction of the quartz particles in vitro and in tissues is apparent if it is due to the presence of high polymers of orthosilicic acid on the surface of the particles. The method would then constitute a histochemical reaction of much value, particularly if it becomes clear that high polymers of orthosilicic acid not only form in the tissues but also play the important mucopolysaccharide-like role ascribed to them by Holt.

It will be recalled that unphagocytosed dust in the centre of the foci occasionally fails to stain metachromatically with thionin. This failure is difficult to explain. Possibly protein is present in sufficient concentration to block the reaction (French and Benditt, 1953), though none is detectable by the Millon and Romieu reactions.

It may be concluded that quartz forms another exception to the idea of "true" metachromasia: the purple colour does not mean the presence of sulphated mucopolysaccharide. It may be due to colloidal silicic acid on the surface of the quartz particles. The other methods provide evidence that mucopolysaccharide appears early in the development of the silicotic focus and disappears late in its evolution. This is discussed in Sections B and C.

B. SPECIFICITY OF METHODS

It is necessary to review briefly the validity of some of the methods employed in this study, particularly those used for demonstrating mucopolysaccharides, in order to determine how much reliance may be placed on each.

1. Metachromasia. The metachromatic reaction has been the method used in most studies on the connective-tissue ground substance, particularly in healing wounds. Metachromasia means the staining of certain tissue components in a colour different from that of the dye itself. Though discovered in 1875 (Heschl, 1875), and given its name by Ehrlich in 1877, there is still considerable controversy regarding the physico-chemical basis of the phenomenon. Sylvén (1941) regarded metachromasia as "true" only when the sections were dehydrated with alcohol promptly after staining. By thus eliminating "false" metachromasia, the

method was supposed to be rendered specific for sulphated mucopolysaccharides in tissue sections. This view must now be rejected, for other acidic radicles in colloidal compounds are able to elicit the reaction, albeit more weakly than the sulphate; for example, carbonyl and phosphate groups can do so (Landsmeer, 1951), and other substances may be added to the list (Walton and Ricketts, 1954), including particulate quartz (Curran, 1952 and 1953) and ribonucleic acid (Penney and Balfour, 1949; Gomori, 1952a, p.72). However, it is still true to say (Pearse, 1951 and 1953, p.149) that acid mucopolysaccharides are most likely to evoke the reaction in sections, though it is very improbable that hyaluronic acid can do so (Sylvén and Malmgren, 1952).

The intrinsic metachromasia of the quartz, which was present over the pH range of 2.5 to 7, obscured any metachromatic reaction on the part of the mucopolysaccharide in the quartz focus. However, in the lesions produced in the mouse peritoneal tissues by 2:4 diamino-6:7 dibenzyl pteridine, there is a hyaluronidase-labile material which stains strongly by the Alcian-blue and Hale methods and is very weakly Schiff-positive, but it does not exhibit metachromasia (Curran, 1953). These results resemble so closely those of the mucopolysaccharide in the quartz focus that it may be inferred that the latter also is probably

not metachromatic.

2. Alcian blue. This method has been available only recently (Steedman, 1950) and was not used in the first experiments. Extensive use of the dye confirms the original findings. Gomori's modification of the method (1954) has no advantage over that given by Pearse (1953, p.436). With a short staining period, Alcian blue demonstrates the mucopolysaccharides of both epithelial and connective tissue origin, the latter particularly well. The results recorded in Part IV of this thesis show, in fact, that, although the method is an empirical staining one (Braden, 1954), it is the most satisfactory of all. It is unfortunate that it and the Hale method have so far been rarely used for studying lesions like the healing wound, for I have found that both techniques demonstrate the presence of a non-metachromatic mucopolysaccharide not only in this type of lesion but also in granulation tissue in several other sites, in the stroma of actively-growing tumours, and in normal connective tissues.

The mucopolysaccharide in the quartz focus stains well with Alcian blue. Previous sulphurylation of the section (Kramer and Windrum, 1953) sometimes intensifies the reaction but does not increase the area which contains stainable mucopolysaccharide.

3. Colloidal iron. Hale (1946) described briefly a method depending on the combination of dialysed iron with the sulphate group of mucopolysaccharides or with the uronic-acid groups of hyaluronic acid and subsequent colouration by the Prussian-blue reaction. Though he claimed that the iron would not combine with neutral polysaccharides or with proteins, the specificity of the method has been characterised as doubtful (Pearse, 1951), and proteins in smear preparations may give a positive reaction (Davies, 1952). A modification has been extensively used (Rinehart and Abul-Haj, 1951) and has been claimed to give a more precise demonstration of the acid mucopolysaccharides. It did not prove superior to the original Hale method, nor did that given by Gomori (1954). Despite criticisms, the Hale technique and its offspring have proved of considerable value in recent years (McGill and Holman, 1954) and its non-specificity, as shown by the hardly comparable conditions of the smear technique, is not prominent in properly stained sections. Grishman (1948) found the method useful in an extensive study of the mucopolysaccharides of various normal tissues, and hyaluronidase was used with success in conjunction with it. She concluded that it can demonstrate hyaluronic acid satisfactorily. Thus, in the vitreous humour there was abundant non-metachromatic, Hale-positive, hyaluronidase-

labile, substance.

The results are somewhat variable, so that experience and the use of numerous sections are required for reliable results. The diffuse pale-blue background is readily masked by a red counterstain such as neutral red or safranin, while the true positive reaction is an intense blue, which contrasts with and is intensified by the counterstain. Use of the latter is best avoided when assessment of the depth of staining is required after the use of hyaluronidase. In any case, the counterstain itself may show marked affinity for mucopolysaccharides or some compound in close association with them, and so obscure the result. This has been evident at times in work on cartilage matrix, but it was not a feature of the present experiment. A similar reaction occurs with Alcian blue, and the effects of sulphurylation are the same with both methods.

In sections, many different mucoid materials are Hale-positive, sometimes including mast-cell granules. Carnoy fixation was recommended by Hale for water-soluble acid polysaccharides, but Bouin's fluid and the other aqueous fixatives proved equally satisfactory; similar appearances were obtained with tissues processed by the

freeze-drying technique. The material in the quartz focus stains intensely, both within the cells and extracellularly, and/parallel results those obtained with Alcian blue.

4. Periodic acid-Schiff method. Schiff's reagent applied to paraffin sections following oxidation by periodic acid is a specific test for vicinal-OH or -OH and NH_2 groups, which occur only in carbohydrates and in a few amino-acids. There are two exceptions: (a) altered fatty acids may resist dehydration and embedding and give a positive Schiff reaction, to some extent even without oxidative pretreatment. Ceroid is one example. (b) elastic fibres in certain locations may react, again often without oxidation.

Many substances in tissues contain one or both of the linkages from which periodic acid can produce aldehyde, and McManus (1946) in his original description claimed histological rather than histochemical value for the method. Nevertheless, it has proved of great value in the study of the tissue polysaccharides and mucins since its introduction.

Hotchkiss (1948), who shares the credit with McManus for introducing the method into histology, claimed in his original description that hyaluronic acid gives a vigorous reaction. He used a "spot" test. This claim seemed to be confirmed when it was found that structures

such as basement membranes and cartilage matrix were strongly Schiff-positive. In consequence, there formed a very widespread impression that the P.A.S. method was of great value in staining connective-tissue mucopolysaccharides. This no doubt influenced Gersh and Catchpole (1949) in their choice of it for studying the origin of the ground-substance; they even postulated a secretory cycle in the fibroblast on the results obtained with it.

However, the method has been found by others to give a weak and sometimes negative reaction with various connective-tissue mucins (Halmi and Davies, 1953), including those of the umbilical cord (Lillie, 1949), while Grishman (1952) found that it gave results exactly opposite to the metachromatic method, by staining epithelial mucins brilliantly and those of the connective tissues very poorly or not at all. This led her to question the validity of the results obtained with it by Gersh and Catchpole (1949). Significantly, too, very few observers have claimed an effect on Schiff-positive structures in tissue sections with use of hyaluronidase (Balasubrahmanyan, 1953).

Other indirect evidence is available of the unsuitability of the P.A.S. method for ground-substance. "Spot" tests fail to stain very pure samples of hyaluronic

acid (Glegg, Clermont and Leblond, 1952; Davies, 1952), and even with the less pure samples which are Schiff-positive, hyaluronidase has no effect (Davies). Chemically, too, hyaluronic acid consumes little periodic acid (Meyer and Fellig, 1950); its glucuronic acid and N-acetyl-D-glucosamine units appear to be linked in such a manner that there are no free 1.2-glycol or α-amino alcohol groups. Similar results are reported for chondroitin sulphate, namely very little consumption of periodic acid and negative "spot" tests (Jorpes, Werner and Aberg, 1948).

These results are all in accord with the view which is now being recognised, that hyaluronic acid cannot be demonstrated by the P.A.S. method, while chondroitin sulphate gives a negative or very sluggish reaction in sections (Glegg et al., 1952). This deficiency of the P.A.S. method is recognised in the two standard books of histochemical methods in common use: Gomori (1952a, p.63) points out that the chondroitin-sulphuric acid in cartilage matrix stains faintly or not at all, while Pearse (1953, p.151) concludes that the ground-substance of the connective tissues does not stain convincingly. Despite these facts, the method is still chosen, apparently specifically, for work on the connective-tissue mucopolysaccharides (Benditt and French, 1953).

In view of the prevailing opinion, it was disturbing to find in the early experiments that the mucopolysaccharide which appeared to be present in the silicotic focus was feebly Schiff-positive. It is now obvious that this is the result to be expected with connective-tissue mucopolysaccharides.

5. Hyaluronidase. It is claimed that hyaluronidase can specifically abolish the staining reactions due to hyaluronic acid and chondroitin sulphate (Gomori, 1952a, p.67), but the enzymes available till now have been relatively impure, and great care is required in the interpretation of results obtained after their use on tissue sections. Many hundreds of sections were employed in the present attempt to determine the precise origin and nature of the mucopolysaccharide demonstrated in the focus. The great majority were treated with hyaluronidase of testicular origin, which affects both sulphated and non-sulphated mucopolysaccharides (Meyer, Chaffee, Hobby and Dawson, 1941). Other sections were treated with the filtrate of a 24-hour culture of Cl. welchii; the bacterial enzyme possibly acts on hyaluronic acid and chondroitin sulphate, but Humphrey (1946) found its effect on chondroitin sulphate to be negligible. A number of sections were treated with a 1:500 aqueous solution of the

venom of Bothrops jararaca, which is claimed to be specific in its action on hyaluronic acid (Campani and Reggianini, 1950). Pneumococcal and streptococcal hyaluronidases, reputed to act specifically on hyaluronic acid, were not available.

The results obtained and their interpretation must be reviewed in the light of recent criticisms of the use of enzymes as histochemical reagents (Benditt and French, 1953; French and Benditt, 1953). Of the possible sources of error described, two are especially important: (a) the enzyme protein may exercise a blocking effect on the stainable groups of the mucopolysaccharide. This had been evident early in the present experiment from the reduced intensity of the red counterstain after enzyme treatment of a section, and full allowance was always made for a possible similar effect on the staining of the mucopolysaccharide. Control sections were incubated in 5 per cent. solution of plasma to provide another, inactive, protein solution. (b) testicular hyaluronidase has a trace of proteolytic activity, shown by its power to clot milk, and destruction of a protein to which the stainable mucopolysaccharide is attached might release the carbohydrate, even in the absence of a true depolymerase. It is worth

noting from their results that trypsin, and to a lesser extent pepsin, though causing the release from cartilage matrix of hexosamine-containing polysaccharide which produced turbidity with acidified albumin solution, caused only slight reduction in metachromasia of the cartilage matrix, while testicular hyaluronidase lacked proteolytic activity in the 2½-hour test period, as shown by its failure to liberate tyrosine into the incubating medium. From this, it may be concluded that if the period of incubation is kept reasonably short, important fallacies can be eliminated. Pearse (1953, p.329) agrees that, in these circumstances, it is valid to use hyaluronidase to confirm the mucopolysaccharide nature of the substance investigated; but the use of testis enzyme alone does not permit one to determine with certainty that the labile substance is hyaluronic acid. Twenty to 30 minutes usually sufficed to removed completely the extracellular mucopolysaccharide of the quartz focus, though the convenient overnight incubation time was often used.

In summary, therefore, the mucopolysaccharide in the quartz focus stains strongly by the Alcian-blue and Hale methods and feebly by the periodic acid-Schiff technique. It is probably not metachromatic, but is labile to

hyaluronidase when extracellular. None of the staining methods can be regarded as truly specific, but a substrate that stains by two or more is very probably mucopolysaccharide in nature, and the effect of hyaluronidase confirms this. The results are characteristic of connective-tissue mucopolysaccharide. The failure of hyaluronidase to act on the intracellular material may be a consequence of fixation of the tissue, for it seems that enzyme can remove labelled mucopolysaccharide from the cytoplasm of the living chondrocyte (p. 205) but not from the fixed cell (p. 42). On the other hand, the mucopolysaccharide in the koniophage cytoplasm may be a non-labile precursor of the extracellular material.

C. ORIGIN OF MUCOPOLYSACCHARIDES

The relation of mucopolysaccharides to fibrous tissue formation is clearly of fundamental importance. At present the most widely held theory of fibrogenesis is probably that stated by Meyer (1947 and 1951). The young fibroblast secretes hyaluronic acid, a chondroitin sulphate, and a precursor of collagen into the surrounding tissue spaces. By local acidification in the immediate neighbourhood of the cells, the native soluble collagen denatures into the insoluble fibre, on the surface of which lies a sheet of the polysaccharides. The regularly-spaced acidic group

of the polysaccharide chain forms the template on which the fibres are built up. With ageing of the fibres, the polysaccharide layer becomes thinner and the hyaluronate is replaced more and more by chondroitin sulphate. Proof of origin of the mucopolysaccharides has proved difficult to obtain, however, and three sources may be considered:

1. Tissue fluids. Nageotte (1922) believed that the materials necessary for formation of connective tissue are derived from the tissue fluids, which in turn receive their contributions from the metabolism of the entire body. He categorically denied the origin of these substances from the connective-tissue cells, and was able to demonstrate that collagen fibres can be precipitated from a cell- and fibre-free acid solution of collagen by neutralisation or addition of salt (Nageotte and Guyon, 1930). This observation has been confirmed (Huzella, 1941) and heparin has been shown to be active in this way (Morrione, 1952). The relationship of this in vitro chemical reaction to what happens in the tissues is difficult to determine, but it should not be difficult to integrate Nageotte's humoral theory with the more orthodox fibroblast theory; the fibroblast would be unable to elaborate the substances required for formation of connective tissue unless it received the raw materials from the tissue fluids. At

least one hypothesis reconciles the facts of fibrillogenesis as at present determined with known processes of synthetic fibre formation, and explains formation of collagen in sites relatively isolated from cellular activity (Kramer, 1952). It suggests that mucopolysaccharides may act as anionic detergents and, by breaking down linkages in the globular form of collagen synthesized by the fibroblast, promote curling and some degree of orientation of the collagen molecules; the collagen protofibrils crystallise as the carbohydrate moiety is lost by dissociation, resulting either from a change in the local electrolyte concentration or from enzyme activity. In some measure supporting this idea, I have found that hyaluronic acid and chondroitin sulphate exert a detergent-like action on the growth of the diphtheria bacillus and alter a granular growth into a diffuse turbidity.

2. The Mast cell. According to Simpson (1950), Staemmler (1921) attributed to mast cells the function of supplying the cement substance (Kitt-Substanz) of the ground material of connective tissue. This view received support subsequently from Sylvén's work on healing of cutaneous wounds (1938-1939; 1941). Sylvén believed that the ground-substance in healing wounds was derived

from mast cells by discharge of their granules, for mast cells were fewer or had fewer granules where metachromasia was greatest. However, no correlation exists between the formation and appearance of metachromatic ground-substance and the presence of the mast cell in healing wounds in scorbutic guinea-pigs (Bunting and White, 1950). Lately, the ideas of Staemmler and of Sylvén have been revived in a slightly modified form by Asboe-Hansen (1950a) and (1953), who points out that heparin and hyaluronic acid are both composed of glucosamine and glucuronic acid, though hyaluronic acid contains no sulphate. Mast cells may contain a sulphated mucopolysaccharide closely related to both but identical with neither; suitably altered in form, this substance is then liberated as a hyaluronidase-sensitive mucopolysaccharide similar to connective tissue ground-substance. He claims that histochemical methods show not only a correlation between the number of mast cells and the quantity of hyaluronic acid in tissue, but also the formation of new hyaluronic acid from mast cells following injection of hyaluronidase into the tissues.

Though there is some support for these results (Cavallero and Braccini, 1951), several criticisms may be

levelled at Asboe-Hansen's work:-

(a) His results depend largely on the metachromatic reaction to demonstrate hyaluronic acid in paraffin sections, whereas hyaluronic acid itself is probably never shown by this technique (Meyer, 1947; Sylvén and Malmgrem, 1952), though a metachromatic substance of unknown composition may accompany it (Davies, 1952).

(b) He reasons that since heparin is Schiff-negative, and since mast cells contain Schiff-positive material, this cannot be heparin but may be a precursor. However, heparin is not always Schiff-negative (Gomori, 1952b).

(c) Paraffin sections were used. It is now claimed that artefacts inherent in this type of section make it useless for studying the liberation and dissolution of the contents of mast-cell granules into their environment (Schoch and Glick, 1953; Devitt, Samuels, Pirozynski and Webster, 1954). This matter is discussed on p. 130

There was no evidence from my present experiments that mature mast cells play any part in the formation of collagen in the quartz focus. They were conspicuously absent from the region of the developing foci, even from foci situated in the subcutaneous tissues where mast cells are very numerous.

There may be a link between the mast cell and the koniophage, however. A proportion of the koniophages come from the adventitia of arteries. In the connective tissues of the rat and mouse (Bensley, 1952), and of man (Montagna, Eisen and Goldman, 1954), mast cells are arranged in concentric circles around the walls of blood vessels, where they arise from perivascular cells indistinguishable from fibroblasts. Either the stem cells of mast cells are located in the walls of blood vessels whence they migrate into surrounding tissue, or the stimulus for differentiation of mast cells radiates from blood vessels into the surrounding tissue (Fawcett, 1953). These facts suggest that the mast cell is derived, like the locally-produced koniophage, from undifferentiated reticulum cells (Maximow, 1927), similar to the multipotent cells of the reticular syncytium of the spleen, bone marrow and lymphadenoid tissue, which exist in the connective tissues even of the adult, especially near the blood vessels. Presumably these may differentiate either to mast cells, full of heavily-sulphated mucopolysaccharide, which then play their role in the tissues, or along a slightly different path to the fibroblastic koniophage, with its less abundant or

less sulphated cytoplasmic mucopolysaccharide. By this view, the mast cell is differentiated almost completely towards mucopolysaccharide formation, whereas this function is less active in the koniophage which retains fibroblastic activities. In this context, a significant observation is recorded by Drennan (Drennan and Beare, 1954); from a careful study of the human mast-cell naevus he concluded that young mast cells are capable of forming reticulin. Asboe-Hansen's idea may be at least partly correct, despite the inadequacy of the techniques used by him for confirming it.

3. The Fibroblast. Soon after the connective tissue cells had been discovered by Schwann (1847), Kolliker (1861) expressed the view which has persisted up to the present, that the intercellular substances of the connective tissues are formed by the fibroblasts. It has, however, proved extremely difficult to obtain direct evidence of this process. In histological preparations, with only the metachromatic reaction available, results were conflicting, and though this method has been claimed to demonstrate hyaluronic acid and to show its origin from the fibroblast (Campani and Reggianini, 1950), the use of ribonuclease suggests that metachromasia of

fibroblast cytoplasm is in fact due to ribonucleic acid (Penney and Balfour, 1949). This view is now widely accepted (Grossfeld, 1954).

The arrival of the supposedly polysaccharide-specific P.A.S. technique promised to be a great help in tackling this problem, and Schiff-positive cytoplasmic granules were described in fibroblasts in situations where ground-substance was forming (Gersh and Catchpole, 1949). However, it was soon evident that the method's specificity was doubtful, and Klemperer (1950), after reviewing some of the evidence against the fibroblast as the source of ground-substance (Von Ebner, 1896-1897; Baitsell, 1915 and 1916; Nageotte, 1922), stated that in his opinion there was no proof yet that fibroblasts form mucopolysaccharides.

Till recently, the fibroblast was studied mainly in healing wounds of skin, but in addition to the new approaches offered by tissue culture (Grossfeld, Meyer and Godman, 1955) and electron microscopy (Porter and Vanamee, 1949; Gross, 1950), a number of newer histological techniques have been devised for studying this cell in the last few years, including implantation of materials such as gauze pledgets (Lattes, Blunt,

et al., 1953) and polyvinyl sponge (Boucek and Noble, 1955). Nevertheless, in a recent review of the problem (1953) Bunting and Bunting conclude that the origin of ground-substance from the fibrocyte seems probable but is as yet not definitely proved.

I originally chose to work with quartz because of its fibrogenic powers, and the surmise that mucopolysaccharide should be found in the silicotic focus was confirmed. My results contribute some information relevant to the fibroblast-ground substance controversy.

(a) It appears that the fibroblastic koniophage forms mucopolysaccharide, and that this cytoplasmic material is liberated in quantity into the focus, not only from dead cells but also by "secretion" from living cells.

(b) The scanty cytoplasmic granules present, which are strongly Schiff-positive and probably similar to those on which Gersh and Catchpole (1949) based their concept of a ground-substance secretory cycle in the fibroblast, have no apparent significance and are not related to the abundant mucopolysaccharide revealed by other methods.

(c) Though it was not possible to determine in sections the exact chemical identity of the mucopolysaccharide in the quartz focus, there is evidence that hyaluronic

acid forms at least part of the material. Its staining reactions are typical of connective-tissue mucopolysaccharide; that is, it stains well with Alcian blue and colloidal iron but weakly with the P.A.S. routine, and is only occasionally faintly basophilic. Hale (1946) claimed that his method could stain hyaluronic acid in sections. The reaction to hyaluronidase is also suggestive; the enzyme from Cl. welchii has a negligible effect on chondroitin sulphate (Humphrey, 1946), and that from snake venom acts specifically on hyaluronic acid (Campani and Reggianini, 1950). Lack of metachromasia, too, is to be expected with hyaluronic acid. Thus Grishman (1948) believed that the Hale-positive, hyaluronidase-labile, non-metachromatic mucopolysaccharide, which she found in the vitreous humor was hyaluronic acid, for the vitreous humor contains no sulphated mucopolysaccharide (Meyer, 1947).

For these reasons, hyaluronic acid may be considered to be present in the quartz focus as it develops. However, sulphated mucopolysaccharide is probably also present, as will be shown in Part III.

A recent experiment (Buck, 1953) provides the closest parallel to the results obtained in the silicotic

lesion. When rabbit tendon is cut, ground-substance begins to appear between the cells and the young fibres of the new tissues after about 4 days and subsequently increases greatly in amount. It is still present in a somewhat reduced concentration after a year. The persistence of mucopolysaccharide here for so long a period contrasts with its behaviour in skin wounds (Sylvén, 1941; Penney and Balfour, 1949; Campani and Reggianini, 1950), where it appears after 24-48 hours and disappears by 12 or 14 days.

Little is known about the functions of the mucopolysaccharides of the connective tissues, but several may be postulated:-

(a) They form a true "ground-substance", filling the tissue spaces, for example in the aorta, but especially filling pathological defects (Bunting and Bunting, 1953).

(b) They are flexible-chain polymers of high negative charge with an affinity for cations and water molecules. They may, therefore, play a critical role in regulating the metabolism of inorganic ions and water. A change in concentration or molecular size would greatly modify the capacity of the connective tissue to bind water and salt, and so the ground-substance may act as a selective

and controlled barrier between the circulation and parenchymal cells. I have found that chondroitin sulphate *in vitro* functions as a cationic exchange resin with calcium, and Sylvén (1950) has suggested that the mucopolysaccharides of the hair bulb may act as labile sulphur-bearing compounds, supplying the sulphur required for the synthesis of the keratin amino-acids. The ability of hyaluronic acid to form hydrophilic colloids which bind the water of the tissues (Meyer and Rapport, 1951) is concerned in the swelling of the sexual skin in monkeys (Duran-Reynals, Bunting and Van Wagenen, 1950) and perhaps in myxoedema (Brewer, 1951).

(c) Sulphated mucopolysaccharides may play a fundamental role in the mechanism of inflammation, by causing release of histamine and a consequent increase of capillary permeability (Meyer, 1953). Changes of considerable magnitude occur in the hyaluronic acid of the tissues in inflammation (Barer, 1952). This role is mentioned again in Part II.

(d) Sulphated mucopolysaccharides may prevent the irregular production of connective-tissue cells (Balazs and Holmgren, 1949), and so prevent the growth of tumour tissue. There may be antagonism between sulphated and sulphur-free mucopolysaccharides.

(e) A protective effect on surfaces liable to minor trauma is revealed by the healing effect of chondroitin sulphate on gastric ulcer (Crandall and Roberts, 1933), and on erosion of the gizzard in chicks (Bird, Oleson, Elvehjem and Hart, 1938).

(f) The role postulated by Meyer for mucopolysaccharides in collagen formation has already been mentioned. The evidence presented here suggests that they play an essential part in the formation of the silicotic focus. They appear to be secreted by the fibroblastic koniophage into its environment. This cell differs in some respects from the conventional histological picture of a fibroblast, but the only true criterion is apparently satisfied; it forms abundant reticulin and collagen (fig. 15, p. 26 ; fig. 27, p. 40).

The silicotic focus in the mouse closely resembles the human lesion, and changes comparable with those described here probably occur also in the human silicotic focus, for the inflammatory reaction is fundamentally identical in all mammals.

D. THE DUAL ROLE OF THE KONIOPHAGE

At present most histologists believe that fibroblasts are differentiated cells which do not give rise to other

types of free cells of the connective-tissue substance. The fibroblast is recognisable on morphological grounds and even more dogmatically on the results of "intravital" staining. By the latter technique, the cytoplasm of the fibroblast, typically elongated or star-shaped, usually remains colourless when, for example, neutral red is employed. In contrast, the tissue macrophages (histiocytes) store abundant dye. However, as Cappell (1929) states, "The capacity for vital staining is not a hard-and-fast attribute of certain cells alone, but is rather to be regarded as an indication of the functional activity of the cells at the time of examination, which may be modified under the influence of environmental conditions" (p.628), and "Frequently it is a matter of considerable difficulty to determine to which group a particular cell belongs, even in the normal resting state. When the cells have been stimulated by injection of vital stains, this difficulty is greatly increased, and cells of apparently intermediate character are frequently met with" (p.614). Again, all the relations of the fibroblast to other mesenchymal and reticular cells have not yet been determined (Maximow and Bloom, 1953, p.56); thus, in the loose connective tissue of mammals, under physiological conditions, transitional forms

between macrophage and fibroblast are rare, but in inflamed tissue the sharp limits between the cells are effaced in certain stages (pp. 57 and 58), and there is evidence that fibroblasts can turn into macrophages and vice versa (p.63). Tissue culture results support this view (Bloom, 1931), and it is possible by altering chemical conditions to turn fibroblasts into macrophages, or reticulum cells into macrophages and fibroblasts (Cameron, 1952, p.108). The use of metallic-impregnation techniques with tuberculous granulation tissue also suggests the possibility of macrophage-fibroblast transitions (Marshall, 1953).

Without doubt insufficient attention has been given in the past to the nature and strength of the stimulus applied to the tissues when classifying the types and functions of the cells involved in the reaction. When, as with quartz, the stimulating effect is powerful and sustained, the rigid boundaries erected by the histologist are broken down; the macrophages which ingest quartz particles are themselves fibroblastic thereafter. Quartz is unique in its power of stimulating to collagen production those cells which ingest it in particulate form (fig.15, p. 26).

Willis (1948, p.642) holds typically strong and clear views on what is termed the intermutability of mesenchymal tissues: "In the past, students of normal histology have been too apt to assume that the different kinds of tissues and cells in the adult are permanently-determined invariable structures, each a distinct immutable species capable of producing by proliferation cells of its own kind only. A study of pathological histology, i.e., of what the various cells can be and do in all manner of abnormal environments, soon corrects this error and shows that great transformations of cellular structure — metaplasias — are possible in most tissues. The cells have much wider potencies for differentiation than are ever displayed in health". These views are in accord with the results presented here. The phagocytic and fibroblastic propensities of the cells are both so well developed that the terms phagocyte and fibroblast are equally applicable. Koniophage is a useful term, though it does emphasize only the phagocytic activity.

When Kettle (1932b) postulated deposition of collagen round silica-containing phagocytes by a process of gelation of an intercellular colloidal sol, apparently without fibroblastic intervention, he was obviously concerned with the same phenomenon as recorded here. Assuming that

the cells ingesting the quartz must therefore be exclusively phagocytic, he was exercised to explain the origin of the collagen fibres, and in effect adopted Nageotte's idea (1927) regarding formation of collagen fibres, which denies the participation of the fibroblast. Only one feature supported the "extracellular" theory of fibre formation in the present experiments, namely the spread of the reticulin and collagen fibres far ahead of the main mass of cells (fig. 27, p. 40). However, careful examination, requiring even serial sections, always revealed cells in the vicinity of these fibres, though the cells did seem able to induce fibre formation at a distance presumably by secretion of all the materials, including mucopolysaccharide, into their surroundings.

Recent results with the electron microscope agree with these views (Wassermann, 1954); in some sites, fibrils probably form within the exoplasm of the fibroblast and are dispensed by the cells together with a matrix, while in others the cell may be slow in the production of fibrils and may deliver first a certain amount of exoplasmic material in the form of aggregates of macromolecules from which fibrils and ground-substance afterwards emerge. In other words, the same process may take place either in conjunction with the cell or outside its border depending

on whether fibrils and ground-substance are produced simultaneously or one after the other.

In this way, the "classical" theory is reconciled with Nageotte's assertion that only the tissue fluids can supply the materials necessary for formation of collagen, for the fibroblast must obtain the raw materials from the tissue fluids before reorganising and liberating them into its environment as fibrils and ground-substance.

Another cell with dual capacities like the quartz koniophage is the endothelioid cell of the tubercle follicle. It phagocytoses the tubercle bacilli and, stimulated by bacterial phospholipid, forms the reticulin network throughout the follicle (Fresen, 1949-1950; Marshall, 1953). The endothelioid cell can also store pigment (Fresen) and take up finely-divided carbon (Markham and Florey, 1951).

The remarkable persistence of the chemiotactic influence exerted by quartz is noteworthy. Exceptionally, and usually in the subcutaneous tissues, the influence fails and only a capsule of cells and collagen forms. It is, in fact, this progressive emigration of cells which gives the quartz lesion its unique and macroscopically readily recognisable proliferative nature (fig. 3, p. 14).

Perhaps cells are killed over this long period in numbers sufficient to release chemiotactic "break-down" products of tissue. In the foci which show few signs of reaction for the first few days (fig. 16, p. 27), necrosis of tissue is initially slight and the reaction slowly increases only as cells migrate and are killed. Belt and King (1945) believed that in experimental pulmonary lesions a secondary stimulus such as infection often appears to be necessary, to activate the dust foci. According to them, this secondary stimulus breaks down the silica-laden cells and liberates again the quartz which then causes a renewed inflammatory reaction. Their findings may not be relevant to the case in point, for in the present experiments the quartz was inert even when extracellular. The phenomenon was responsible for much of the difficulty, as will be seen in Part II, in assessing the inhibitory effect of cortisone and ACTH on the inflammatory response to quartz.

E. RETICULIN-COLLAGEN RELATIONSHIP

The relationship between reticulin and collagen has been disputed since Mall (1888) failed to obtain gelatin by boiling lymph-node reticulin. Reticulin differs from collagen in many respects:- morphology; formation of a

branching network; failure to exhibit birefringence when examined by polarised light; failure to swell in the presence of dilute organic acids; poor fuchsinophilia when stained by Van Gieson's method; and argyrophilia when appropriately treated with silver. These are the main points of difference, and many believed that they were sufficient to prove that the two fibres were quite distinct (Foot, 1928). However, the apparent conversion of reticulin to collagen in infective granulomata and in other conditions has been pointed out (Foot, 1925; Miller, 1927). In healing wounds too, the two types of fibre are laid down in close association, and it was debated whether the reticular fibres undergo a chemical change in order to mature to non-argyrophilic collagen fibres or whether the differences were due to purely physical factors. For example, reticulin may be embedded in polysaccharide or a polysaccharide may be adsorbed on reticulin and this other material may then be responsible for the difference in staining reactions, even though the chemical structure of reticulin and collagen are in fact identical. Examination of the "fibrinoid" material in the rheumatic and rheumatoid nodules (Glynn and Loewi, 1952) lends support to this view. In "fibrinoid", the collagen fibres are

disintegrated, and there is at the same time an influx of polysaccharide material. This polysaccharide becomes at some time or other more intimately related to the disintegrated fibres, which are then strongly positive to silver impregnation.

The almost invariable association of reticulin with collagen in the body accounts for most of the difficulties in investigating the precise nature of reticulin. In the quartz focus, the appearances are straightforward, with relatively few elements compared, for example, with a healing cutaneous wound, and it provides an excellent lesion for studying reticulin-collagen relationship. The spatial arrangement is ideal, with a "growing edge" where the elements are forming (fig. 13, p. 25 ; fig. 27, p. 40). Moreover, the changes occur slowly, allowing ample time for determining the importance of the state of maturity of the fibre. Each focus consists after a few days of a relatively pure culture of koniophages at the periphery, and an argyrophil network enclosing the individual cells begins to appear in 48-72 hours (fig. 13, p.25). By 7 or 8 days the fibres begin to exhibit red colouration by Van Gieson's method, which proves to be a surprisingly reliable and almost histochemical method

for collagen. At the same time, the intense argyrophilia of the fibres slowly decreases till eventually the fibres stain by this method only the golden-yellow colour of collagen. The collagen fibres initially branch and form a network enclosing the cells (fig. 24, p. 37), but as the focus matures and the cells atrophy they re-orientate and form compact parallel bundles which enclose the focus concentrically (fig. 32, p. 47). Mucopolysaccharide is present between the fibres, but it is not possible by histology to determine the changes which occur on the surface of the reticulin fibre during its transition to collagen. Mucopolysaccharide may be abundant around both types of fibre, so that the differences in the properties of collagen and reticulin are not simply due to the mere presence of mucopolysaccharide. A more intimate association between collagen fibre and mucopolysaccharide than is detectable in sections must be present if this theory be true. This statement is at variance with Pearse's results (1950). He found that normal brown collagen fibres frayed in places into individual reticulin fibres, and where fraying was not complete the broad collagen bands were seen to be made up of individual reticulin fibres arranged in parallel bundles. The process was experimentally reproducible by the action

of Cl. welchii hyaluronidase, which presumably broke down the cement substance between the reticulin fibres. By contrast, in the quartz focus fibres may traverse a zone rich in mucopolysaccharide and yet still retain strong argyrophilia, even though they appear to have adsorbed much of the stainable mucopolysaccharide in their immediate surroundings (fig. 28, p. 41), and the use of hyaluronidase does not increase the argyrophilia of the fibres.

Kramer and Little (1953) have recently shown that the protein of reticulin is closely related to collagen in its reaction to enzymes. The electron microscope appearances of the fibrillar component of each are identical, both possessing the characteristic 640 Angstrom periodicity (Gross, 1950), and the X-ray diffraction patterns are similar. In other words, the fibrillar component of reticulin is almost certainly a variety of collagen. Collagen and reticulin have been compared to rope and linoleum respectively; the fibrous elements in both may be composed of the same material but their arrangement is different, and in reticulin the fibres are embedded in an amorphous matrix (Kramer and Little, 1953). There is nothing in this analogy to contradict

the findings in the silicotic focus, but it is noteworthy that the extensive investigations of these authors, like those recorded here, fail to elucidate the precise physical and/or chemical relationship between the mucopolysaccharide and the fibrous protein which confers on reticulin its special staining characteristics.

CONCLUSIONS

Silicotic foci produced in the peritoneal cavity of the mouse provide valuable material for studying collagen formation. Cells which phagocytose finely-divided quartz, the so-called koniophages, are stimulated to intense collagen formation, and mucopolysaccharide appears both in the cytoplasm and extracellularly.

On the surface of quartz particles there is a layer which stains metachromatically, and which may consist of colloidal silicic acid. In the development of the focus, this substance may play a role similar to that postulated for endogenous mucopolysaccharide in collagen formation; unlike the latter, however, it is unaffected by any enzyme and so can exert an effect for a prolonged period. On this account, the metachromatic reaction may constitute a valuable histochemical method. Reticulin and collagen are almost certainly chemically identical,

and both types of fibre can form at a considerable distance from the fibroblasts. Reticulin matures into collagen, and though the accompanying change in staining reaction is probably related to the mucopolysaccharide in the region of the fibres, the exact nature of the altered relationship of mucopolysaccharide to fibre is not clear.

PART II

THE EFFECT OF CORTISONE AND ADRENOCORTICOTROPHIC HORMONE (ACTH) ON THE DEVELOPMENT OF THE SILICOTIC FOCUS IN THE MOUSE

INTRODUCTION

The dramatic effects of cortisone and adrenocorticotrophic hormone (ACTH) on the manifestations of rheumatic diseases, and on some not classified as rheumatic, emphasized the importance of the connective tissues in a variety of disorders. However, it was apparent soon after the introduction of these hormones for therapeutic purposes that they exerted other important effects of a less desirable nature. Thus, surgical wounds failed to heal (Ragan, Howes, Plotz, Meyer and Blunt, 1949), apparently from inhibition of granulation-tissue formation (Creditor, Bevans, Mundy and Ragan, 1950); among the effects were a diminution of cellular elements, the sparse proliferation of fibroblasts, and the relative lack of ground substance. In animal tissues, the hormones exerted a powerful inhibitory action on the inflammatory reaction in lesions which included wounds of skin (Ragan et al., 1949), turpentine granulomata (Shapiro, Taylor and Taubenhaus, 1951), the tuberculin reaction (Harris and Harris, 1950), and implanted gauze pledgets (Lattes, Jessar, Meyer and Ragan, 1953). They also greatly retarded the phagocytic response to particulate materials such as carbon (Spain,

Molomut and Haber, 1950).

It was apparent that the quartz focus was a suitable lesion in which to assess the various effects of the hormones on the inflammatory reaction.

MATERIALS AND METHODS

Quartz lesions were produced in mice exactly as described in Part I of this thesis (p. 8). Cortisone acetate (Merck) was used, diluted with sterile saline so that 1 ml. contained 2 mg. The ACTH was Acthar (Armour) brand. The following experimental groups of animals were used:-

A. CORTISONE AND THE DEVELOPING FOCUS

1. Twenty mice received 0.4 mg. cortisone daily for 14 days. Four hours after the second injection 20 mg. quartz were given intraperitoneally. The animals were killed 1, 2, 3, 5, 7, 8, 10, 14, 21, 31, 48, 62, 84, 112 and 122 days after the quartz injection.

2. Fifteen mice were given quartz and cortisone as series 1, but in addition from the 14th to the 90th day each received a daily maintenance dose of 0.1 mg. cortisone. Animals were killed 1, 14, 21, 28, 31, 48, 62, 84, 112 and 122 days after the quartz injection.

3. Forty five mice received 1 mg. cortisone daily for 4 days and thereafter 0.5 mg. daily for 42 days.

Each was given 20 mg. of quartz intraperitoneally and 5 mg. subcutaneously on the ventral surface after the 4th dose of cortisone. Animals were killed immediately and at intervals of 5, 15, 30 and 90 minutes, 4 hours and 24 hours after the injection of quartz. Others were killed after 2, 4, 7, 10, 15, 22, 32, 39, 55, 80, 116 and 145 days. In this experiment, the dosage of cortisone was much higher than in series 1 and 2. The effect of the cortisone on the subcutaneous focus and also on the earliest reactions here and in the peritoneal sac was studied.

B. CORTISONE AND THE MATURE FOCUS

Fifteen mice received 20 mg. quartz intraperitoneally. After 90 days, a 14-day course of cortisone (0.4 mg. daily) was given. Animals were killed 7, 31, 62, 84, 91, 92, 93, 94, 96, 102, 106, 113, 129 and 158 days after the quartz injection.

C. ACTH AND THE DEVELOPING FOCUS

This series was identical with A1 except that each mouse received 1 mg. ACTH subcutaneously morning and evening in place of cortisone.

D. CONTROLS

Twenty four mice were treated in all respects as series A1 except that sterile normal saline was substituted

for cortisone. Another 35 mice acted in similar fashion as controls for series A3.

In all experiments, tissues were removed and treated as described in Part I (p. 9).

RESULTS

A. CORTISONE AND THE DEVELOPING FOCUS (GROUP A)

The mice in the earlier experiments (A1 and A2) all received cortisone a day before the quartz injection, and some (A2) got a small maintenance dose for 3 months. In the later experiment (A3), the dosage was increased, given for 3 days before the administration of quartz, and continued for as long as the resulting high mortality would allow. Despite this intense course of treatment with cortisone, the results in no way differed from those in the earlier experiments (A1 and A2), and all three series will be considered together.

SYSTEMIC EFFECTS. These testify to the effectiveness of the dosage of the hormone. Cortisone causes atrophy of the thymus, spleen, adrenals and other tissues, and there is consequently marked loss of weight which is slowly regained, so that by 6 or 7 weeks even the animals still on a maintenance dose of cortisone are of the same average weight as the controls. The subcutaneous quartz foci often ulcerate through to the surface, but

this never occurs in the control animals. Eosinopenia is present. Mortality is high with large doses of cortisone; 8 mice died in series A3, which received 1 mg./day for 4 days and then 0.5 mg./day for 42 days. The causes of death are various, but generalised infection is common; in some there is cellulitis of the abdominal wall, while others have multiple abscesses, commonly in the kidneys.

LOCAL EFFECTS

Macroscopic Appearances. There was no detectable effect from cortisone in animals killed immediately after injection of quartz. In particular, the subcutaneous gelatinous bulla was identical in controls and treated.

By 30 minutes, however, hyperaemia of the tissues, already well developed in the controls, is absent in the cortisone series. Vascular congestion is sometimes just detectable in these after 1½ hours, but is far less than that in the controls.

By 4 hours, it is notable that the gelatinous exudate found in peritoneal cavity and subcutaneous tissues of controls is absent in the treated animals.

By 24 hours, it is apparent that cortisone is delaying fixation of the dust by the peritoneal tissues. This is particularly noteworthy on the abdominal wall;

here, dust nodules rarely form and at most are minute, less than 1 mm. diameter, while large aggregates have already formed in the controls (fig. 1, p. 12). These large foci are accompanied by abundant free fluid in the sac, but fluid exudate never amounts to more than a trace in the peritoneal or subcutaneous tissue of the treated mice.

By 3 days, free dust, rarely found in the controls, is invariably still present in the cortisone series. In these, it persists, sometimes for more than 3 weeks. The relatively small amounts of dust taken up by the tissues form small lesions which appear "dry" on the surface when compared with the smooth and more "fleshy" aspect of the foci in the controls (fig. 2, p. 12 ; fig. 3, p. 14) which are also, on average, several times larger. Already these control lesions show the proliferative response typical of quartz (fig. 37, p. 108).

By 7 days, a specific and characteristic lesion develops in many cortisone-treated mice. A thin fibrous membrane or plaque, "pearly" in appearance, forms on the abdominal wall, and occasionally on organs such as the kidneys or spleen. Its presence allows instant recognition at post-mortem of the cortisone-treated animal. Found any time after a week, it is often particularly well-developed

from 70 to 90 days. It may be extensive, even 2 cms. across, and after 3 months it tends to develop a finely nodular aspect (figs. 34 to 38, pages 106 to 108). It replaces the large heaped-up proliferative mass found on the abdominal wall of the controls. This is never seen in cortisone-treated animals. Cortisone exerts a somewhat similar effect in the subcutaneous tissues. The wrinkled, bosselated type of focus there becomes a smooth-surfaced, flattened lesion under its influence.

When cortisone administration is stopped (series A1), the mice soon regain their expected weight, and within 7 or 10 days the quartz lesions in the tissues are indistinguishable from the controls. They quickly enlarge and become more fleshy, and ultimately densely fibrous. However, no matter how long the period, even up to 4 months, that has elapsed since the animal received cortisone, there is often the specific and well-developed fibrous plaque on the abdominal wall.

In the mice which received a daily maintenance dose of cortisone (series A2 and A3), the quartz foci continue to be smaller than the corresponding control lesions, up to approximately 7 weeks. Thereafter, it is not possible to detect any reduction in size from the hormone's administration.

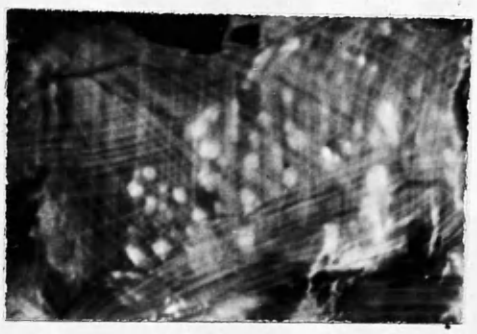


Fig. 34. The thin fibrous plaque on the serosal surface of the abdominal wall of a cortisone-treated mouse, which received intraperitoneal quartz 75 days previously, has become finely nodular. Compare with fig. 37. X 3.

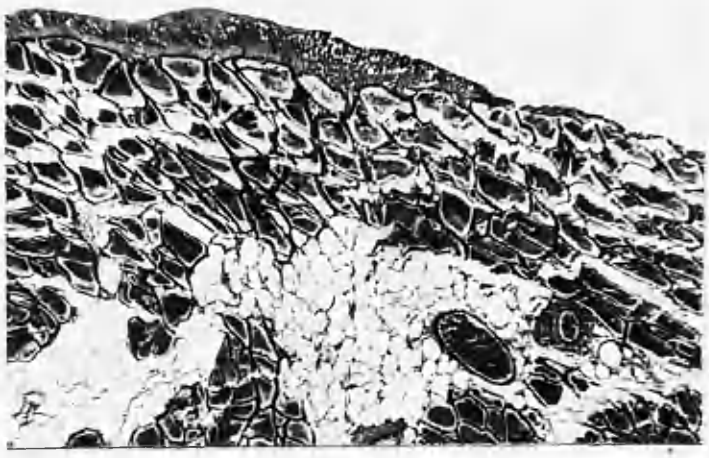


Fig. 35. The plaque on the abdominal wall of a cortisone-treated mouse consists of dense reticulin and collagen. Gordon and Sweets's method X 60.

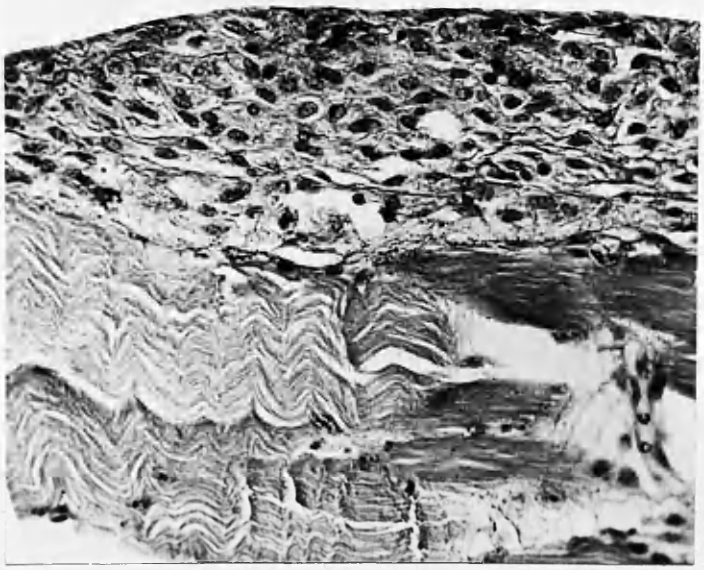


Fig. 36. The plaque on the abdominal wall surface consists at first of quartz-laden koniophages, which are ultimately converted into dense collagen even though cortisone administration is continued. Haematoxylin and eosin X 400.

Fig. 37. This heaped-up ("proliferative") lesion on the abdominal wall of control animals is not seen in cortisone-treated mice. X 5.

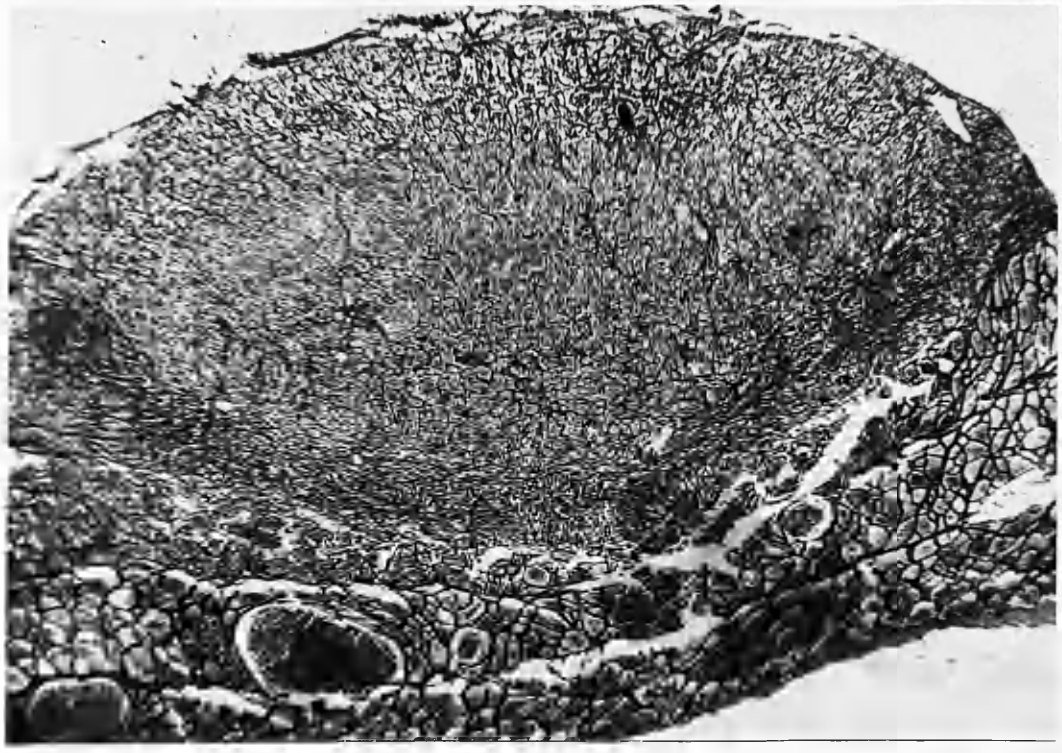


Fig. 38. This shows the microscopic structure of the control lesion on the abdominal wall. Compare with fig. 35. Gordon and Sweets's method X 60.

Microscopic Appearances. In contrast with the striking effects of cortisone, which are macroscopically readily recognised, the microscopic differences are somewhat less marked.

As pointed out in Part I (p. 22), the fact that occasional quartz foci show very little reaction even after several days (fig. 16, p. 27) makes the assessment of the effects of cortisone and ACTH more difficult, and necessitates the study of a very large number of lesions, in order to determine the effects of the hormones. Animals killed immediately after receiving quartz show no effect from cortisone, but by 1½ hours, treatment with it leads to a marked reduction of the vascular response and of the quantity of fibrinous exudate around the larger foci.

By 24 hours, this diminution in the acute inflammatory response under its influence is pronounced, and comparatively very few cells are present around the treated lesions (fig. 39, p. 110 ; fig. 40, p. 111). All types of cell are so affected. The effect on the cellular response is particularly evident in foci located in the loose areolar subcutaneous tissues, which, unlike the peritoneal tissues, are sparsely cellular but, when injected with quartz, become the seat of a very pronounced leucocytic

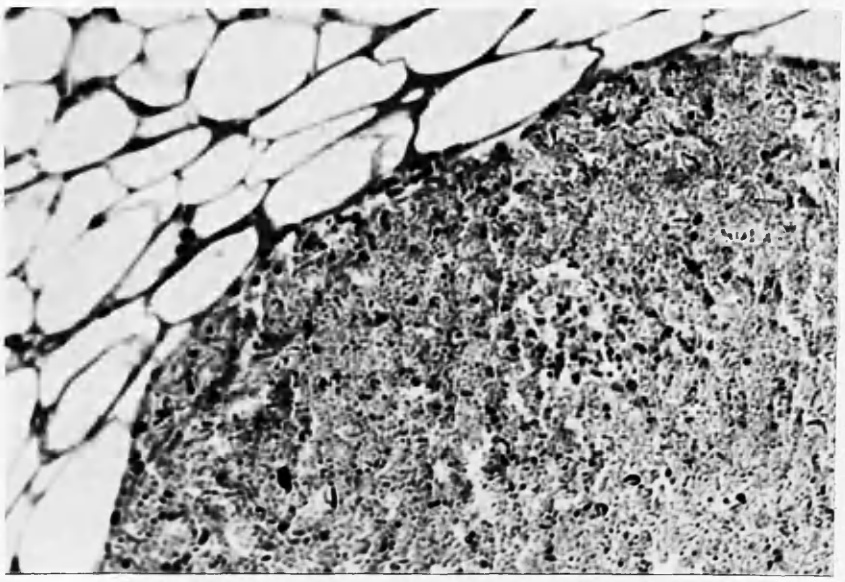


Fig. 39. There is no evidence of reaction to the quartz, injected 24 hours previously. Haematoxylin and eosin X 400.

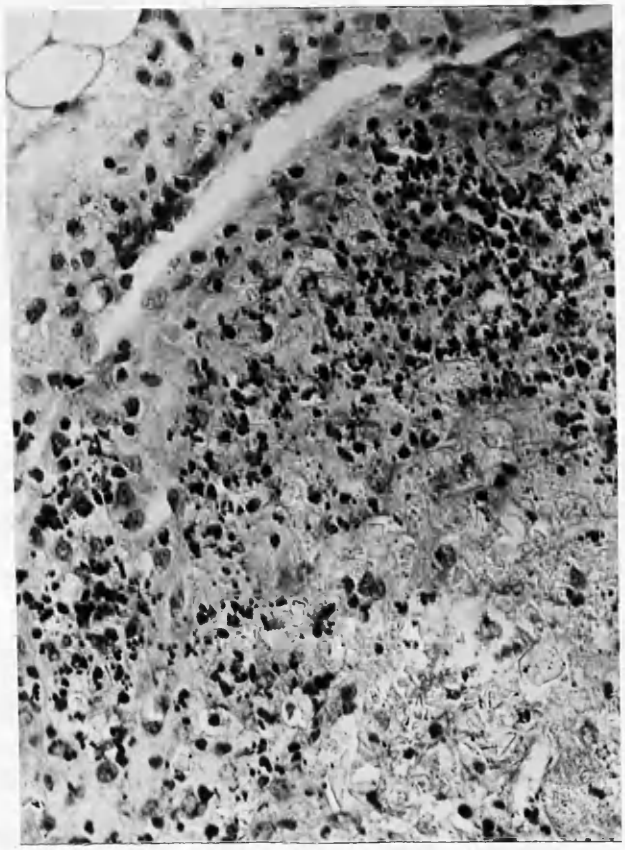


Fig. 40. In the control animal, a marked inflammatory reaction is evident by 24 hours and many cells have invaded the dust (see also fig. 10, p. 21 , and fig. 11, p. 23). Haematoxylin and eosin X 400.

response in the control animals (fig. 10, p. 21).

By 4 days, the dilatation of blood vessels has usually subsided, but a delayed vascular reaction is often noted in the treated animals. The earliest reticulin fibres are now present between the koniophages in both treated and control mice.

By 7 days, it is evident that cortisone reduces the number of cells emigrating from the adventitia of arteries, but koniophages are now fairly numerous in the region of the dust collections, and in fact this type of cell is, as usual, overwhelmingly predominant in each focus (fig.17, p. 29). Cortisone inhibits neither the phagocytic nor the fibroblastic function of the koniophages, each of which is distended with quartz particles, and the first collagen fibres are already present around it. There is, therefore, no delay in formation of connective tissue fibres under cortisone's influence. However, the lesions are, on average, much smaller, contain less dust and fewer cells, and the quantity of new connective tissue is considerably less than in the controls. The result of cortisone administration, therefore, is a quantitative rather than a qualitative change. This finding applies also to mucopolysaccharide formation. This substance is detectable in all treated animals after a week, both in

the cytoplasm of the koniophages and also extracellularly (fig. 28, p. 41). In keeping with the reduced cell response around the dust collections, however, the total quantity secreted is noticeably less.

Subsequently, the effect of continued administration of cortisone can be detected up to the 8th week. Though the smaller lesions still become wholly cellular within a few weeks, the influence of the hormone is manifested by a marked diminution in koniophage response in all the larger foci. Lesions from treated mice examined after 7 weeks are not microscopically different from the controls.

The flat plaque on the serosal aspect of the abdominal wall of the animals of the cortisone series is as distinctive microscopically as it is naked-eye (figs. 34 and 35, p. 106). It is cellular in the first few weeks (fig. 36, p. 107), but thereafter reticulin fibres increase rapidly and it finally becomes densely collagenous.

The numerous mast cells in the cutaneous and peritoneal tissues of the mouse were examined with care, but it was not possible to discern a specific action of cortisone on them.

B. CORTISONE AND THE MATURE SILICOTIC FOCUS (GROUP B)

These animals had many mature lesions consisting of concentric dense collagen bundles (fig. 32, p. 47). It is not possible to show any effect of cortisone on these

lesions.

C. ACTH AND THE DEVELOPING SILICOTIC FOCUS (GROUP C)

The results are variable. In general, the effects on the quartz lesions are similar to those in the cortisone-treated animals. Thus, occasional mice have free dust in the peritoneal cavity after 7 or 8 days, while in some there is a large flat abdominal plaque. However, in other animals the foci are indistinguishable from the controls, and a few have large masses on the abdominal wall.

DISCUSSION

In the early experiments (A1 and A2), the dosage of cortisone was based on that used by Selye (1949). It was possible to increase it for short-term experiments (series A3), but in view of the mortality it was reduced for those of longer duration (series A2). Magarey and Gough (1952) gave 2.5 mg. of cortisone to mice and all died within 17 days. It would appear from the present results that a daily maintenance dose of 0.1 mg./day, which can be continued for several months, is as effective as 0.5 mg./day, a dosage which produces a fairly high mortality within a few weeks. The systemic effects of the cortisone provided good evidence of the pharmacological adequacy of the doses employed, and were the same as reported

by several authors (Antopol, 1950; Molomut, Spain and Haber, 1950).

It is convenient to consider the effect of cortisone and ACTH on each of the elements which compose the quartz focus at the various stages of its development.

A. BLOOD VESSELS. One of the important effects of cortisone and ACTH is on vascular endothelium. Kendall in 1941 thought that the primary function of the adrenocortical steroids was to control the permeability of cells and the transfer of inorganic ions and water between the cells and the extracellular space. The control of permeability by cortisone affects endothelium as well as other cells. Best and Taylor (1950) emphasised cortisone's property of maintaining the impermeability of capillary endothelium. The experimental use of cortisone has provided support for these concepts. For example, it was found to reduce capillary leakage in the exteriorised meso-appendix of rats (Balourdas and Chambers, 1952), while ACTH lessens the oozing from burned surfaces of patients (Whitelaw, 1951). Cortisone, by maintaining endothelial impermeability, prevented haemoconcentration after thermal stress in rabbits (Moon and Tershakovec, 1954 a and b), and not only largely prevented the increase in capillary permeability produced

by intradermal injection of protein-digestion products but also diminished local oedema and leucocytic infiltration at the site of the injection. Cortisone may show a number of biological effects by the change it causes in vascular permeability (Rebuck, Smith and Margulis, 1951a; Spain and Molomut, 1952).

The mechanism by which cortisone decreases the permeability of membranes is as yet unsettled, but has been ascribed to the formation of increased amounts of hyaluronidase inhibitors, with a consequent protective effect on viscous ground-substance (Seifter, Baeder and Begany, 1949). It may stimulate mast cells to produce anti-hyaluronidase-active lipoproteins (Sommers, Edwards and Chute, 1954).

Apart from affecting the permeability of capillaries, cortisone reduces the formation of new capillaries in healing wounds (Ragan et al., 1949).

Several effects seen in the quartz focus may be examined in the light of these results. Firstly, in both the subcutaneous and peritoneal sites, the virtually complete suppression of the gelatinous inflammatory exudate, which normally develops within a few hours and disappears after about 3 days, probably depends on cortisone's ability to prevent the decrease in permeability

which normally occurs in acute inflammation. The early capillary dilatation was also virtually absent, but was sometimes replaced by a delayed reaction about the fourth day. Secondly, though the quartz lesion does not call forth a very great proliferation of capillaries, it was evident that there was no prolonged inhibition of new capillary formation, for after a week or two scanty vessels were detectable as usual among the collagen fibres at the periphery. Lastly, the cellular response was altered, and this must now be discussed.

B. THE KONIOPHAGE has two distinct activities, the phagocytic and the fibroblastic, and each will be considered in turn.

(1) Effect of cortisone and ACTH on phagocytic activity of the koniophage.

To assess the phagocytic power of the cells of the reticulo-endothelial system is a complex and difficult task. This is readily apparent from the conflicting nature of the published results of experiments designed to measure the effect of cortisone and ACTH on this system. The earliest results with cortisone appeared clear-cut; there was inhibition of phagocytosis of carbon (India ink) in mice (Spain, Molomut and Haber, 1950), and of tubercle bacilli in guinea-pigs (Carlisle, Gibson and Schmatolla, 1950). The phagocytic activity of neutrophil leucocytes

was found to be decreased in patients receiving cortisone and ACTH (Crepea, Magnin and Seastone, 1951), and ACTH not only diminished phagocytosis in experimental "skin windows" in human subjects (Rebuck, Smith and Margulis, 1951b), but completely prevented pigment phagocytosis in a patient with sympathetic ophthalmia (McLean, 1951).

The results obtained with quartz were not in accord with this earlier view that cortisone impaired the phagocytic activity of individual cells but subsequent reports showed that this view was not the whole truth. An actual increase in phagocytic ability of the reticulo-endothelial cells of cortisone-treated rabbits exposed to inhalation of virulent tubercle bacilli was even claimed by Lurie, Zappasodi, Dannenberg and Swartz (1951), though increased phagocytosis was unaccompanied by a comparative digestive ability on the part of the defensive cell body. Similarly, the hormone significantly enhanced the phagocytosis of staphylococcus aureus and colloidal thorium dioxide by macrophages, but failed to reduce the mortality in mice given Klebs. pneumoniae (Marcus, Esplin and Hill, 1953). Macrophagic activity was not reduced in mice injected with T. Evansi or India ink (Friebel, 1952), while absorption of India-ink particles from the peritoneal cavity was unaffected (Magee and Palmer, 1953). Large doses of cortisone also failed to alter the efficiency of the reticulo-endothelial (Kupffer) cells in the

liver, but definitely depressed the ability of these cells to destroy the micro-organisms they had engulfed (Clawson and Nerenberg, 1953).

Gell and Hinde (1953) concluded that cortisone does not directly interfere with the function of macrophages, but that it may suppress activity of mesenchymal cells by altering their environment, by reducing tissue or capillary-wall permeability. Animals receiving cortisone have a capacity for phagocytosing particulate material similar to that of the controls (Benacerraff, Halpern, Biozzi and Benos, 1954), but when the cells of the reticulo-endothelial system are loaded with particles, the hormone markedly inhibits recovery of its granulopectic activity, possibly by inhibiting multiplication of the cells which will replace macrophages no longer functional.

From this rather conflicting evidence, it appears that the effect of cortisone on the phagocytic response to quartz in the omental and cutaneous tissues may be mediated through several mechanisms, all of which must be closely linked.

Reduction of phagocytic ability might be expected, but there was no evidence of alteration, either enhancement or inhibition, of the phagocytic power of the individual koniophage. The cytoplasm of each cell of this type in the vicinity of the dust quickly filled with

quartz. There was, of course, no need to consider any "digestion" of the engulfed dust particles. The failure to demonstrate an inhibition of phagocytic activity was unexpected in view of the published work at that time, but is more readily understood in the light of some of the results recorded subsequently.

Maintenance of capillary and tissue impermeability has been mentioned (p. 116) as probably causing the virtual abolition by cortisone of the gelatinous exudate that is normally present around a quartz focus for the first 3 days. If lymph fails to pass through the capillary walls then there must also be a greatly reduced emigration of cells such as the monocyte. Those cells which do succeed in leaving the capillaries would be restricted in their wanderings through the comparatively unyielding ground substance. The hypothesis is undoubtedly very important, but it was not possible to assess from sections the part which this factor of capillary impermeability played in diminishing the number of phagocytes available.

The anti-mitotic effect of cortisone on primitive reticular cells is thought to be of much importance. Many koniophages originate in undifferentiated adventitial cells and in the taches laiteuses, and cortisone markedly reduces cellular proliferation in these sites. In

consequence, fewer cells migrate from them, and after the supply of macrophages in the tissues has been exhausted with only part of the dose of quartz inside cells, no secondary wave of phagocytes is available to deal with what remains. This cause of failure of granulopectic activity is in accord with the report of Benaceraff et al. (1954), but cortisone also prevents reduction in viscosity of the ground-substance with the consequent failure of cells to leave blood vessels and to migrate through the tissues, and this must also play an important part.

Diminished granulopectic power alone fails to explain the failure of the cortisone-treated animal to immobilise the dust within a few days. The tissues and the quartz are mutually adhesive, and most of the dust is quickly bound up in large acellular masses, particularly on the serous surface of the abdominal wall. In no instance among treated mice was a typical abdominal mass found, and at most one saw an occasional minute focus, less than 1 mm. in diameter. In order to prevent large aggregates from forming, cortisone must exert an influence on the adhesive attraction of the tissues for quartz and of the dust particles for each other. It is difficult to postulate how cortisone exerts this effect. Staining methods reveal no change in the

mucopolysaccharides of the connective tissues, and the formation of the subcutaneous gelatinous bulla is certainly unaffected by the heaviest cortisone dosage. These negative findings, however, do not preclude the possibility that cortisone not only makes the ground-substance less permeable but also less sticky and adhesive.

It has been suggested in Part I (p. 56) that a layer of colloidal silicic acid exists on the surface of the quartz particles, and that this layer might play a part in causing the particles to adhere. But it is difficult to see how cortisone can directly influence this inorganic gel. It is much more likely to alter the state of the ground-substance mucopolysaccharides.

The great delay in immobilising the quartz may be responsible for the formation of the cortisone plaque by permitting prolonged contact between dust and a wider area of abdominal-wall serosa than usual, so that finally a thin layer comes to adhere over most of the surface.

(2) Effect of cortisone and ACTH on the fibroblastic properties of the koniophage and on connective tissue.

Connective tissue can be regarded as comprising two extracellular components, fibrils of collagen and amorphous ground-substance. These will be considered separately. The fibroblast is conveniently discussed in connection with the ground-substance.

Collagen fibrils. Cortisone, apart from any effect it may have on the formation of new fibres, has been thought to exert a lytic action on mature fibrils. Experimentally, atrophy of collagen has been produced in skin treated directly with adrenal cortical extract, and therapeutically cortisone was used to treat keloid and Dupuytren's contracture. The results of the present experiments, however, show that the collagen of the mature (90-day) focus is quite unaffected by heavy and prolonged dosage with the hormone.

Mucopolysaccharide and the fibroblast. More evidence is available of an effect on the ground-substance and on the fibroblast. Asboe-Hansen (1950b and 1952) observed loss of the free metachromatic ground-substance from the skins of patients receiving ACTH, and believed that the hormone could also remove pathological deposits of hyaluronic acid from the tissues. His use of metachromasia and hyaluronidase to demonstrate hyaluronic acid are open to criticism, however. Despite some support for his results (Cavallero & Braccini, 1951), the evidence, reviewed on pages 115and 116, very strongly suggests that cortisone and ACTH, far from dissolving the connective-tissue mucopolysaccharides, make them much less permeable. In this indirect way they exert many of their effects on the

inflammatory reaction. In the present experiments, though alteration in permeability and consistency of ground-substance could be inferred from indirect evidence, there was no alteration in the subcutaneous gelatinous bulla in which the indigenous tissue mucopolysaccharides are directly implicated.

Many facts indicate that cortisone inhibits the new formation of ground-substance, in lesions of the skin (Ragan et al., 1949; Creditor et al., 1950; Michael and Whorton, 1951) and in the subcutaneous tissues (Shapiro, Taylor and Taubenhaus, 1951). Upton and Coon (1951) found, however, that cortisone and ACTH not only had no effect on this in wound repair in scurvy but they also failed to diminish the number of fibroblasts and the amount of collagen in the wounds of healthy animals. Their dosage of 4 mg./100 g. body-weight was perhaps inadequate for the guinea-pig, an animal which is apt to give anomalous results in cortisone experiments. However, as with phagocytosis, the earlier clear-cut ideas were not sustained; a complete lack of cortisone effect on the early stages of repair (Lattes, Jessar, et al., 1953) and on the synthesis of mucopolysaccharide in the cock's comb (Schiller, Benditt and Dorfman, 1952) were reported, while

no weakening or macroscopic alteration of the suture line of end-to-end anastomoses of the colon in dogs receiving ACTH was demonstrable (Geoghegan and Brush, 1954). Lattes, Jessar et al. (1953) made an important discovery which appears to explain at least some of the discrepancies. The cortisone-induced depression of repair was modified by spontaneous infection. Tissue damage by bacteria was thought to release substances, probably mucopolysaccharide in nature, necessary for the initiation and continuation of the reparative processes which would otherwise have been inhibited by cortisone. When infection was controlled by antibiotics, the hormone produced the most constant and most marked inhibition of inflammation and repair. Infection was regarded as particularly important in superficial lesions, like the healing wound. This idea is supported by the fact that the inhibitory action of cortisone on the healing of incised cutaneous wounds in rats can be nullified by painting or injecting the lesions with tissue-culture media containing embryo extract (Montgomery and Green, 1954).

Infection can be excluded as an important factor in the present experiments, for, though the tissues of animals dying of generalised infection were examined, the results are not included; in any case they showed no significant difference from the others of the cortisone series. However, the inhibitory effect of cortisone on repair is probably lessened in the silicotic focus by the breakdown products of tissue killed by the quartz dust.

With *in vitro* experiments, results have tended to be more uniformly negative, lending weight to the belief that cortisone exerts its effects in the tissues indirectly and in a complex fashion. Cortisone acetate differs in its behaviour from cortisone "free alcohol" (Kaufman, Mason and Kinney, 1953; Grossfeld and Ragan, 1954), and only the acetate is considered here. In concentrations even up to twenty-five times the so-called therapeutic level, it failed to influence growth or mitotic activity in fibroblasts in tissue culture (Steen, 1951), and similar results were reported by Kaufman *et al.* and Grossfeld and Ragan. One discovery was especially interesting; though very high concentrations of cortisone retarded proliferation of fibroblasts in culture slightly for about 48 hours, the inhibitory effect was not apparent after 3 to 4 days and the rate of collagen synthesis by the growing tissues was

not decreased during a 12-day period (Gerarde and Jones, 1953).

Another approach was employed by Layton (1951a). Chick-embryo tissues and granulation tissue from wounds in chick embryos incorporate labelled sulphate into the ground-substance of connective tissues *in vitro* and cortisone reduces the synthesis of these sulphated mucopolysaccharides. Strictly speaking, this only shows that the hormone exhibits a retarding effect on the exchange of the ester sulphate group in the chondroitin sulphuric acid.

In view of all these reports, an inhibitory effect on the activities of the fibroblastic koniophage was expected, but surprisingly there was no delay in the appearance around these cells of the first reticulin fibres by 60 or 70 hours and of collagen by 7 or 8 days. Similarly, mucopolysaccharide appeared in their cytoplasm at the usual time and in comparable concentrations, though the extracellular material was less. However, all of the lesions were much smaller, and undoubtedly much less collagen and mucopolysaccharide formed in a given period under cortisone treatment.

These results differ in some respects from those obtained by Magarey and Gough (1952) who found that cortisone

could cause a definite delay in the appearance of collagen fibres in the silicotic focus in the rat. They also noted a curious proliferation of capillaries in the peritoneal cavity of the rabbit, but this result was not obtained by them in later experiments (Gough, 1954, personal communication).

It would appear that caution is still needed in drawing any general conclusions regarding the effect of cortisone and ACTH on the synthesis of mucopolysaccharides and collagen.

C. OTHER CELLS

The ability of cortisone and ACTH to prevent the customary increase of permeability of tissues and particularly of capillary-wall in inflammation exercises many remote effects, and is undoubtedly an important factor in reducing the number of cells around the silicotic focus in the early phases.

Polymorphs, very numerous for the first few days in the control lesions, were insignificant in number in treated animals. The reduction was particularly obvious around foci in the subcutaneous tissues whose normal cell population is comparatively small. The hormone has a similar effect on polymorphs in a variety of lesions, including cutaneous wounds (Ragan et al., 1949), tuberculous abscesses (Carlisle

et al., 1950) and inflammatory allergic lesions (Dougherty and Schneebeli, 1950).

Lymphocytes are difficult to define with certainty in tissue sections. As Cappell (1929) points out, cells resembling them are particularly numerous in the taches laiteuses and in the perivascular tissues, where in many cases they may in fact be the primitive undifferentiated reticular cells of Maximow or the "adventitial" cells of Marchand. There was reduced cellular proliferative activity in these sites, but no histological evidence of direct damage to lymphocytes, as has been claimed (Rebuck et al., 1951a)was evident. No attempt was made to confirm the lymphocytopenia which cortisone produces (Rebuck et al.; Dougherty, 1952).

Mast Cells. In tissue cultures of embryonic skin, the free wandering cells, which include the mast cell, are most sensitive to the hormone (Paff and Stewart, 1953). In view of the suggested importance of this cell in fibroplasia (Sylvén, 1941), a cortisone effect was sought. It has been claimed that the hormone can induce cytoplasmic vacuolation, conglomeration and altered staining reaction of the metachromatic granules, disruption of the cell membrane and granule scattering, and even destruction of the mast cell (Stuart, 1951; Cavallero and Braccini, 1951;

Asboe-Hansen, 1954), but it is not clear what this implies. Asboe-Hansen (1952) links it with diminution of hyaluronic acid in the connective tissues following cortisone administration, but it has been connected also with heparin production (Wilander, 1938).

Mast cells are numerous in the tissues of the mouse, especially in the skin. Rupture of the cell membrane with scattering of granules was often seen, but not more frequently in the cortisone series than in the controls. It has been claimed that these appearances of mast-cell degeneration in sections are absent in carefully-prepared mesenteric spreads and are to be regarded as artefacts inherent in the paraffin-section technique (Devitt, Samuels, Pirozynski and Webster, 1954). Schoch and Glick (1953), using a full-thickness skin biopsy technique, also think that the appearances are artificial, but Smith and Lewis (1954) consider that the matter is not yet settled.

CONCLUSIONS

In the silicotic lesion in the mouse, cortisone and to a lesser extent ACTH profoundly alter the initial acute inflammatory response and greatly delay the fixation of dust by the peritoneal tissues. The form of the focus is sometimes altered, with the formation of a specific

cortisone lesion. Microscopically, the effects are less obvious, and no proof was obtained that the hormones directly affect the phagocytic and fibroblastic powers of the individual koniophage, nor do they delay the time of appearance of mucopolysaccharide and fibrous tissue. However, the production of mucopolysaccharide and collagen is much less in cortisone-treated than in control animals. Disturbance of the activities of local tissue cells by cortisone appears to be relatively slight, whereas the number of cells reaching the focus is greatly reduced under its influence. The modification of the inflammatory reaction thus produced is quantitative rather than qualitative.

PART III

THE UPTAKE OF LABELLED SULPHATE BY THE QUARTZ FOCUS IN THE MOUSE

INTRODUCTION

Despite the availability of the newer histochemical staining methods for demonstrating mucopolysaccharide in tissue sections, the position is still unsatisfactory. Absolute specificity can be claimed for none, and it is probable that all are comparatively insensitive. Moreover, the one method that is founded on the most rational basis, the periodic acid-Schiff technique, is unsuitable for connective tissue mucopolysaccharides. It stains chondroitin sulphate very feebly and hyaluronic acid not at all.

The use of labelled sulphate seemed to offer an entirely new approach to the problem of demonstrating mucopolysaccharides. Layton (1950a) showed by chemical extraction methods that embryonic tissues and granulation tissues from wounds of embryonic muscle absorbed inorganic sulphate and bound it in the mucopolysaccharide of the newly-formed connective tissue. His methods provided no information on the cytological distribution of the ion retained within the tissues; autoradiography is required for this purpose.

If the koniophages in the quartz focus form sulphated mucopolysaccharide, as would be expected from

Meyer's (1947) hypothesis of collagen formation, autoradiography should show that these cells take up the labelled sulphate. The investigation of this matter forms the substance of the present Part III of this thesis. Later work on the distribution of labelled sulphate in normal tissues is described in Part IV.

MATERIALS AND METHODS

Quartz foci were produced in 22 mice by the methods described in Parts I and II, and lesions ranging in duration from 8 to 103 days were studied. The isotope used was S35 which has a half-life of 87.1 days and releases β particles of 0.167 Me V. energy. The S35 was injected subcutaneously or intraperitoneally as sulphate ion at pH 7 in an average dose of 4 μc/g. body-weight. Thirteen mice were killed 4 to 18 hours after the injection and usually at 15 hours. The other 9 mice were killed 2 to 18 days after receiving the S35. The tissues were fixed for 24 hours in alcohol or a mixture of equal parts of alcohol and acetone, but Bouin's fluid, 10 per cent. neutral formalin, and Carnoy's solution were found to be equally satisfactory, despite the belief that they cause occasional failure of development of the film (Doniach and Pelc, 1950). Double-embedding was used,

first in 2 per cent. celloidin for 24 hours then in paraffin for one and a half hours in the vacuum embedding oven. Sections, often serial, 6μ thick, were floated on water at 40° C., and those required for autoradiography were mounted on gelatine-coated slides.

The Doniach and Pelc (1950) technique with fine-grain Kodak stripping film was used. Exposure times ranged from 15 to 70 days. The film was developed in Kodak D-19b developer for 20 minutes at 20° C. The haematoxylin counterstain used in the original method tended to mask the lowest concentrations of activity, and Sawyer's light green-safranin O method (1940) was modified for use with this technique and gave the best compromise between histological detail and autoradiographic clarity. The slides were overstained for 4 hours or more in the following solution:-

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

Hydrochloric acid was omitted as it occasionally loosened the emulsion from the slides. Removal of excess stain was effected in Sorensen's buffer at pH8 for approximately 30 minutes, and the sections were treated subsequently with 0.1 per cent. aqueous safranin O for

4 hours or more. The autoradiographs were rinsed in water and then differentiated in 0.001 N HCl until the emulsion was pale green with nuclei red, and cytoplasm light green. They were mounted in D.P.X. after 18 hours' soaking in equal parts of xylol and D.P.X. Floating the sections for autoradiography on water no doubt had the desirable effect of causing the loss of the very soluble forms of S35 present in the tissues, such as the inorganic or ethereal sulphates. However, some sections were floated on diacetin, to avoid loss of water-soluble substances (Bourne, 1952), and then coated with 1 per cent. celloidin in ether/alcohol, equal parts. Since water was essential for floating the stripping film, the celloidin layer was used for protection of the section during the brief time it was in contact with water. It was removed by ether/alcohol after development. Sections adjacent to those used for autoradiography were stained by haematoxylin and eosin and by the methods of Van Gieson and Gordon and Sweets. Mucopolysaccharides were identified by the Alcian-blue, Hale and periodic acid-Schiff methods, and metachromasia was usually demonstrated by cresyl-fast violet.

A number of sections were treated by testicular

hyaluronidase (Benger's Hyalase) before being used to prepare autoradiographs. The enzyme was employed in 0.025 per cent. solution for 15 hours at 37° C., and control sections were treated with boiled enzyme.

Quartz foci of 60 days' duration were removed from 4 mice and treated by the in vitro method described in Part V (p. 190).

RESULTS

In 8 of the mice killed within 18 hours of receiving S35, the quartz foci have taken up the labelled sulphate in considerable concentration (figs. 41-43, pages 138 to 140). The isotope is located in the areas occupied by koniophages. The strongest positive results are found in 75-day quartz lesions (fig. 42, p. 139). There is no uptake in collagen, even when this is rich in stainable mucopolysaccharide, or in plasma cells and lymphocytes. Where the koniophages are widely separated, usually near the centre of the focus, and when the level of radioactivity is fairly low, it is possible to confirm that the ion is confined to the cytoplasm of the cells (fig. 44, p. 140). In 5 animals of this group, no uptake is found in any of the quartz lesions. In the positive animals, all foci show uptake and even small koniophage collections may be located by their radioactivity.

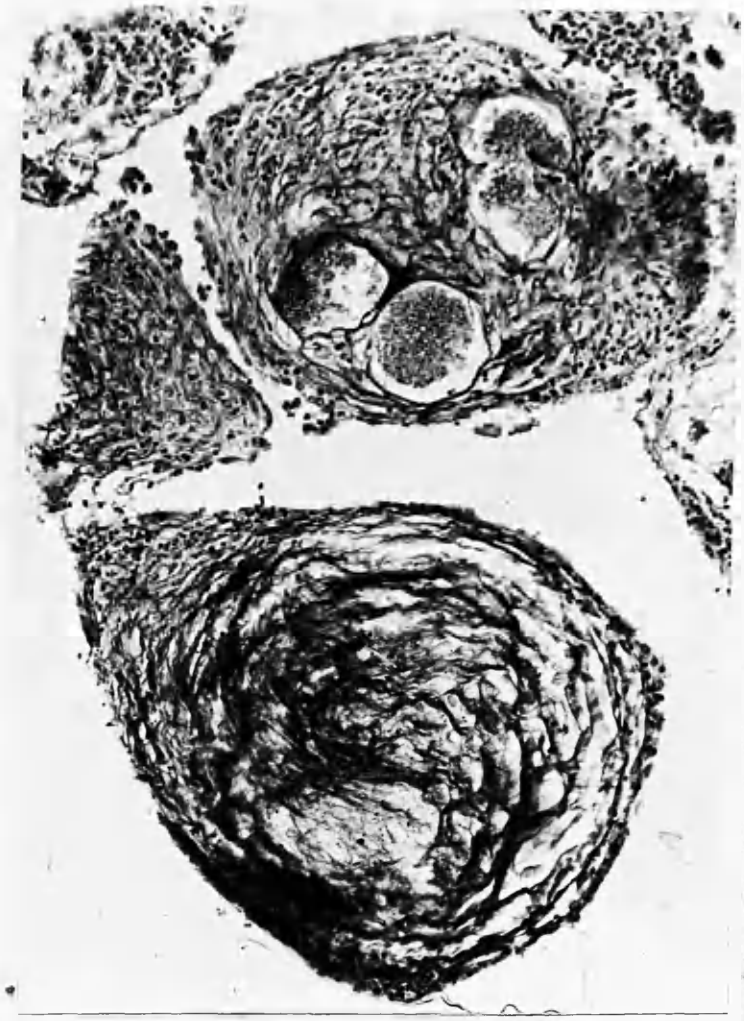


Fig. 41. This shows three quartz foci of 75 days' duration. The centre of the largest focus is densely collagenous, but koniophages persist at its periphery and are very numerous in the other two foci. An unusual feature of the focus at the top is the presence of two large blood vessels, cut in transverse section. Iron haematoxylin and Van Gieson X 160.

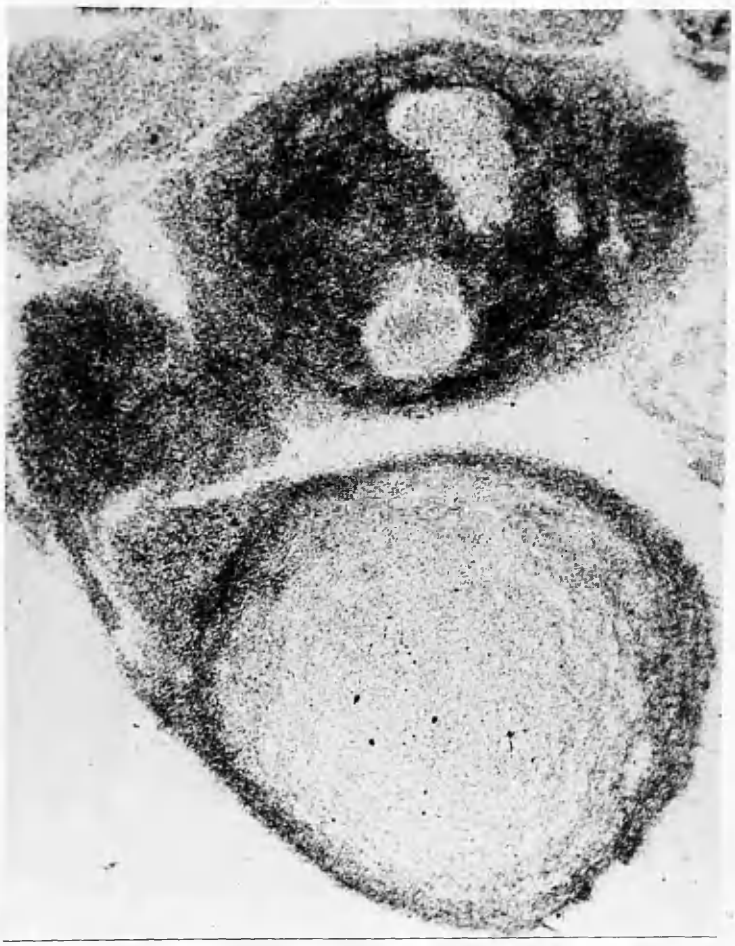


Fig. 42. Radioactive sulphate is present in fairly high concentration in the areas composed of koniophages. There is little evidence of uptake in the central collagen of the largest focus, though mucopolysaccharide can still be demonstrated by stains in this area. Autoradiograph X 160.

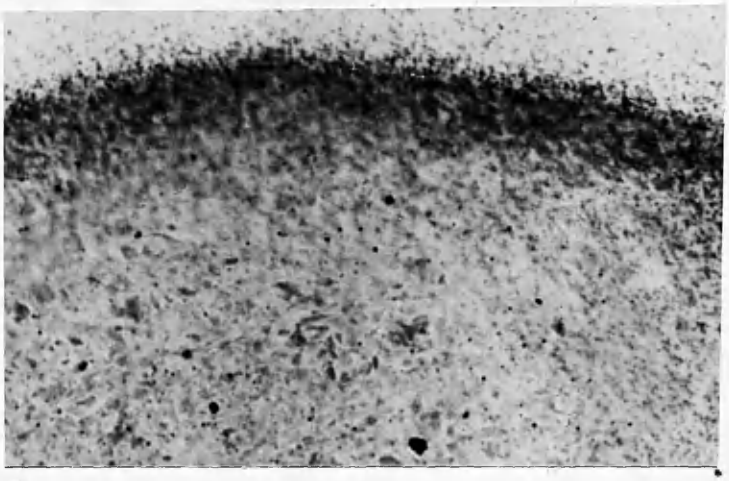


Fig. 43. Radioactivity is confined to the peripheral cellular layer of the largest focus shown in fig. 42. Autoradiograph X 800.

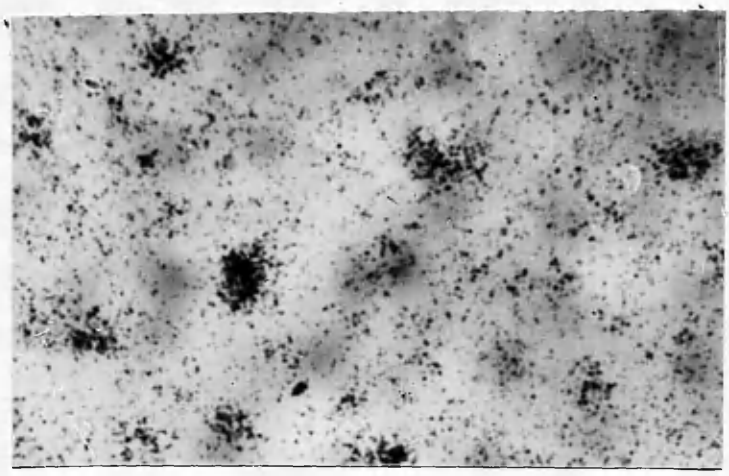


Fig. 44. In areas where the koniophages are widely separated and where the uptake of sulphate is comparatively moderate, it is possible to show that the isotope is located within the cells. Autoradiograph X 800.

No convincing evidence of sulphate uptake is detected in the mice killed more than 2 days after receiving S35.

The silicotic foci treated with sulphate *in vitro* for 2 days give a positive result similar to that obtained by injecting the ion into the living animal.

DISCUSSION

The significance of utilisation of sulphate ion by the koniophage depends on the specificity of the method. This is considered in Parts IV and V, where the evidence is strongly in favour of a specific uptake of the ion by cells forming mucopolysaccharide. There is little evidence of direct exchange of the sulphate with that present in the tissue mucopolysaccharides; it is taken up by cells whose anabolic processes incorporate it into sulphated mucopolysaccharides, which, initially cytoplasmic in location, are secreted into the surrounding tissues. By analogy, therefore, the evidence presented here makes it probable that the koniophage forms sulphated mucopolysaccharide which is then liberated into the quartz focus, a finding in accord with the results obtained by stains (fig. 28, p. 41).

The present experiment underlines the advantages

of the quartz focus in studying fibroplasia. It is easy to study separately the individual elements in the lesion (fig. 26, p. 39 ; fig. 27, p. 40). For example, many foci consist wholly of koniophages, and these cells are seen to take up the sulphate. On the other hand, collagenised foci show no uptake, even when they still contain considerable quantities of stainable mucopolysaccharide (fig. 41, p. 138 ; fig. 42, p. 139), and lymphocytes and plasma cells are also inactive. In occasional foci, discrete koniophages may be found and S35 can be located in their cytoplasm (fig. 44, p.140). The difficulties inherent in other lesions were encountered in an experiment on hepatic fibrosis done in conjunction with Dr. Patrick of this department. Cat-gut was implanted in mouse livers and the sulphate ion could be located in the granulation tissue which formed around the foreign material. However, the different elements composing this tissue were so intermingled that it was not possible to determine which contained the sulphate. The problem is being pursued further by Drs. Patrick and Kennedy.

Hyaluronidase applied to sections from which autoradiographs were then prepared was without effect.

This is in agreement with the comparative failure of the enzyme to affect stainable mucopolysaccharide within fixed cells (fig. 28, p. 41 ; fig. 29, p. 42).

No radioactivity was found within foci when these were examined some days or weeks after administration of sulphate. Presumably radioactive mucopolysaccharide is liberated by the koniophages, but the "turn-over" of the sulphate radicle is sufficiently rapid to decrease the level of radioactivity to a level difficult to detect by autoradiography.

CONCLUSIONS

The koniophage in the quartz focus takes up labelled sulphate. It is probable that the ion is incorporated in sulphated mucopolysaccharide synthesized by the cell.

PART IV

THE DISTRIBUTION OF THE SULPHATED MUCOPOLYSACCHARIDES IN THE MOUSE USING LABELLED INORGANIC SULPHATE (S^{35})

INTRODUCTION

This part of the thesis records experiments undertaken to investigate the uptake of labelled sulphate by various mammalian tissues. The mouse was chosen as the experimental animal, because a relatively small dose of the isotope produced a high concentration in the tissues of such a small animal, and the distribution of the ion could be studied readily in practically all of the tissues in complete sagittal or coronal sections of the new-born mouse.

MATERIALS AND METHODS

Thirty-eight albino mice were used, divided into two groups according to age. Fifteen animals investigated within 3 days of birth from one group. The other 23 were adult mice of ages 3 - 9 months.

The method of administration of S35 and staining and autoradiographic techniques were exactly as described in Part III (p. 134), except that each mouse of group I was fixed intact in alcohol for 1 - 2 hours then sliced sagitally or coronally. Sulphurylation of some sections prior to staining for metachromasia (Kramer and Windrum, 1953) was occasionally performed.

RESULTS

Tables I and II show the distribution of S35 in the

tissues of the new-born mouse, as identified by the stripping-film autoradiographic technique. For comparison, in table I the results of staining adjacent sections with Alcian blue are given. The concentrations of the isotope are indicated very roughly from 0 to +++ on the basis of the intensity of blackening of the overlying film: low concentrations are +, high concentrations +++. The staining results are similarly estimated.

TABLE I

DENSITY OF S35 IN AUTORADIOGRAPHS COMPARED WITH DENSITY OF ALCIAN BLUE STAINING IN COMPARABLE SECTIONS

TISSUE	DENSITY OF S35 IN AUTO-RADIOGRAPH	DENSITY OF ALCIAN BLUE STAINING
SKIN		
Mast cells	+++	+
Reticular layer of dermis	+	+
Hair bulb and papilla	++	++
Hair (whisker): Hyaline and dermic layers of the follicle	++	++
SKELETAL SYSTEM		
Cartilage cell: cytoplasm	+++	+ to ++
Matrix of cartilage	++	++ to +++
Bone lamellae	++	+++

TISSUE	DENSITY OF S35 IN AUTO-RADIOGRAPH	DENSITY OF ALCIAN BLUE STAINING
EYE		
Cornea	++	++
Sclera	+	+
Lens	-	+
Lens capsule	+	+
Ciliary body	+	+
Retina: inner plexiform layer . .	+	+
Lachrymal gland: stroma . .	+	++
acinar cells	-	-
CARDIO-VASCULAR SYSTEM		
Valve cusps	+	++
Aorta and venae cavae: tunica media	++	+
Arteries and veins, including coronary vessels: tunica media	++	+
Endocardium: connective tissue	+	+

TISSUE		DENSITY OF S35 IN AUTO-RADIOGRAPH	ALCIAN BLUE STAINING
ALIMENTARY SYSTEM			
Unerupted tooth:	cells and matrix of dental papilla . . .	++	+
	superficial pro-dentinal layer . . .	++	+++
	odontoblasts' cytoplasm	?	?
Salivary gland:	acinar cells . . .	−	+ to ++
	peri-ductal connective tissue . .	++	++
	stroma	+	+
Stomach:	mucus-secreting glands .	+++	+++
	proventriculus: submucosa	+	+
	micro-organisms on mucosal surface . . .	+	++
Small and large intestines:			
	goblet cell cytoplasm and mucus	+++	+++
Pancreas:	acinar cells: . . .	+	+
	islets of Langerhans .	−	−
Liver:	giant cells (? megakaryocytes). . .	++	++
	bile duct epithelium . .	+	+

TISSUE	DENSITY OF S35 IN AUTO-RADIOGRAPH	DENSITY OF ALCIAN BLUE STAINING
RETICULO-ENDOTHELIAL AND HAEMATOPOIETIC SYSTEMS		
Spleen: giant cells (? megakaryocytes)	+++	+
fibrous septa	+	+
Marrow: megakaryocytes	++	++
RESPIRATORY SYSTEM		
Connective tissue of lung	+	+
Respiratory epithelium	-	+
GENITO-URINARY SYSTEM		
Convoluted tubules of kidney	+	+
Granulosa and thecal cells in ovary	+	-
Liquor folliculi	+	++
Vaginal wall: connective tissue	+	+
Vas deferens: connective tissue	+	+
NERVOUS SYSTEM		
Brain	+	+
Peripheral nerves	++	+

TABLE II

CONCENTRATION OF S35 IN AUTORADIOGRAPHS

+++	++	+
Mast cells	Megakaryocytes of marrow	Connective tissues of salivary and lachrymal 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	Valve cusps
Goblet cells of intestine: mucigen and cytoplasm	Hair: hyaline layer of the follicle and connective tissues of bulb and papilla	Peripheral nerves and brain
Mucin in lumen of intestine	Unerupted tooth: papilla and superficial prodentine	Lens capsule
Giant cells (? megakaryocytes) of spleen	Aorta, venae cavae, and small arteries and veins: tunica media	Bacteria of proventriculus
	Brain: superficial acellular zone	Acinar cells of pancreas
	Cartilage: matrix	
	Cornea	

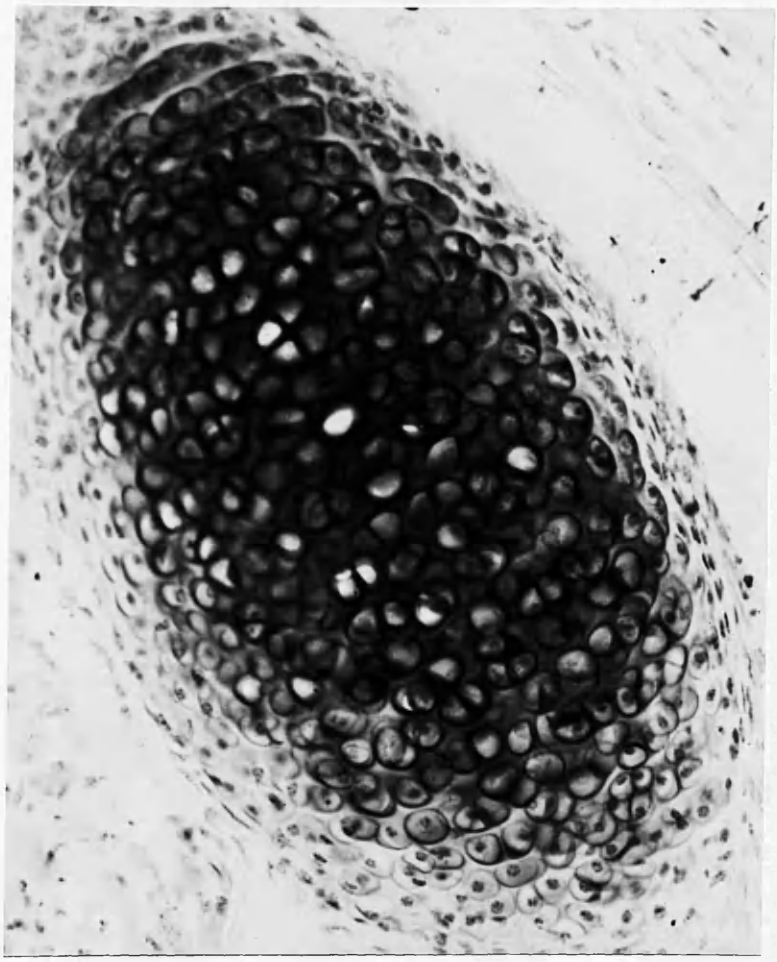


Fig. 45. The rib is still wholly cartilaginous. Metachromatic mucopolysaccharide is present in the cytoplasm of the cells and in the matrix. A number of cells have been dislodged in cutting. Cresyl-fast violet X 200.

Fig. 46. Sulphate is present in the matrix and in the cytoplasm of the cartilage cells of the rib. A lower concentration is present in the perichondrium. Autoradiograph X 200.

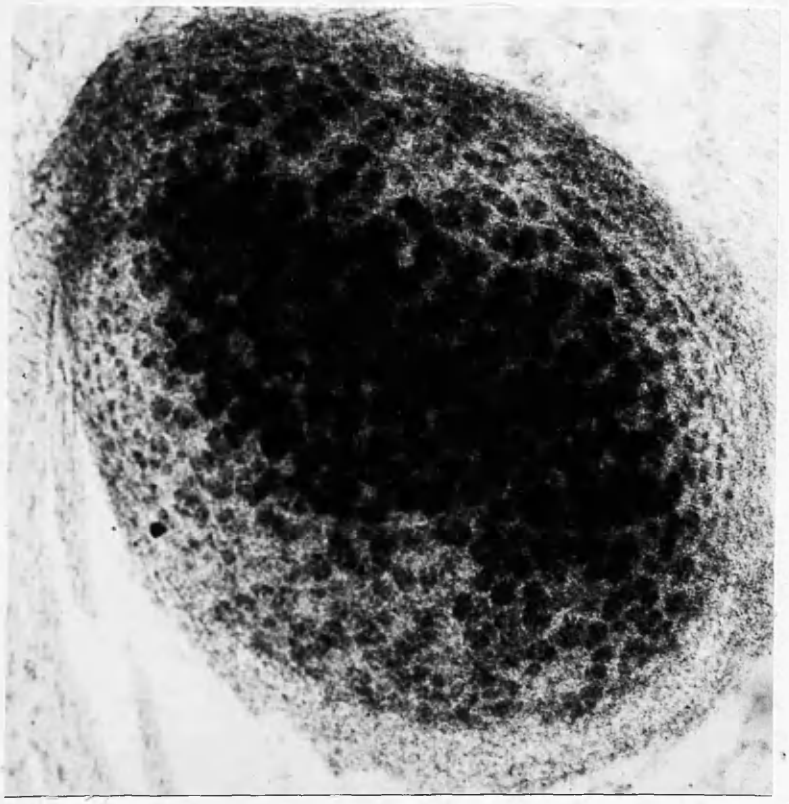


Fig. 47. Incubation of the section of rib with hyaluronidase prior to autoradiography removes most of the S35 from the matrix and perichondrium but leaves the cellular concentrations largely unaffected. The younger peripheral cells metabolise only small amounts of the ion. Autoradiograph X 200.

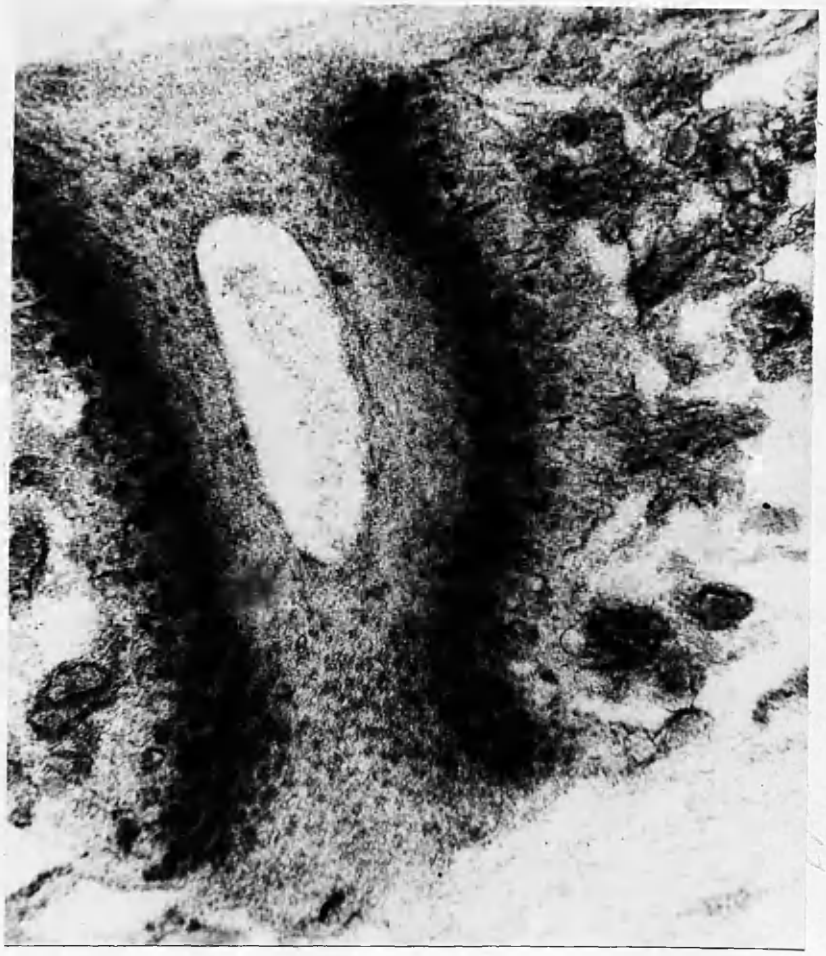


Fig. 48. This shows parts of two vertebral bodies and an intervertebral disk. The section has been treated with hyaluronidase prior to autoradiography. The mature cells of the epiphysial cartilage contain much sulphate, but the younger cells nearer the disk are comparatively inactive, as are the large cells of the hypertrophic zone. The newly-formed peripheral layers of the bone lamellae of the vertebral bodies retain S35. Similar appearances are present in the long bones. Autoradiograph X 150.

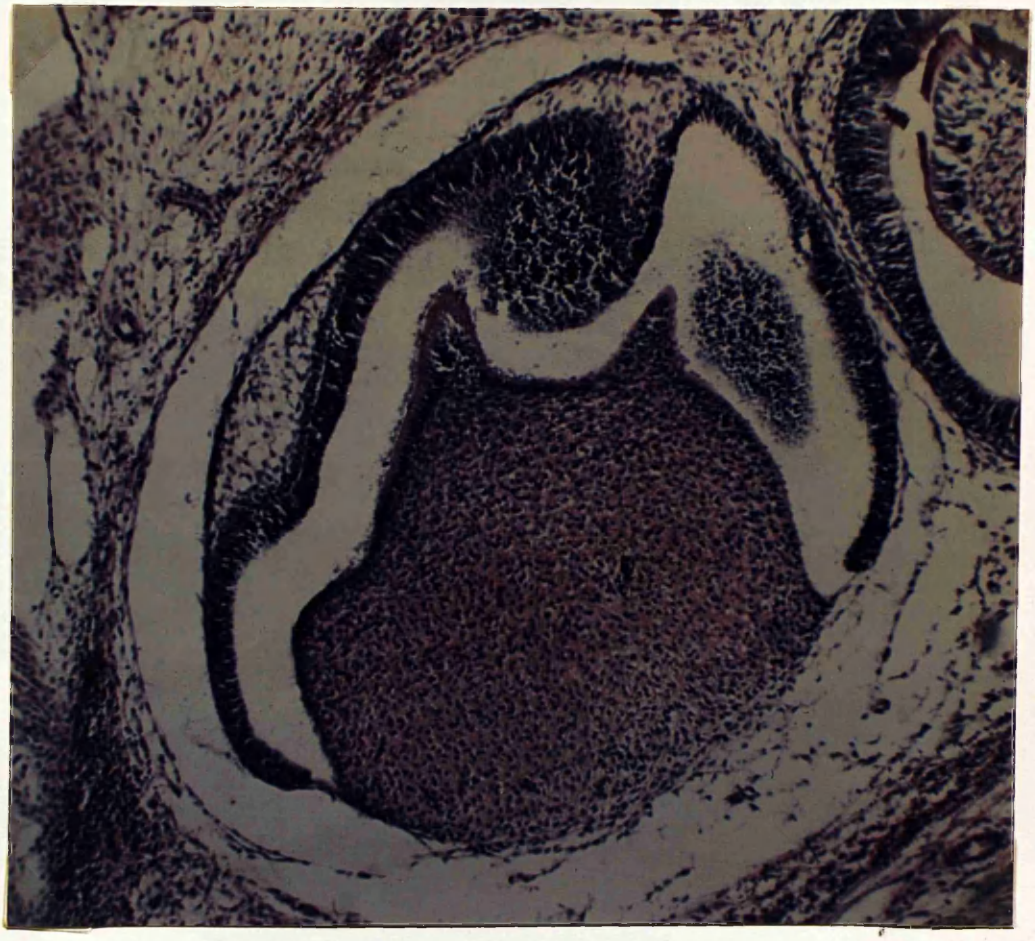


Fig. 49. In this unerupted tooth, mucopolysaccharide is present throughout the matrix of the dental papilla and in high concentration in the layer of pro-dentine on the surface of the papilla. Periodic acid-Schiff X 100.

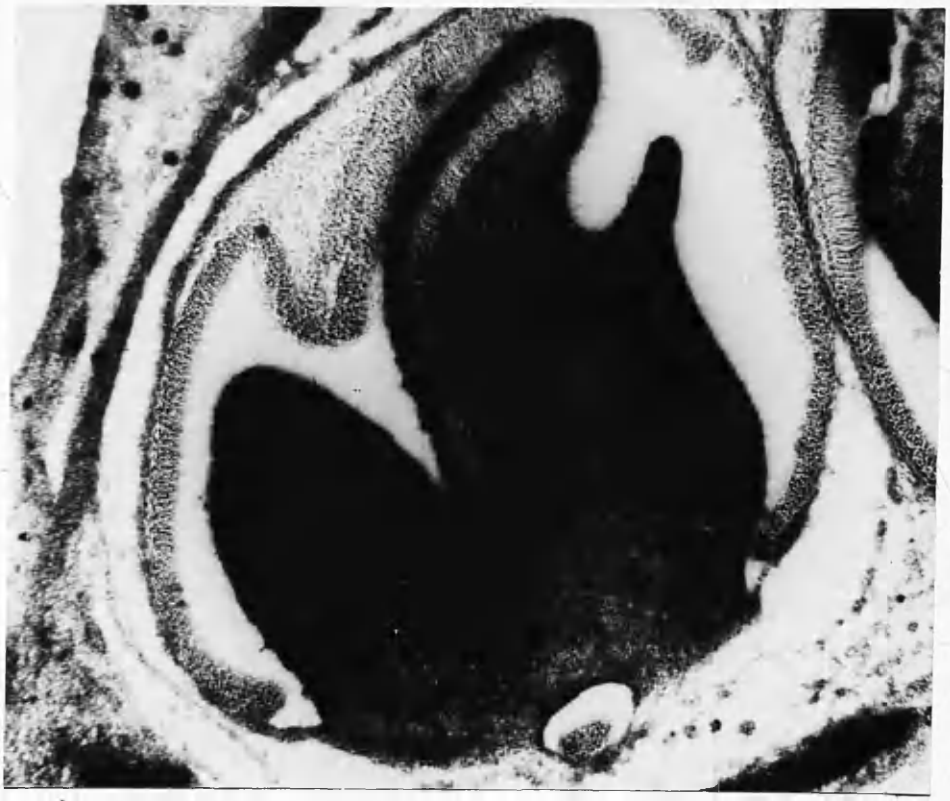


Fig. 50. The papilla of the unerupted tooth is strongly positive, as is the pro-dentinal layer. At one point the odontoblast layer is seen. The enamel organ is negative, but mast cells are prominent in the neighbouring tissues. Autoradiograph X 100.

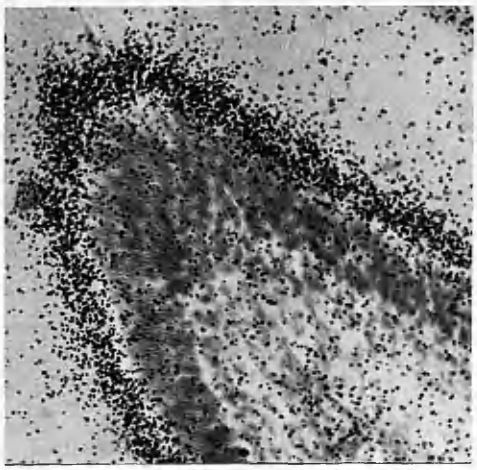


Fig. 51. The concentration of sulphate is high in the layer of pro-dentine. Autoradiograph X 200.

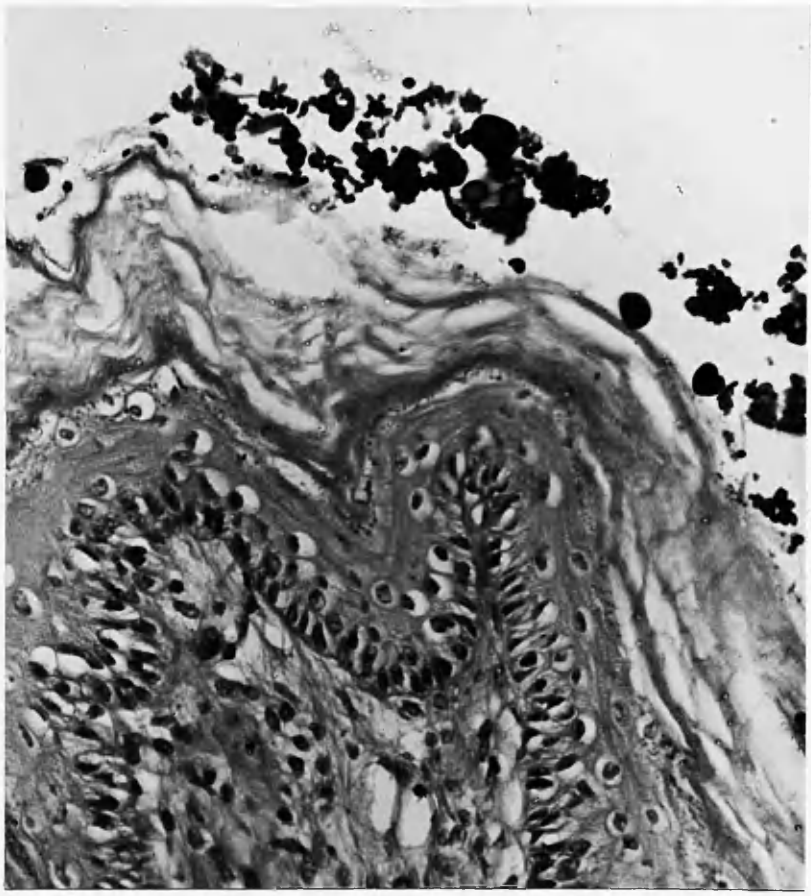


Fig. 52. In the proventriculus, a mixed bacterial flora is established on the mucosal surface within a day or two of birth. The organisms contain mucopolysaccharide. Periodic acid-Schiff X 600.

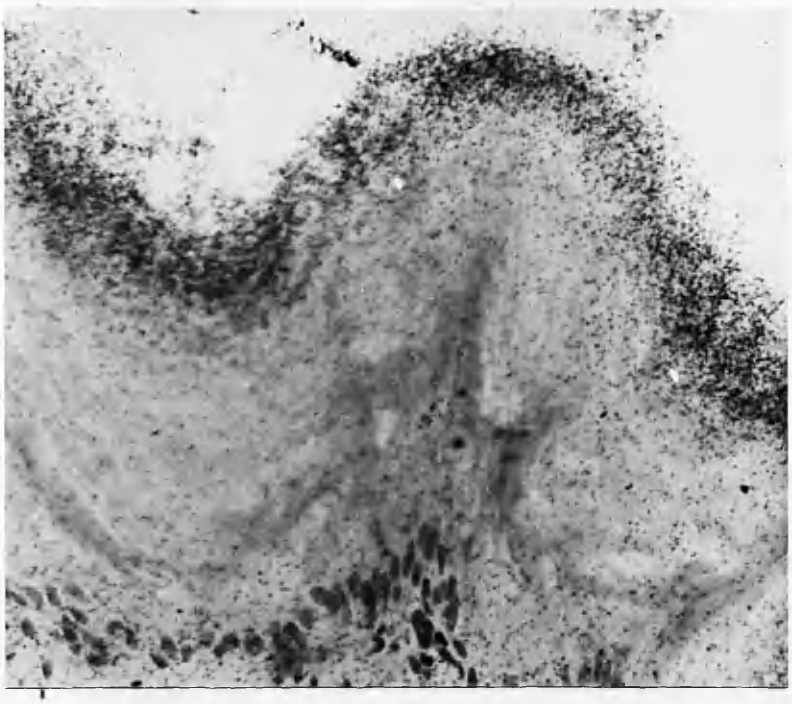


Fig. 53. The bacteria on the mucosal surface of the proventriculus metabolise labelled sulphate. Autoradiograph X 600.

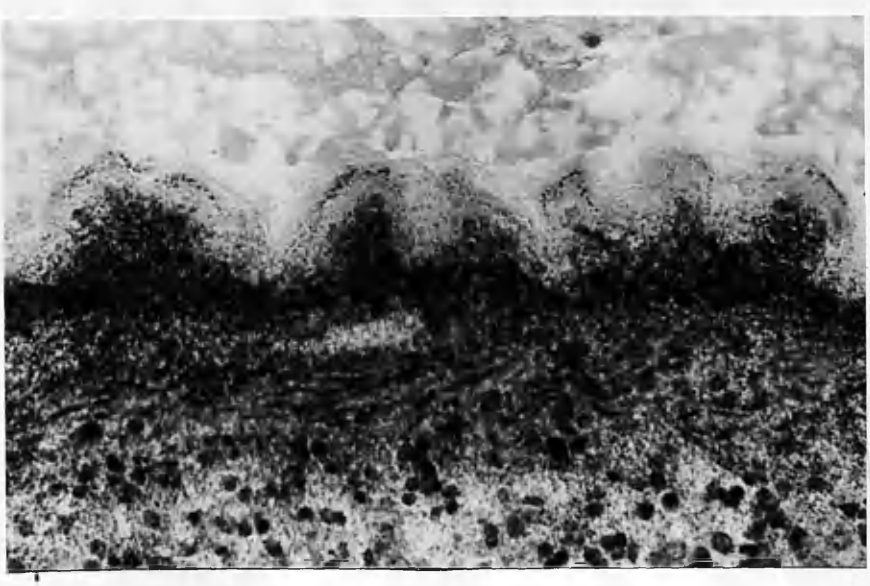


Fig. 54. In the new-born mouse, uptake of sulphate in the proventriculus is confined to the mucopolysaccharide present in the submucosa. Autoradiograph X 300.

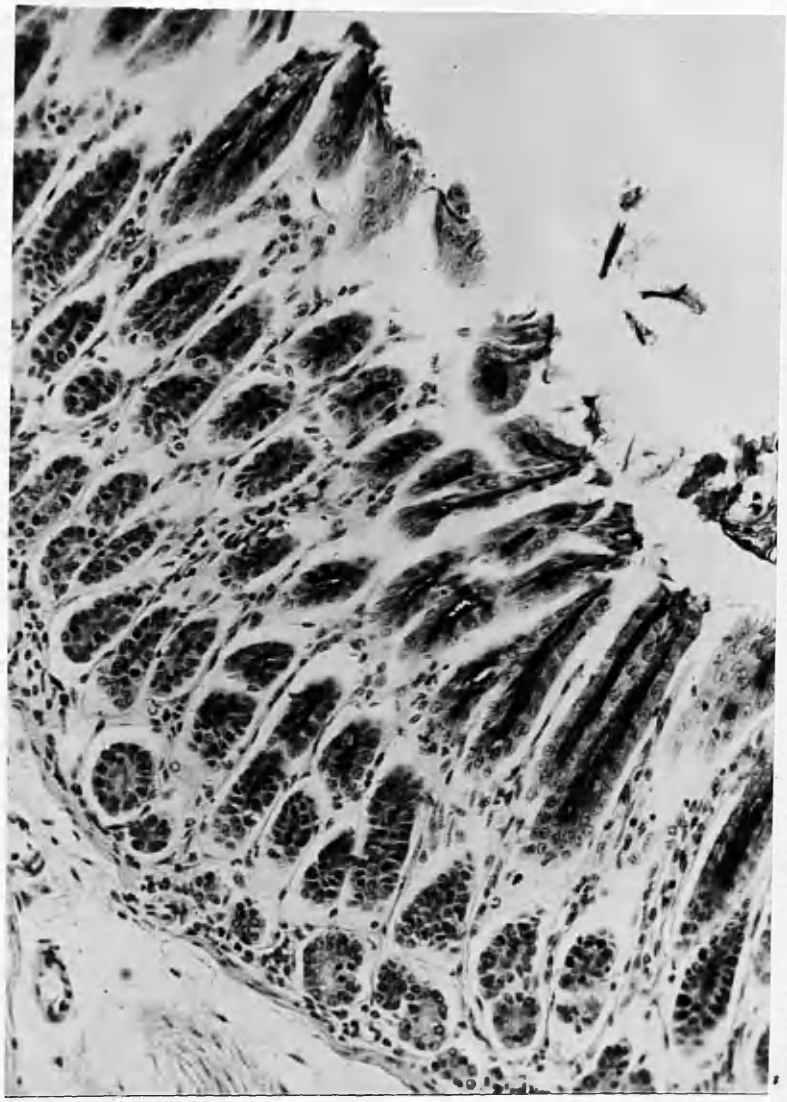


Fig. 55. In the distal part of the mouse stomach, the mucin secreted by the more superficial glands is strongly Schiff-positive. Periodic acid-Schiff X 200.

Fig. 56. In the distal part of the mouse stomach, only the mucopolysaccharides of the basal glands contain much S35. Autoradiograph X 150.

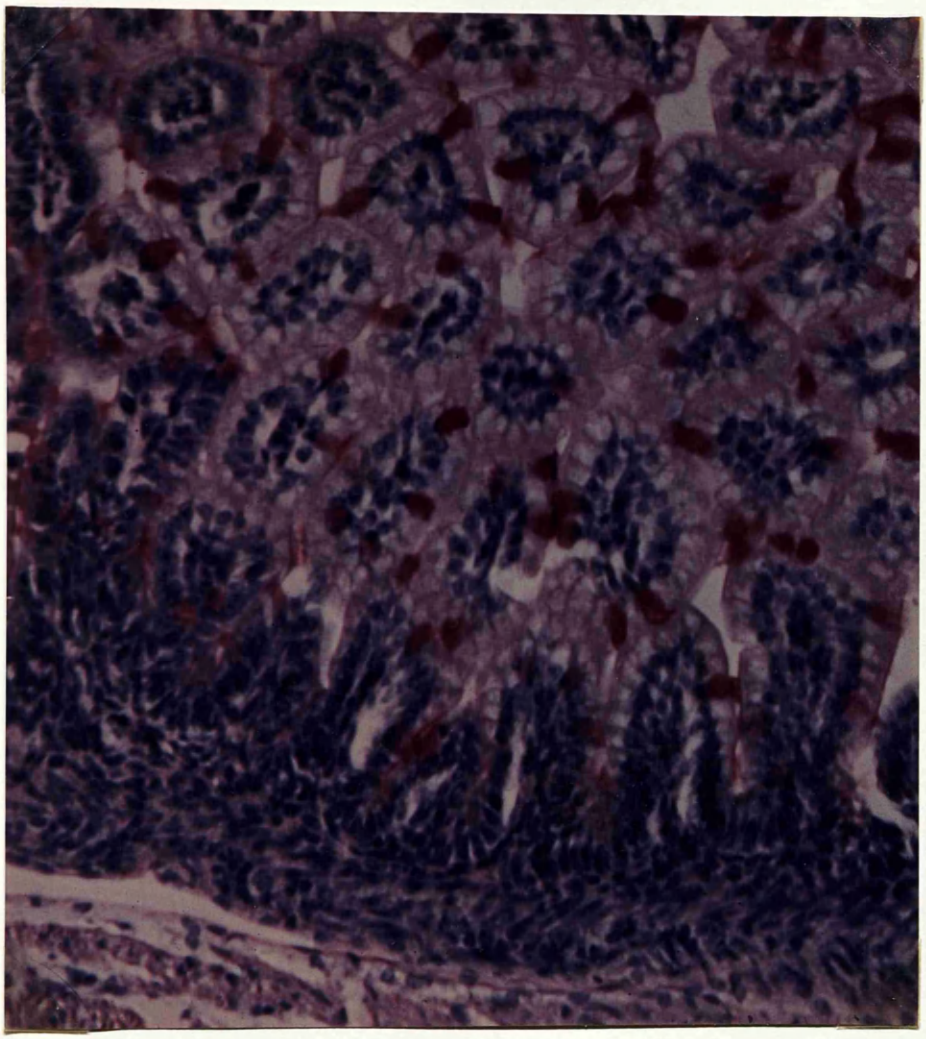


Fig. 57. The mucin secreted by the goblet cells of the small intestine is strongly Schiff-positive. Periodic acid-Schiff X 400.

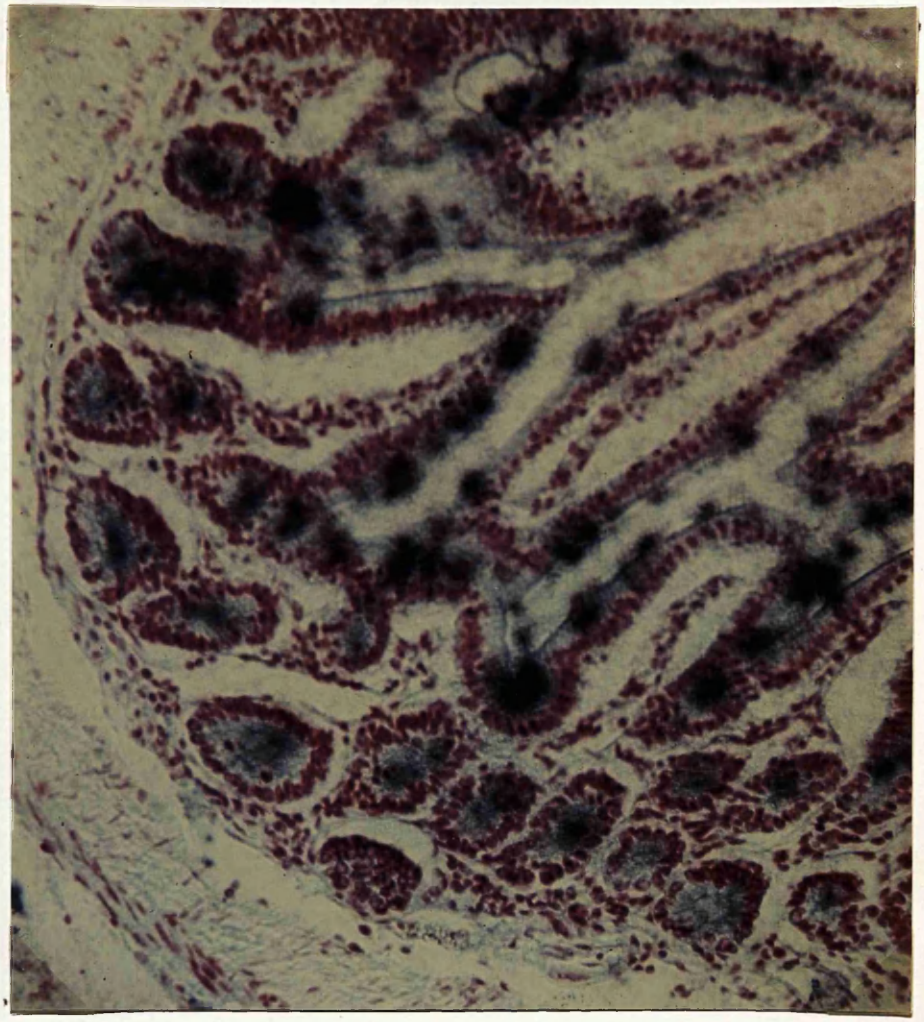


Fig. 58. The mucin in the goblet cells of the small intestine is strongly radioactive from the presence of sulphate. Autoradiograph X 400.

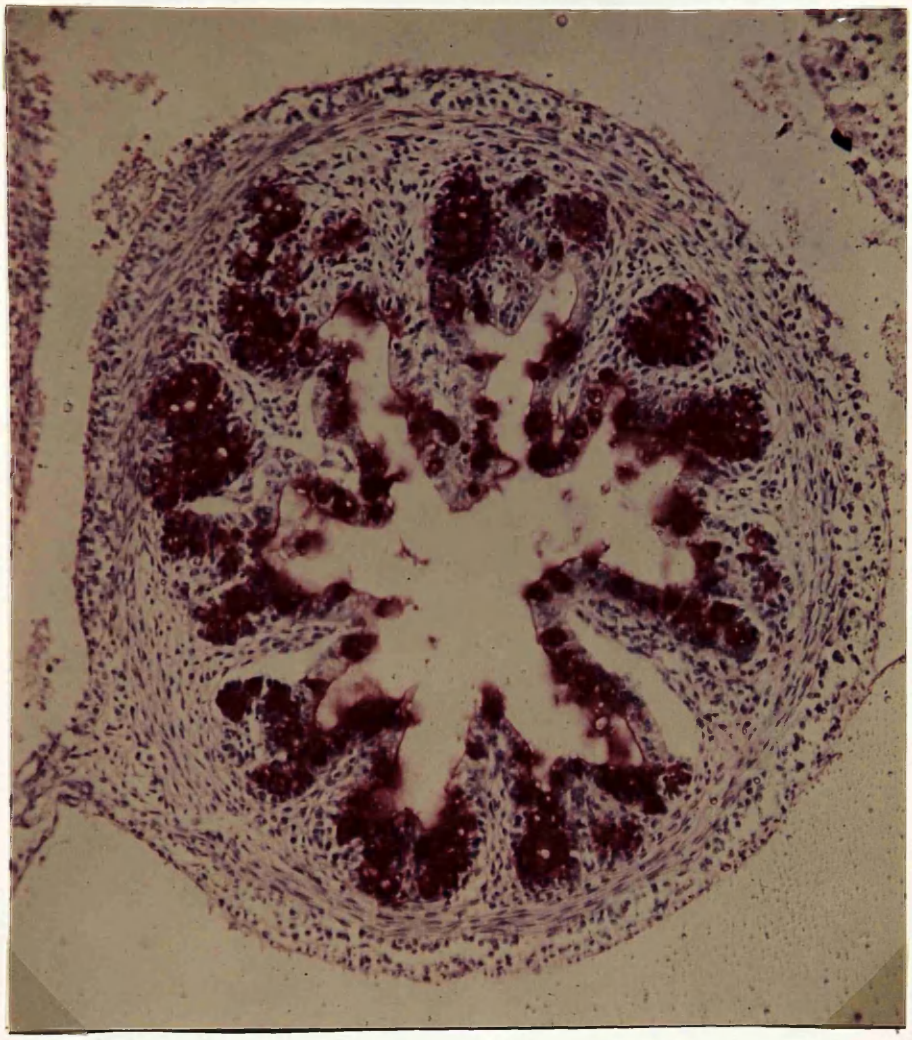


Fig. 59. The glands of the colonic mucosa secrete large quantities of mucin. Periodic acid-Schiff X 200.

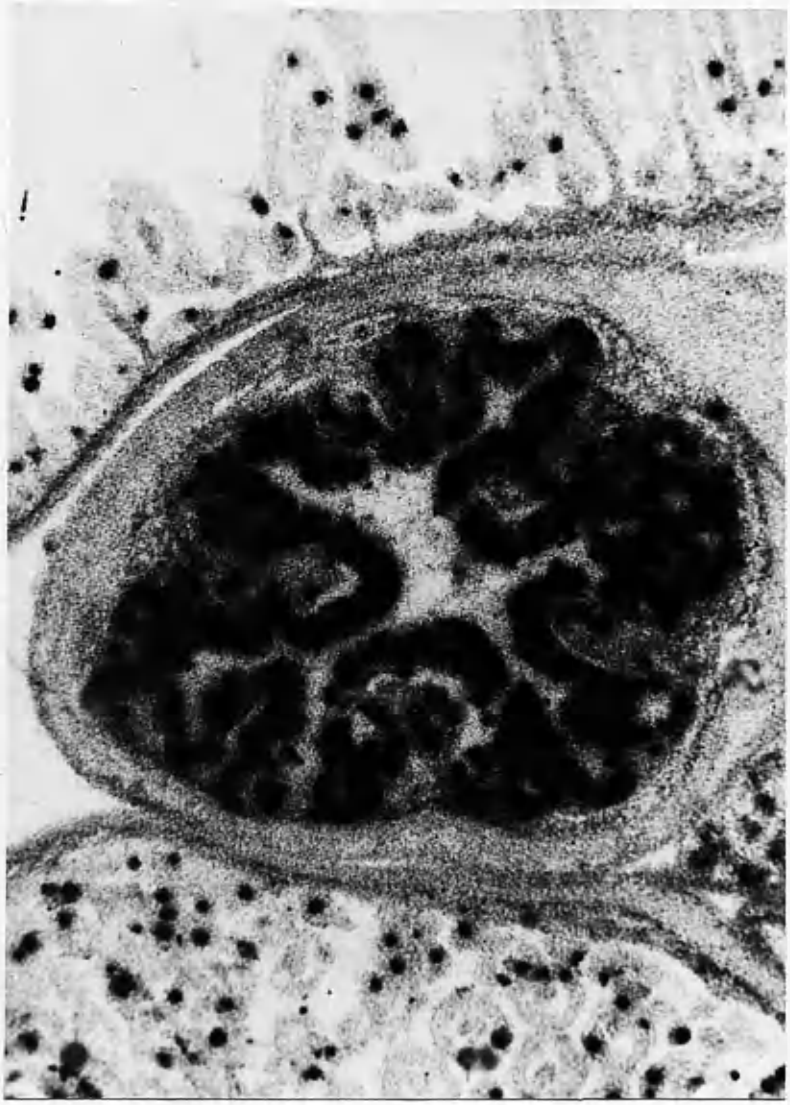


Fig. 60. This shows the large quantity of sulphate-containing mucin secreted by the mucosa of the colon. The individual goblet cells of the small intestine are also very active. Autoradiograph X 125.

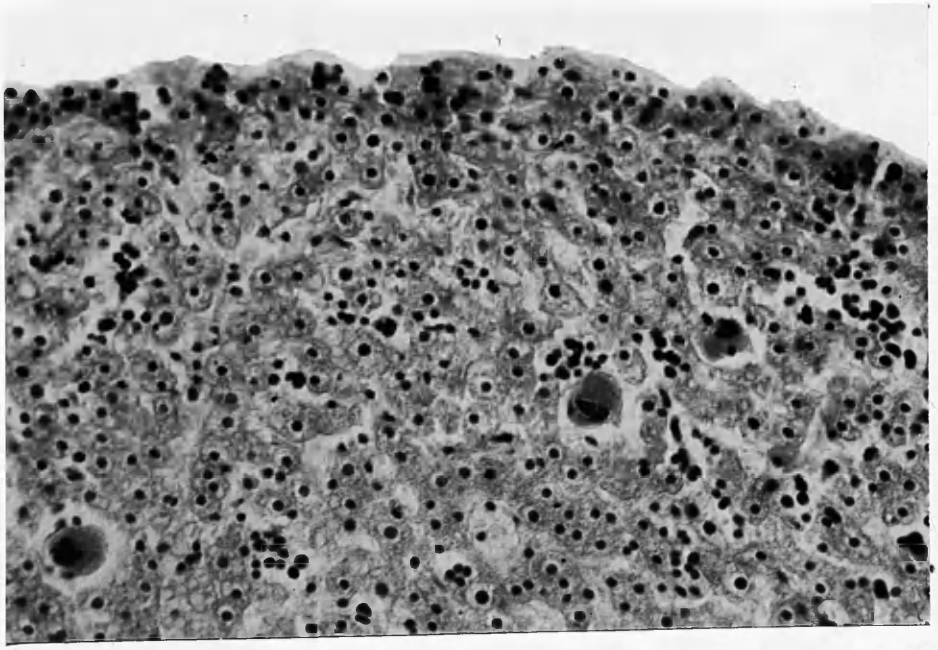


Fig. 61. Giant cells are scattered throughout the liver. They are probably megakaryocytes. Haematoxylin and eosin X 350.

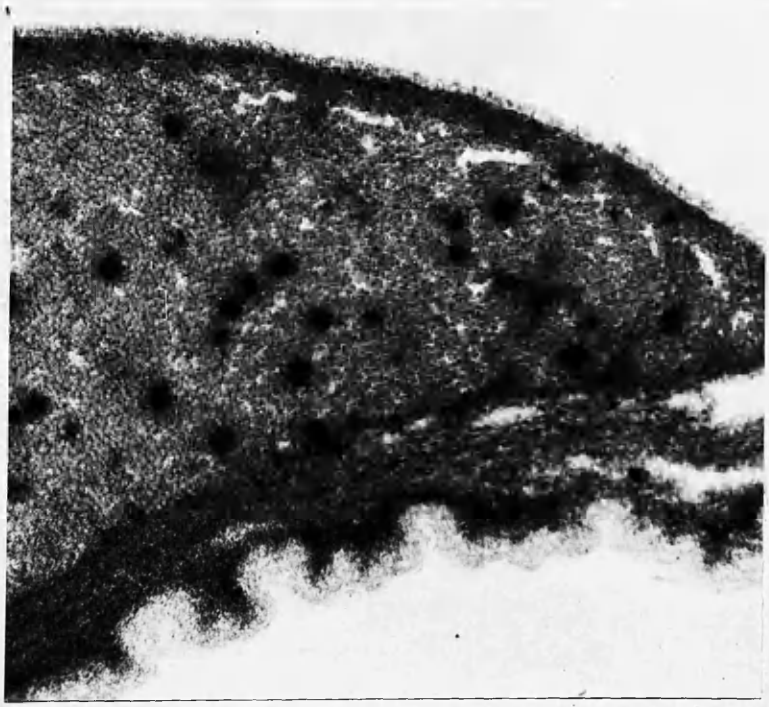


Fig. 62. Similar giant cells in the spleen take up a high concentration of sulphate. A lower concentration of the ion is located in the submucosa of the pro-ventriculus. Autoradiograph X 100.

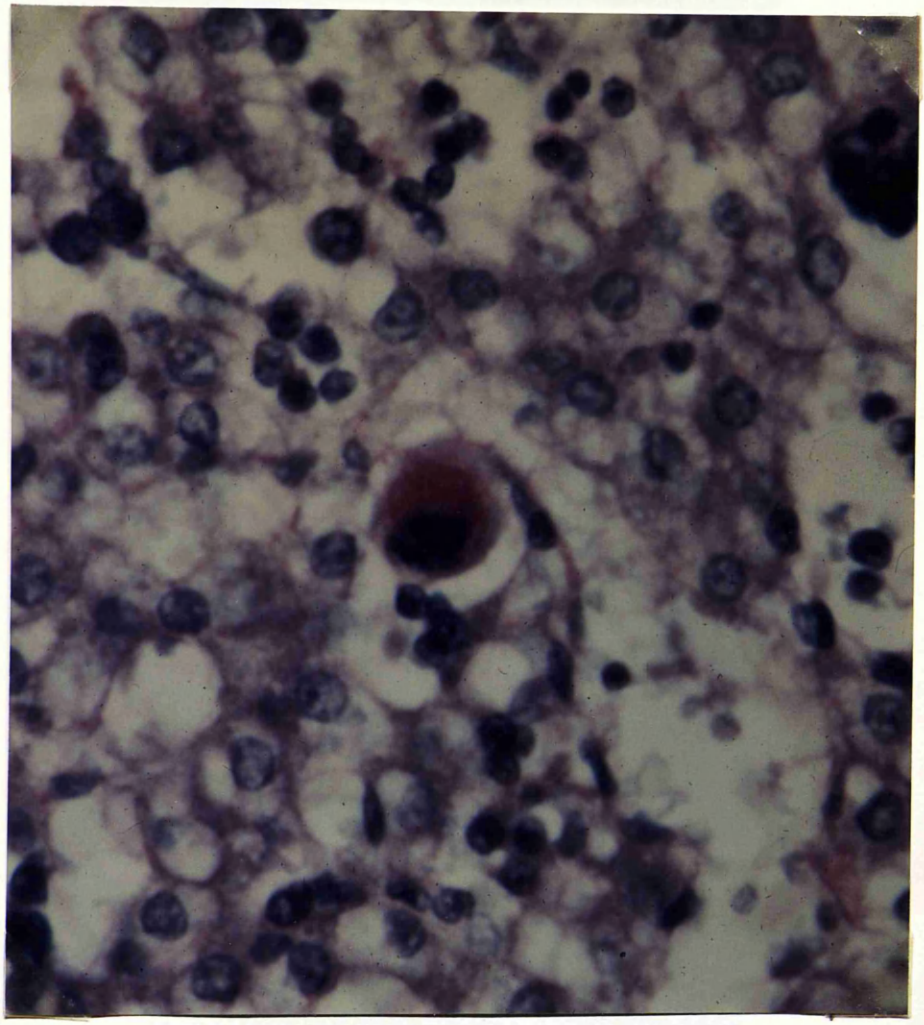


Fig. 63. The giant cells in the liver and spleen are usually mononuclear and the cytoplasm contains stainable mucopolysaccharide. Periodic acid-Schiff X 800.

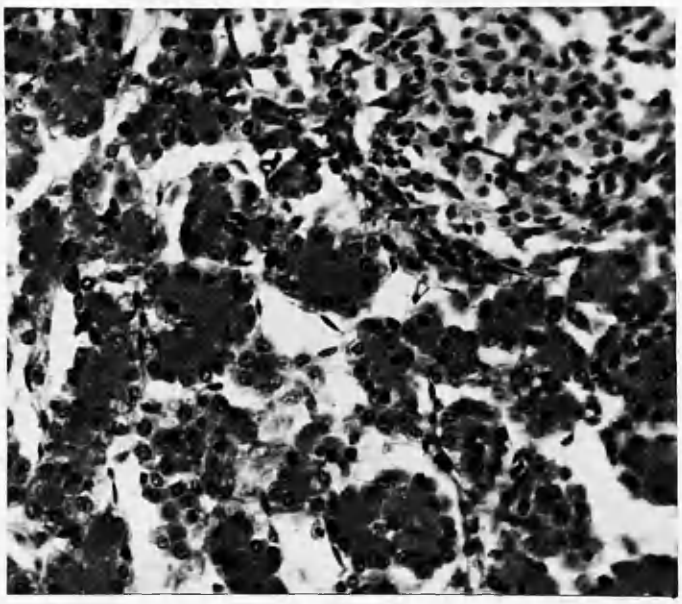


Fig.64. The acinar cells of the pancreas contain mucopolysaccharide in their cytoplasm. Periodic acid-Schiff X 200.

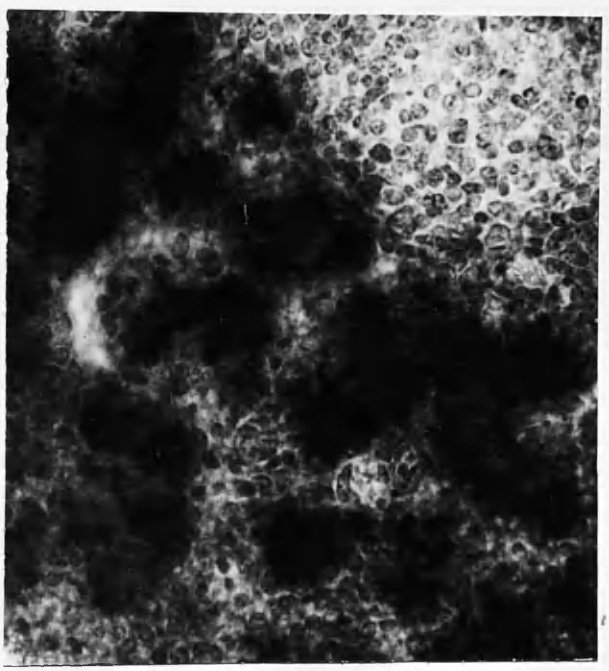


Fig. 65. The acinar cells take up S35, but the islet cells do not utilise the ion. Autoradiograph X 200.

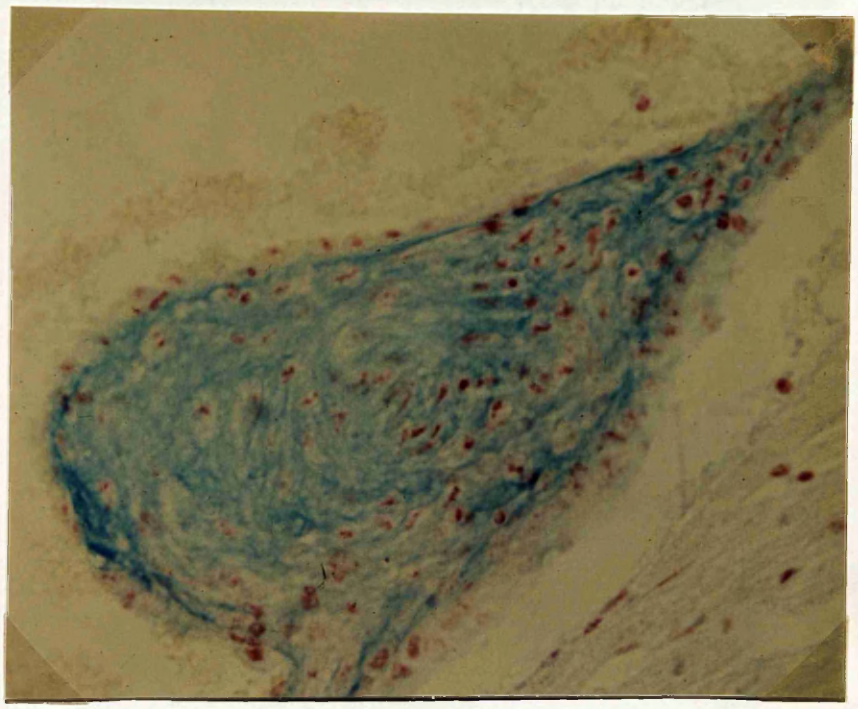


Fig. 66. In the aortic valve cusp of the mouse, there is a considerable quantity of mucopolysaccharide. Alcian blue X 1200.

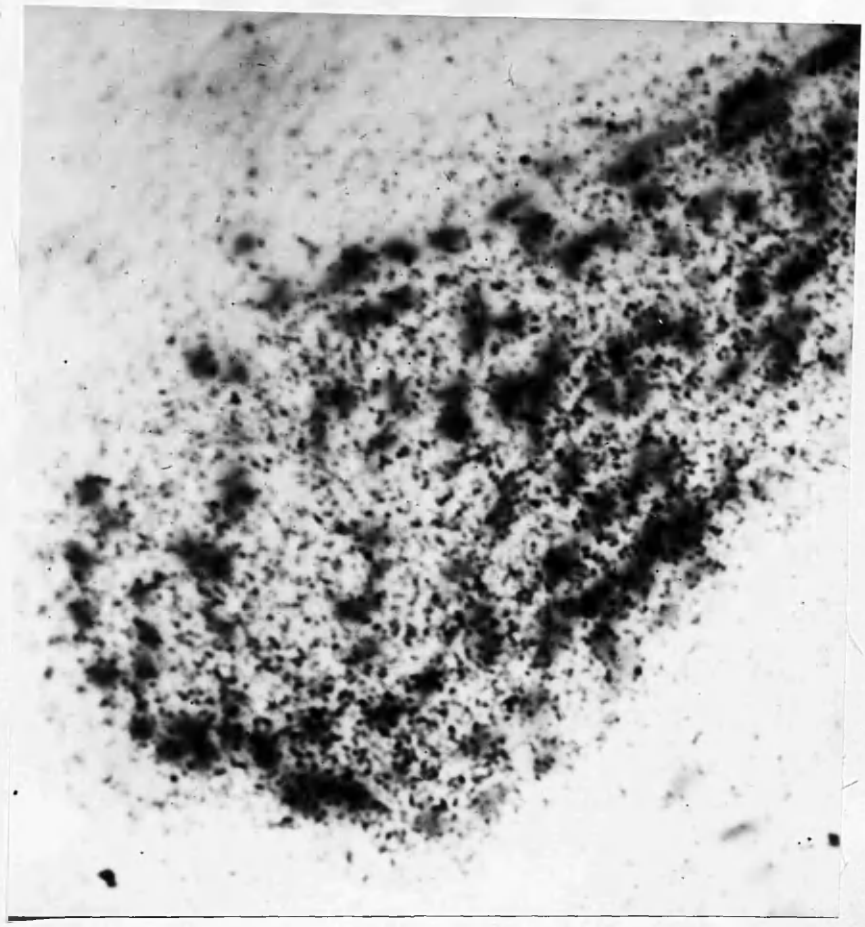


Fig. 67. Sulphate ion is incorporated in the mucopolysaccharide in the aortic valve cusp. Autoradiograph X 1500.

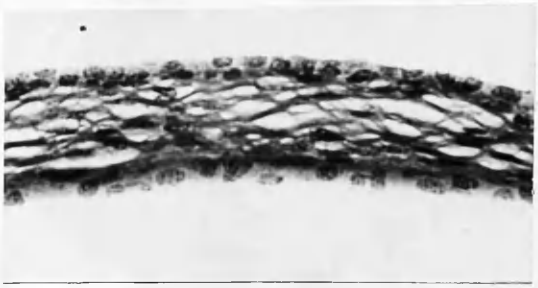


Fig.68. Mucopolysaccharide, greatly shrunken by fixation, is abundant in the cornea of of the new-born mouse. Periodic acid-Schiff X 400.

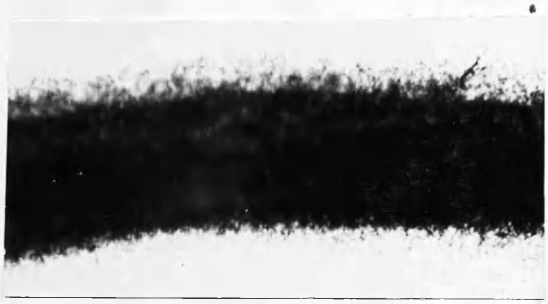


Fig. 69. A high concentration of sulphate is retained in this substance. Autoradiograph X 400.

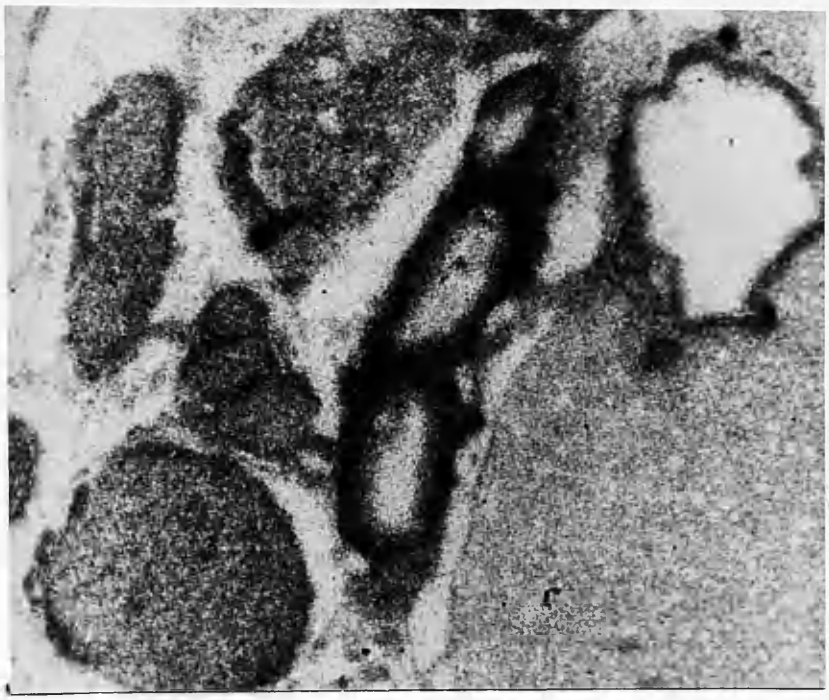


Fig. 70. In the brachial plexus, the nerves are weakly positive, but the medial coats of vein and artery are more active. Autoradiograph X 160.

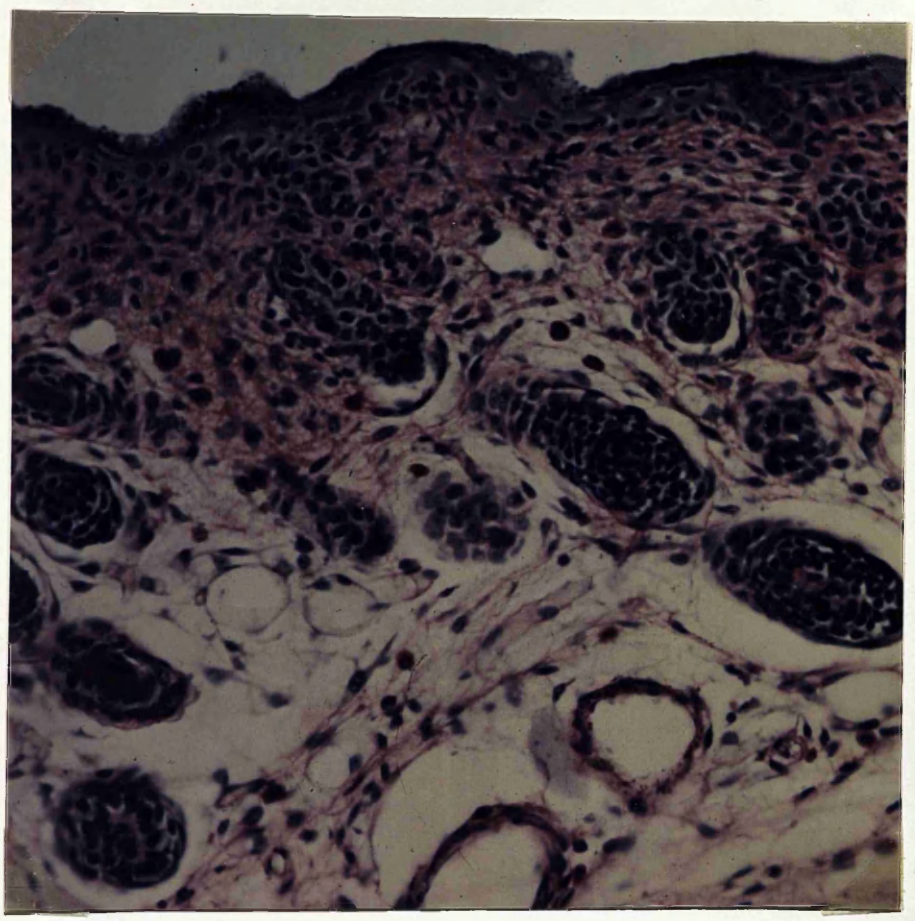


Fig. 71. In the skin, Schiff-positive mucopolysaccharide is present in the reticular layer of the dermis and in hair bulb. Some may also be detected in the mast cells and connective tissue fibres. Periodic acid-Schiff X 75.

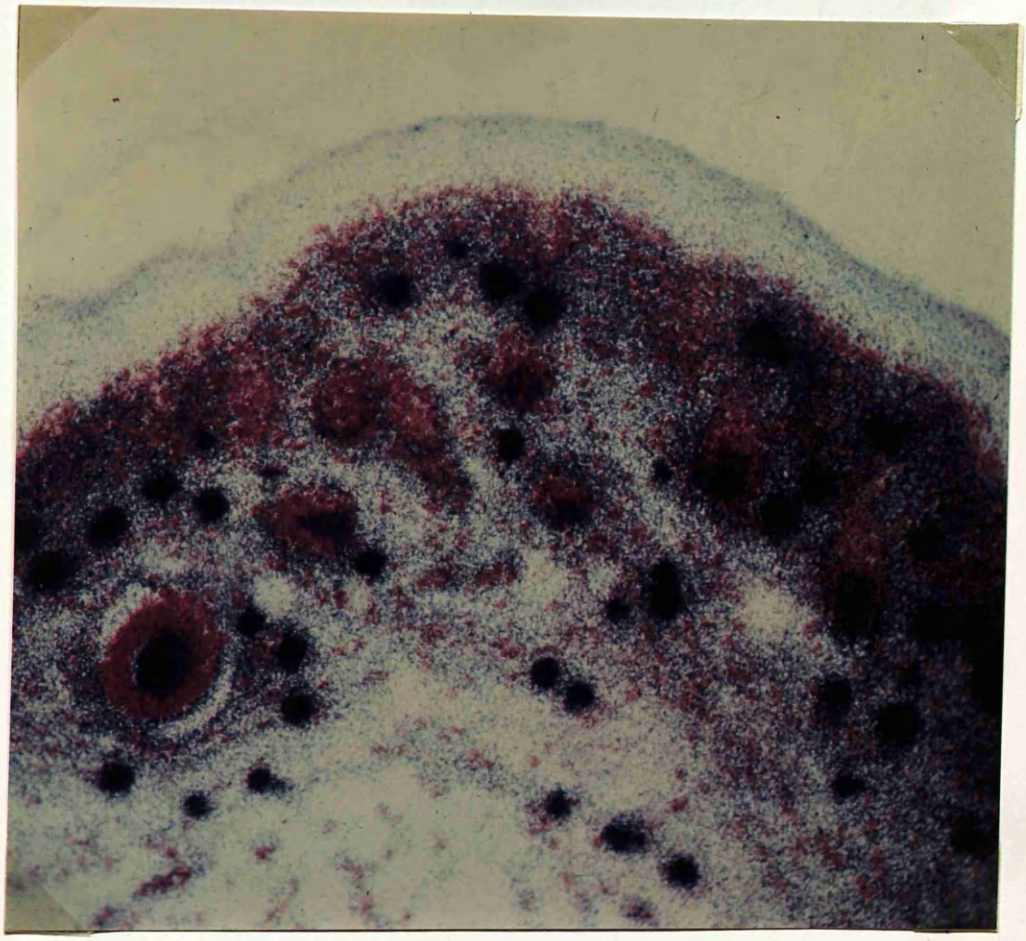


Fig. 72. In the skin, S35 is incorporated in the mucopolysaccharides of the reticular layer of the dermis and of connective tissue. Mast cells show very high activity. Autoradiograph X 75.

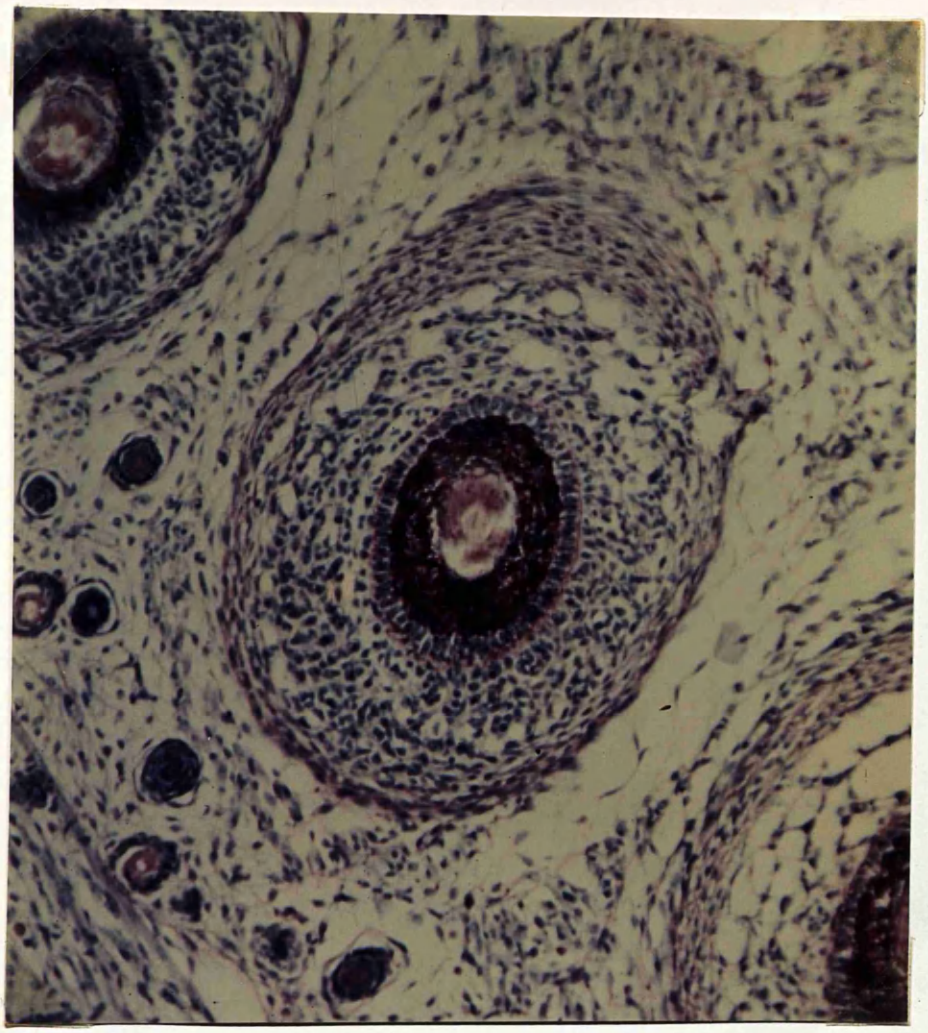


Fig. 73. In this section of hair (whisker), the keratin of the shaft stains feebly, the glycogen in the cells of the external hair sheath very intensely. The hyaline and dermal layers of the follicle are Schiff-positive. Periodic acid-Schiff X 50.

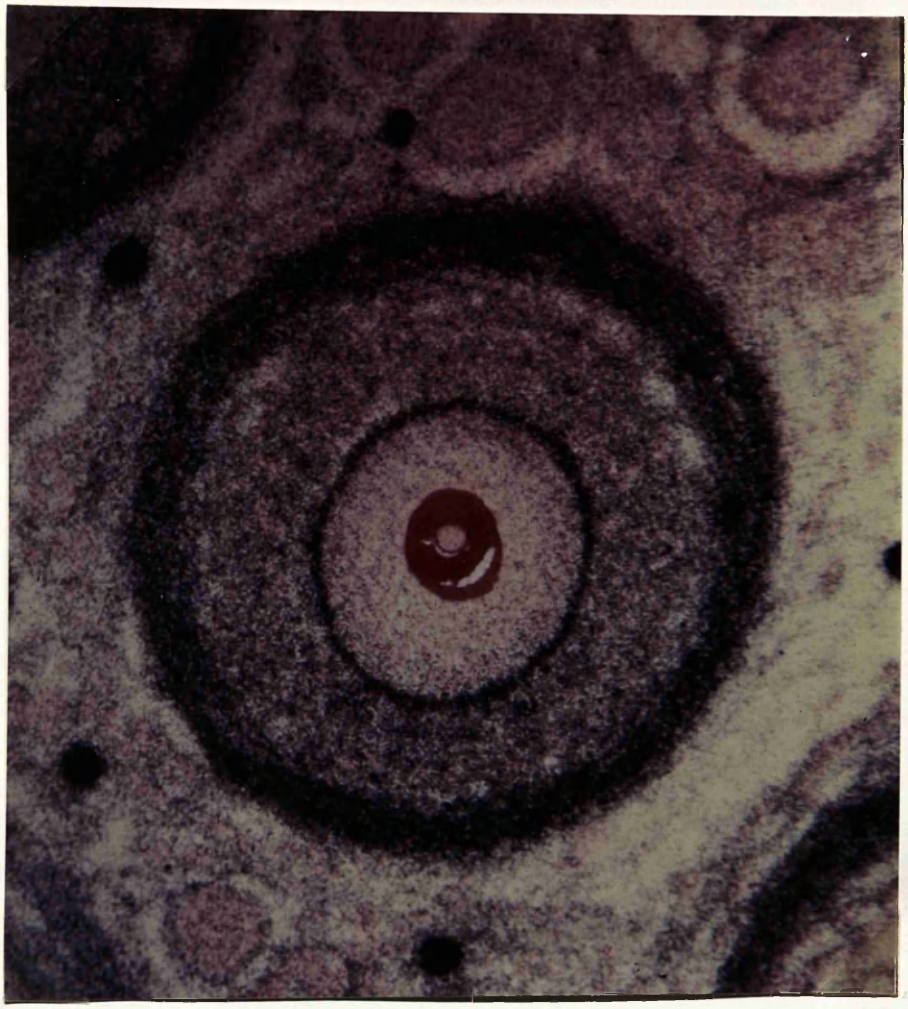


Fig. 74. The hair shaft and the cells of the external sheath are inactive, but the mucopolysaccharides of the hyaline and dermal layers of the follicle have retained S35. High concentrations of the ion are present in the four mast cells. Autoradiograph X 50.

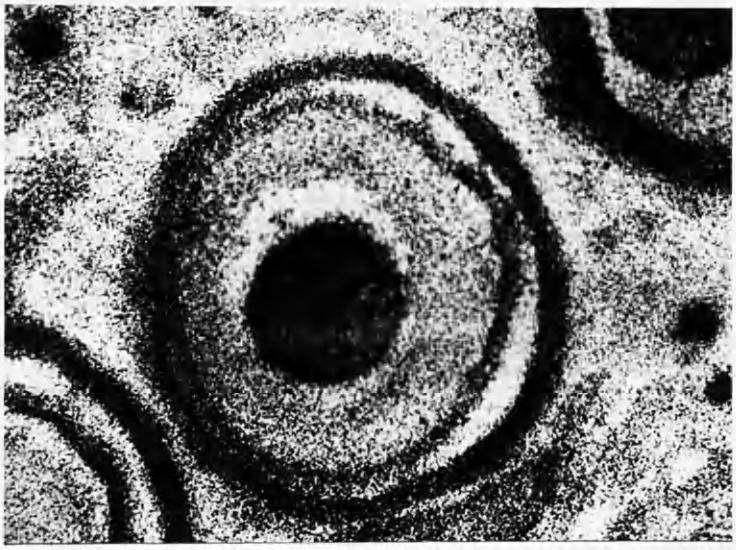


Fig. 75. In this section of hair (whisker), the mucopolysaccharide which is present in the bulb contains a high concentration of sulphate. The hyaline layer of the follicle is also positive. Autoradiograph X 40.

In every case the isotope is present in the tissues in close association with mucopolysaccharide (figs. 45 to 75, pages 151 to 176), and few deposits of mucopolysaccharide fail to take up the ion. Some of these are mentioned in table I. Of the four histochemical methods used, the Alcian blue method proved the most specific and reliable indicator of mucopolysaccharides. The periodic acid-Schiff routine usually stains the epithelial mucopolysaccharides well, but the picture is complicated by the staining also of keratin and glycogen (fig. 73, p. 174). The latter is very abundant in many tissues of the young mouse. The Hale method gives results similar to those with Alcian blue: sometimes it depicts connective tissue mucopolysaccharides particularly well. The metachromatic reaction with cresyl-fast violet is of use mainly in staining mast cells and cartilage: if sections are dehydrated in alcohol before they are mounted in D.P.X. the method is comparatively insensitive. It must be emphasised that, as with the autoradiographic results, the grouping of staining intensities into three categories is very approximate.

Many sections of each organ were examined from every animal for assessment of the concentrations of S35 and of stainable mucopolysaccharide. Random re-checking of many sections gave results which accorded very closely with those

obtained on first examination. In the young animals, no doubt because of their higher metabolic and growth rates, the tissues always retain higher concentrations of the isotope. For this reason, table I is based largely on the findings in this age group. The concentrations of S35 are lower in the tissues of the older group of mice and the results consequently less consistent. Cartilage presented some difficulty because of the fairly wide range of concentration of stainable mucopolysaccharide in the cytoplasm and matrix of this tissue. The S35 concentration is always very high in mature cells and moderate in the matrix. It is possible by treatment of sections with testicular hyaluronidase before autoradiography to reduce greatly the concentration of S35 in the matrix. The intracellular S35 is unaffected by this treatment (fig. 47, p. 153). Elsewhere, the enzyme removes much of the S35 fixed in connective tissues but has no effect on cellular concentrations.

Table I shows that the correlation between the autoradiographic and staining methods is very close. In every case where the isotope is present, there is stainable mucopolysaccharide. Even when the concentration, as judged by the stains, is low, the autoradiograph is positive.

In fact, the lowest detectable concentration (+) of mucopolysaccharides is more readily recognisable by autoradiography than by any of the staining methods. The second noteworthy feature, seen readily in table II, is the remarkably high concentration of S35 within cells compared with that incorporated in the extra-cellular mucopolysaccharides. Even in the young mice of group 1 whose metabolic and growth rates were high, the concentration in the mucopolysaccharide-rich connective tissue is low as compared with that within cells. Cartilage illustrates this particularly well. Stainable mucopolysaccharide is usually moderate in quantity in the cytoplasm of cartilage cells; much more is present in the intercellular matrix. With autoradiography, however, the matrix usually shows moderate concentrations but within the cytoplasm of mature cells S35 is found in high concentration. Immature cartilage cells do not metabolise S35 and many of the very large cells are also inactive (fig. 47, p.153 ; fig. 48, p. 154). Only the autoradiographic method reveals this important difference in metabolic activity between the cells. The giant cells throughout the spleen and liver show very moderate quantities of mucopolysaccharide, but their uptake of S35 is considerable (figs. 61 and 62, p.165 and p.166 ; fig. 63, p. 167). The autoradiographs with

diacetin-floated sections show no significant difference from those prepared from water-floated sections.

DISCUSSION

When S35 in the form of sulphate ion is given to animals it may follow several metabolic pathways. Most of it is very quickly excreted as inorganic and ethereal sulphates; a fraction is retained in sulphated mucopolysaccharides, and a trace may be found in the sulphur-containing amino-acid cystine, very probably incorporated in this way by the animal's intestinal micro-organisms.

S35 has been administered to man as sulphate ion on very few occasions (Borsook, Keighley, Yost and McMillan, 1937), and the rate of excretion is high. The rat has usually been employed to determine the fate of the S35 given in this form and in this animal more than 90 per cent. of the injected dose is eliminated in the faeces and urine within a few days as inorganic and ethereal sulphates (Laidlaw and Young, 1948; Dziewiatkowski, 1949; Everett and Simmons, 1952). Mice also appear to have a high rate of excretion and so, for autoradiography, it is necessary to kill the mice within a day of injection of the isotope.

The fraction of S35, given as sulphate ion, detectable

in cystine or protein is so small that it is most improbable that the rat or any other mammal can incorporate the element in this form into sulphur-containing amino-acids (Tarver and Schmidt, 1939 and 1942; Dziewiatkowski, 1954). The trace of radioactive cystine found is most probably synthesised by the intestinal bacteria; it is negligible in the autoradiographs.

The fraction of S35 sulphate retained by various tissues has been shown by chemical extraction to be present in an esterified form such as chondroitin sulphate - in skeletal cartilage and intestinal tract (Dziewiatkowski, 1952 and 1953); skin (Bostrom and Gardell, 1953); connective tissue ground substance and collagen (Layton, 1951b); aorta, spleen, kidney, tibia, red marrow, heart and skeletal muscle of chick embryos (Layton, 1952); and in the healing wound in the hen (Layton, 1950b and 1952).

The autoradiographs confirm the presence of S35 in those tissues and show its invariable association with histochemically demonstrable mucopolysaccharides. A few sites, for example, contain mucopolysaccharide but do not take up sulphate. Presumably the compounds present are non-sulphated; this is certainly true in the submaxillary salivary gland (Meyer, 1947). The technique clearly demonstrates that the highest activities are within cells

(table II) which are forming mucopolysaccharides, such as intestinal goblet cells, cartilage cells and mast cells. Fibroblasts in the quartz focus also show this (Part III; Curran and Kennedy, 1955a), and recent observations on the uptake of S35 in vitro by human cartilage show that the activity is strictly confined to the cartilage cells (Curran and Gibson, 1956). The metabolic activity of the cells determines the concentration of S35 within them. The more active tissues of young animals retain much higher concentrations of the element than those of adults, as has been previously shown (Layton, 1950a; Layton and Denko, 1952).

The cells of the external sheath in the upper part of the lower third of the hair follicles of the human mons pubis are known to stain metachromatically (Montagna, Chase and Melaragno, 1951). Sylvén (1950) believed that the polysaccharide-ester sulphates of the growing hair follicles played a role in the supply of sulphurous constituents in keratin synthesis and should be regarded as labile S-bearing compounds, but the present results provided no evidence that the epithelial cells of the external hair sheath metabolise S35 sulphate (fig. 73, p. 174 ; fig. 74, p. 175).

In the eye, a sulphuric acid ester of hyaluronic acid is present in the cornea (Meyer and Chaffee, 1940; Asboe-

Hansen, 1953) and in the autoradiographs it retains a considerable quantity of S35 (figs. 68 and 69, p. 171).

A surprisingly high degree of radioactivity is shown by the mixed bacterial flora of the mouse proventriculus. The flora is established within a day or two of birth. The bacteria also contain stainable mucopolysaccharide (fig. 52, p.157 ; fig. 53, p.158). So much radioactive mucus is present in the intestine beyond this region that it is not possible to comment on the bacterial activity there (fig. 59, p.163).

In the goblet cells of the intestine, S35 is found in fairly high concentration in the cytoplasm when this is present in sufficient quantity: the amount is less than that of the globule of mucigen within the cell. In contrast, "scatter" from the extremely active layer of superficial pro-dentine prevents determination of the activity of odontoblast cytoplasm in the unerupted teeth of the young mice (figs. 50 and 51, p.156).

The giant cells throughout the spleen and liver of mice are possibly megakaryocytes (fig. 61, p.165 ; fig. 63, p.167). It is known that megakaryocytes contain Schiff-positive polysaccharide, possibly glycogen (Pearse, 1953, p. 153), but more probably a "mucoid ground-substance" (Halmi and Davies, 1953). In fact, the presence of this material has been used to differentiate

the megakaryocyte from the Reed-Sternberg cell (Fisher and Hazard, 1954). In the present experiment the cells in the liver and spleen contain Schiff-positive material which is also stainable by the Alcian-blue and Hale methods. They readily metabolise S35 (fig. 62, p.166) but clumps of radioactive platelets were not identifiable. Lajtha (1954) has recently shown in smears that cells of the myeloid series, at an early stage of their differentiation, take up inorganic sulphate and form a substance probably related to the chondroitin sulphates. This could not be confirmed in sections of marrow. The amount of radioactivity in the marrow cells is very small but the bone lamellae contain a great deal of S35 (fig. 48, p. 154), and Layton (1950a) may be correct in suggesting that Singher and Marinelli (1945) and Dziewiatkowski (1949) included some of these in their red-marrow samples when they found much of the element in extracts of this tissue.

The relatively few studies published on the autoradiographic use of S35 give a restricted idea of the degree of resolution and sensitivity obtainable by this technique (Friberg and Ringertz, 1954). Holt and Warren (1953 a and b), using a freeze-drying method, obtained negative results with water-floated sections. They found S35 in blood; I was unable to show this. Odeblad and Bostrom (1952) found in the rat and rabbit diffuse uptake

by all coats of the alimentary tract, heart, muscle, liver, spleen, pancreas, lung and thyroid. In my experience, only brain shows this type of uptake and here the stains show a low but definite concentration of mucopolysaccharide. Odeblad and Bostrom also found uptake by the islets of Langerhans and by respiratory epithelium, but these, in the mouse, appear to be consistently negative. Their "colloidal particles" in liver and spleen probably correspond with the megakaryocytic activity in my results.

Layton and Sher (1953), using S35, showed by chemical extraction that 59 per cent. of the sulphate-containing mucopolysaccharides of articular cartilage is depolymerised by testicular hyaluronidase, whereas only small percentages of those present in soft tissues react in this way. In the present study, autoradiographs of sections pre-treated with enzyme show clear-cut loss of S35 in the matrix of cartilage and to a less extent in the connective tissues (fig. 46, p.152 ; fig. 47, p. 153). The high concentrations present within cells are unaffected (fig. 47, p.153 ; fig. 48, p. 154). This method of using hyaluronidase overcomes some of the objections raised by Benditt and French (1953) and French and Benditt (1953) to the interpretation of results obtained by treatment of

sections with the enzyme prior to staining. The failure of hyaluronidase to attack mucopolysaccharides bound within fixed cytoplasm contrasts with the result obtained by subjecting the living cell in culture to the enzyme's influence (Part V).

CONCLUSIONS

Labelled sulphur (S35), given as sulphate ion to mice, is metabolised by cells producing sulphated mucopolysaccharides.

In most cases where mucopolysaccharide can be stained in the tissues of the mouse, a sulphur-containing fraction is detectable by autoradiography.

The autoradiographic method is capable of a remarkable degree of resolution.

It is an effective means of specifically identifying the presence in tissues of sulphated mucopolysaccharide, and even more significant is its accurate identification of the cells elaborating this group of compounds.

PART V

SOME ASPECTS OF CARTILAGE BEHAVIOUR IN VITRO

INTRODUCTION

Mr. Thomas Gibson, F.R.C.S., of the Department of Plastic Surgery, has been interested for several years in the survival of human cartilage in graft form and in storage. For two important reasons there is a need for a simple, reliable test of vitality of this tissue. Firstly, grafts of living cartilage behave unpredictably, some distorting and others undergoing resorption. Death of the tissue may play a part in these changes, but this has not so far been definitely ascertained. Secondly, experimental work in rabbits suggests that homograft cartilage can survive indefinitely (Craigmyle, 1955). This may be true also of man, and if so, a cartilage "bank" would be of considerable value. To establish it, however, information would be required regarding survival of the tissue in storage.

Histological examination alone is inadequate to determine viability, for, though cellular autolysis is presumptive evidence that the tissue was dead when fixed, the absence of autolysis does not necessarily mean that it was alive. Cellular proliferation after transplantation or in tissue culture, which is irrefutable evidence of vitality in other tissues, either does not occur in the case

of adult cartilage or is such a slow process that it is valueless as a criterion of vitality. Adult cartilage differs from most other tissues in possessing metabolism of a very low rate and almost wholly anaerobic in pattern (Bywaters, 1937). The carbohydrate utilisation of cartilage is, indeed, one of the lowest among all tissues (Laskin, Sarnat and Bain, 1952), partly because of its avascularity and the sparseness of its cell population. Manometric observations are thus unreliable, especially when small pieces are under examination. The various enzymes involved in glycolysis have been demonstrated in cartilage (Albaum, Hirshfeld and Sobel, 1952), but none of these was thought to provide a suitable basis for a test of vitality. Attention was therefore turned to radioactive sulphur (S35), for inorganic sulphate thus labelled is metabolised by chondrocytes in the mouse (p. 153 ; Curran and Kennedy, 1955b). Because S35 has been administered to humans on very few occasions and its long-term effects are unknown, it was necessary to devise an in vitro method. Layton (1949) found by direct measurement after chemical extraction that embryonic cartilage and chondrogenic tumours would take up the sulphate ion in vitro. It seemed feasible, therefore, for human cartilage to show similar activity

which could be demonstrated by the autoradiographic technique.

MATERIAL AND METHODS

TECHNIQUE

A slice of cartilage 0.5 - 1 mm. in thickness is placed in a roller tube containing 2 ml. of Tyrode's solution. The pH of the medium is adjusted, after sterilization, to 7.2 and the tube incubated for 48 hours at 37°C in a roller tube machine revolving at 10 revs. per hour. Each ml. of solution contains 1 µc. of carrier-free sulphate (S35), 100 units of penicillin, and 100 µg. of streptomycin. Thereafter, the cartilage is fixed in alcohol or in 10 per cent. aqueous formalin and paraffin sections are cut at 6 or 8 µ. Autoradiographs are prepared from these by the stripping-film technique (p. 134), using coarse and fine grain films. Adjacent sections are stained with haematoxylin and eosin, Alcian blue and by Hale's method.

Variations in the quantity of the medium, the size of the specimen, and the period of incubation are permissible for simple qualitative results, but the standard technique described here and summarised in table III (p. 201) has been generally adhered to, so that comparative quantitative estimations could be made.

MATERIAL

Most investigations were made on human cartilage, normal and in graft form. Some specimens were obtained at post-mortem examination, and cartilage from animals was also used, mainly for control purposes.

A. Normal cartilage. Two hundred and ninety-nine specimens of human cartilage, removed at operation from ear, rib or nose from subjects ranging in age from 4 to 48 years, have been used. Of these, 101 specimens were subjected to the standard test only (table III, p. 201). The remainder were treated in various additional ways in an attempt to ascertain the relationship of sulphate uptake to tissue vitality and also to determine the permeability of cartilage matrix to electrolytes. The treatments included exposing the cartilage sample to various degrees of heat and cold, to fixation, and to enzyme inhibition, as follows:-

1. Twelve slices were maintained at 4°C. in labelled Tyrode's solution for 2 days; 8 of these were subsequently incubated for 2 days at 37°C., while 4 were immediately fixed and processed.

2. Twenty-three specimens were incubated in labelled Tyrode's solution containing one of the following substances, which, with the possible exception of heparin, are enzyme inhibitors.

(a)	M/5	Mersalyl:	5 specimens
(b)	M/15	sodium malonate:	" "
(c)	M/20	sodium fluoride:	" "
(d)	M/200	potassium cyanide:	" "
(e)	1 per cent.	heparin:	3 "

3. One hundred and five specimens of cartilage were used to determine the times of survival of cartilage at different temperatures and in various media. The details of these are given in table IV (p. 201). When a sample was removed from storage, it was placed in fresh Tyrode solution containing S35 and incubated for 2 days at 37°C., as in the standard technique. Autoradiographs were then prepared as usual.

Where Tyrode or plasma was the storage medium, 6 to 12 tubes were used in each experiment. Each tube contained a 1 mm. slice of cartilage in 2 ml. of the appropriate medium. In the 37° C. experiments, one sample was removed daily and tested, but this was done only at 3 or 4-day intervals when the storage temperature was 4° C. In those cases where the specimen was simply wrapped in a moist saline swab for storage, sampling was twice weekly, and if the tissue was in the form of a relatively large block, the 1 mm. slice for testing was cut from it. In the freezing experiments, the stored specimens ranged in thickness from 1 to 6 or 7 mm.

4. Fifty two specimens were subjected to one of the following procedures prior to testing:

(a) 8 specimens treated for 24 hours with a variety of fixatives, including 10 per cent. formalin, absolute alcohol and cold (4°C) acetone.

(b) 9 slices heated to 100°C.

(c) 4 pieces kept at - 25°C. for 24 hours.

(d) 4 small portions placed in liquid nitrogen for 10 minutes.

(e) A further 21 specimens placed in solid carbon dioxide for periods ranging from 2 hours to 2 years. Two others were placed for 2 hours in 10 per cent. glycerol solution, 2 in 20 per cent. and 2 in 30 per cent., prior to storage for 2 hours in carbon dioxide.

5. The diffusion of electrolytes through cartilage matrix and plasma clot was tested in three experiments.

(a) The volume of labelled Tyrode medium was increased to 10 ml., and in it two rods of cartilage, one without perichondrium, each 1 cm. in diameter and 2 cm. long, were incubated. Thereafter, each was cut into slices, 1 mm. thick, which were processed to paraffin in the usual way prior to preparation of autoradiographs.

(b) Two rods, one without perichondrium, each 5 cms. long and 1 cm. diameter, were used. The lower end of each was immersed in 25 ml. of labelled Tyrode solution in a bottle sealed by a thin rubber diaphragm, which fitted the cartilage closely to prevent spread of the medium up its surface by capillarity. The whole, enclosed in a sealed container (fig. 76, p. 196), was then incubated for 48 hours.

Slices 1 mm. thick were then taken at intervals along each rod, and all the cartilage for 1.5 cm. above the surface of the Tyrode solution was sampled in this way. As in (a), paraffin sections were then cut and autoradiographs prepared, to determine how far up the rod the chondrocytes showed uptake of sulphate.

(c) Two 1 mm. slices of cartilage were enclosed in plasma clots and each incubated as usual in labelled Tyrode solution; the clot was approximately ½ cm. thick.

B. Graft cartilage. Small portions of auto- or homo-graft cartilage which had been inserted some time previously were obtained whenever unsatisfactory results with a graft necessitated further operative repair. In other cases, when serial operations were required, occasional graft samples were obtained. In all, 11 specimens of autograft and 6 of homograft became available and were tested. The oldest autograft was of 37 years' duration, while the oldest homograft was 7 months.

C. Post-mortem cartilage. Twelve specimens of costal cartilage, obtained at post-mortem examination, from subjects ranging in age from 17 to 58 years of age, were tested. The period lapsing from time of death to the time of post-mortem examination ranged from 3 to 60 hours.

D. Animal cartilage. Twelve specimens of costal cartilage from mice ranging in age from birth to 18 months were tested

in addition to 8 from young guinea-pigs and rats.

RESULTS

A. NORMAL CARTILAGE

Without exception, all specimens of normal, freshly-removed, untreated cartilage show uptake of S35, manifested by blackening of the film overlying the tissue section. Radioactivity is concentrated in small areas immediately overlying the chondrocytes (fig. 77, p. 197 ; fig. 78, p. 198), and the radiation appears to have originated from the cytoplasm of the cells and not from the matrix (fig. 79, p. 199), although "scatter" is sometimes marked around each cell. When fixation shrinks the cytoplasm so that it adheres to one part of the lacuna, radiation is found only over this part. The mature chondrocytes show the highest activity; the least mature cells adjoining the perichondrium and the large vacuolated cells towards the centre do not retain the ion to the same degree (see also fig. 48, p. 154). The intensity of the reaction varies according to alterations in incubation and exposure times, but, because of the wide dispersal of the cartilage cells, there was never any doubt about the result being positive or negative.

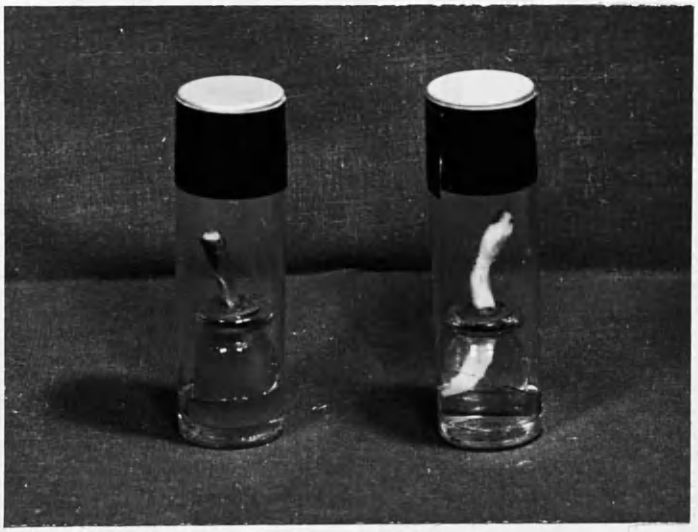


Fig. 76. The lower end of each cartilage rod is immersed in labelled Tyrode solution in a bottle sealed by a thin rubber stopper which fits the cartilage closely and prevents spread of the medium up the surface of the rod. The whole is inside a sealed container. The rod on the left is covered with perichondrium.

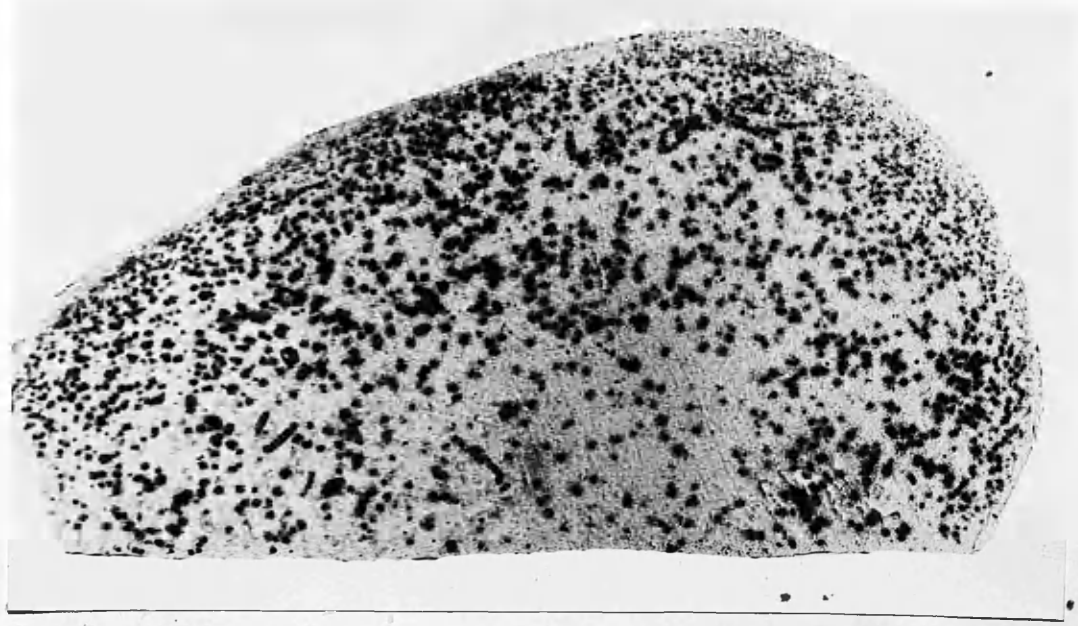


Fig. 77. This section of adult human costal cartilage shows uptake of radioactive sulphate. The radioactivity is located over the chondrocytes, and none is found in the matrix. Autoradiograph X 15.

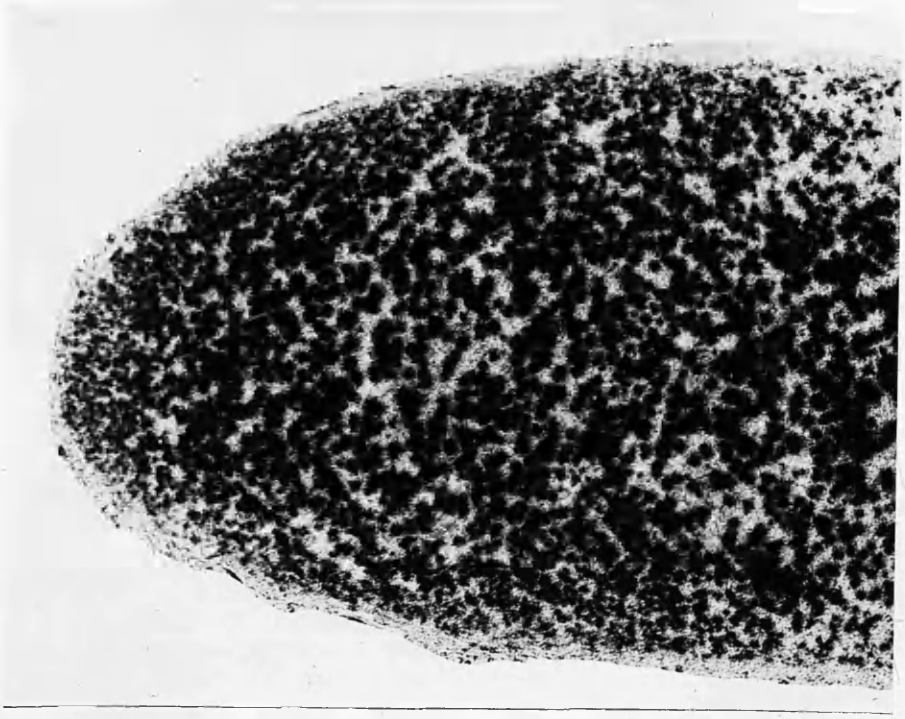


Fig. 78. This section of human costal cartilage from a young adult shows an extremely high uptake of S35 by the cells but none is found in the matrix. Autoradiograph X 20.

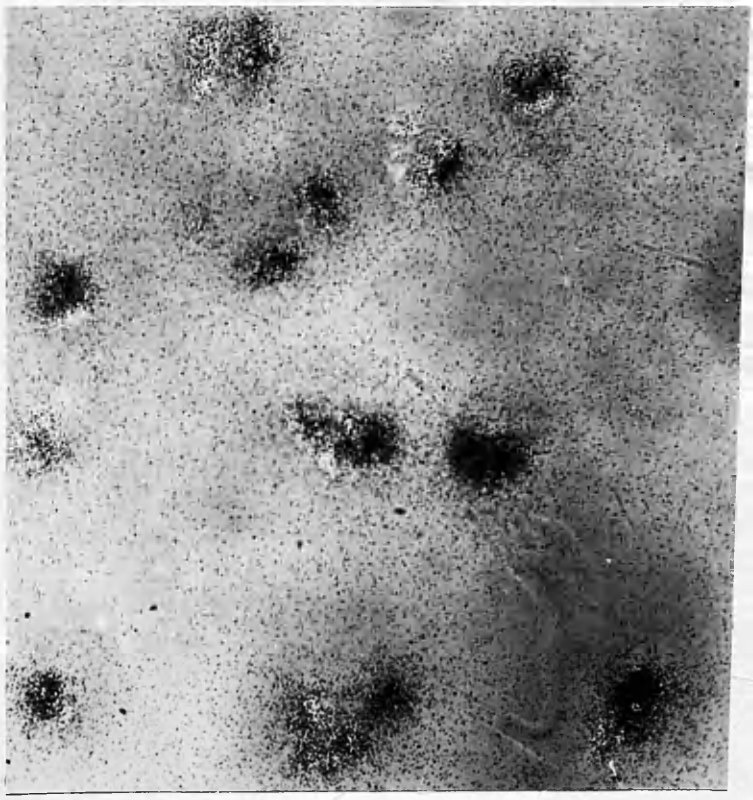


Fig. 79. This section of adult human costal cartilage shows the strictly cellular location of the radioactive sulphate, despite the presence of slight "scatter". The matrix area reveals only a normal background level. Autoradiograph X 150.

A1. No uptake of sulphate occurs at 4°C., but the cartilage is capable of normal uptake if incubated thereafter at 37°C.

A2. The presence of sodium malonate or Mersalyl in the Tyrode's solution greatly diminishes the uptake of sulphate ion by the cartilage; only a trace of activity is detectable over a number of cells. Sodium fluoride and potassium cyanide are without detectable effect in the concentration employed. The presence of 1 per cent. heparin in the medium has no effect on sulphate uptake.

A3. In each storage-survival experiment, a number of positive results are followed by one or two weakly positive, while the remainder are thereafter negative. The results are remarkably consistent, and the maximum times of survival are shown in table IV, p. 201 . It is noteworthy that cartilage will survive at 4°C. for 40 days in Tyrode or 28 days when simply wrapped in block form in a moist saline swab, as judged by this test.

A4. Fixation of the tissue invariably abolishes uptake completely, as does heating to 100°C. Cooling below 0°C., either in the deep-freeze or by means of solid carbon dioxide, also produces a negative result, as does instantaneous cooling in liquid nitrogen. Pre-treatment with various strengths of glycerol solution does not protect

TECHNIQUE

1. ½ mm. SLICE OF CARTILAGE.
2. 2 ml. TYRODE SOLUTION IN ROLLER TUBE.
3. 48 HOURS INCUBATION AT 37° C.
4. FIXATION: PROCESS TO PARAFFIN SECTIONS.
5. AUTORADIOGRAPHS - STRIPPING FILM.
6. EXPOSURE - 15 DAYS: THEN DEVELOPMENT AND FIXATION.

TABLE III.

SURVIVAL TIMES OF STORED CARTILAGE

STORAGE MEDIUM.	STORAGE TEMPERATURE.	SURVIVAL TIME (APPROX.) DAYS.
1. TYRODE.	37° C.	4.
2. TYRODE.	4° C.	40.
3. PLASMA.	37° C.	2.
4. (a) MOIST SALINE SWAB - SLICES.	4° C.	14.
(b) MOIST SALINE SWAB - SOLID ROD.	4° C.	28.
5. CO_2 SNOW.	-70° C.	0. (No effect from glycerol pre-treatment).
DEEP FREEZE.	-25° C.	

TABLE IV.

DIFFUSION OF SULPHATE ION.

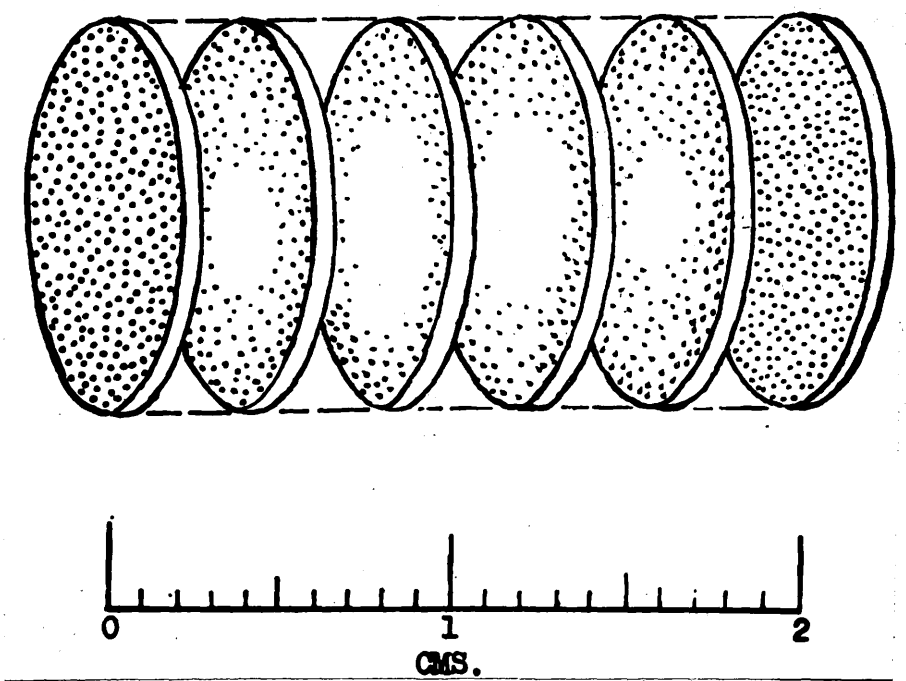


Fig. 80. The rod of cartilage was sampled at 6 points after incubation in labelled Tyrode. This shows the distribution of S35 in the chondrocytes in these 1 mm. slices. The ion has diffused into the rod for a distance of about 5 mm.

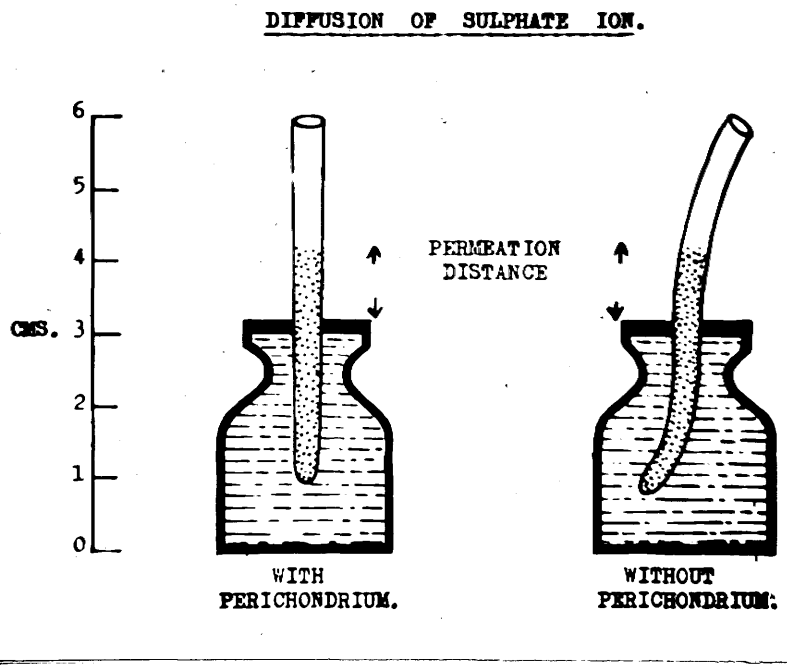


Fig. 81. This shows the uptake of S35 in the cartilage rods (fig. 76). Autoradiographs from sample (1mm.) slices at intervals along each rod demonstrate that the sulphate has diffused upwards for more than 1 cm.

the cells from the effect of freezing (table IV, p. 201).

A5. The rods of cartilage show diffusion of the sulphate, and presumably of the other electrolytes in the Tyrode medium, into the rod for at least 0.5 cm. (fig. 80, p. 202), while the vertical rods show diffusion of the ion upwards for 1 cm. (fig. 81, p. 203). In neither experiment does the presence of the perichondrium have any effect. Cartilage enclosed in plasma clot shows activity equal to the control.

B. GRAFT CARTILAGE

The oldest autograft, inserted 37 years previously, shows uptake of sulphate; in places there is some replacement of cartilage by cellular myxomatous tissue which shows no uptake. The oldest homograft, of 7 months' duration, is also positive.

C. POST-MORTEM CARTILAGE is capable of utilising sulphate up to 60 hours after the death of the subject.

D. ANIMAL CARTILAGE shows uptake similar to the human. In tissue from very young animals, the ion is probably partly located in the stroma.

DISCUSSION

A. Factors influencing sulphate uptake.

It has previously been shown that, after injection of labelled sulphate, the location of the ion in cartilage

is largely cytoplasmic, even in very young mice (p.146). Bostrom (1952) found that, in vitro, chondroitin sulphate alone does not take up the ion. It would appear, therefore, that in the experimental animal sulphate must be metabolised by the chondrocytes before incorporation in the chondroitin sulphate of the matrix, and the present results with human tissue agree exactly with this hypothesis. It is of interest that only a trace of activity is found over each cell when 0.05 per cent. of testicular hyaluronidase is present in the Tyrode medium. This enzyme is able to depolymerize chondroitin sulphate.

One might expect a sulphatase to be involved in the uptake of labelled sulphate, but chondrosulphatases have not been found in the higher animals (Fromageot, 1950). They are present in bacteria, and radio-activity in autoradiographs is occasionally found over clumps of micro-organisms; contamination of this type did not appear to have the inhibitory effect on the cartilage which Layton (1950b) found.

The inhibitory action of Mersalyl confirms the finding of Bostrom and Jorpes (1953) that the uptake of the ion is associated with a sulphydryl enzyme. The inhibiting effect of sodium malonate is significant. The specimens of cartilage tested in this way were from two young subjects, 12 years of age. The various intermediaries involved in the Krebs cycle have been isolated from

cartilage (Follis and Berthrong, 1949) and malonate probably acts on the cells of young cartilage by competitive inhibition of succinic dehydrogenase. It thus appears that respiratory activity is necessary for uptake of labelled sulphate by cartilage cells. Boyd and Neuman (1954) have confirmed this important link by chemical extraction methods in embryonic cartilage of fowls; in this type of tissue the pattern of respiration is almost wholly aerobic.

It has been claimed (Lutwak-Mann, 1940; Bywaters, 1937) that no cytochrome or cytochrome oxidase is present in cartilage. For this reason, the failure of potassium cyanide, in the concentration employed, to affect the reaction is not unexpected.

The enzyme system responsible for sulphate uptake was invariably inactivated by freezing. The lethal effect of freezing was confirmed by the presence of autolytic changes, seen in ordinary stained sections and apparently developing during the test procedure. It appears from this result that sulphate uptake is inseparably linked with the viability of the chondrocyte. In accordance with this view is the failure of cold acetone (4°C.) fixation to preserve the enzyme system responsible for it.

Heparin and heparinoid substances occasionally exert

toxic effects on man, causing alopecia and diarrhoea (Hirschboeck, Madison and Pisciotta, 1954). Perhaps they exert these effects by interfering with the synthesis of sulphated mucopolysaccharides in the tissues by substrate competition. From the present experiment, however, it appears that heparin fails to influence the synthesis of chondroitin sulphate in vitro.

Uptake of S35 by animal cartilage was similar to that by human cartilage and accords with the result in vivo (fig. 47, p. 153 , and fig. 48, p. 154). It reacted in the same way as the human material to inhibitors of respiratory activity. The respiratory pattern of the chondrocyte undoubtedly changes with age, and use of enzyme inhibitors with the test would help to determine it. The koniophage also takes up sulphate in vitro (p. 139), so it should also be possible to examine its respiratory activity in the same indirect way.

Survival of the adult chondrocyte for more than 60 hours after death of the subject must be related to its very low metabolic rate of a largely anaerobic pattern. Quantitative measurements of the normal uptake of different age-groups at intervals up to 3 or 4 days after death might provide the basis of a test for estimating in medico-legal work the time of death.

B. Survival of cartilage in grafts and in storage.

The results listed above in Section A indicate that the enzyme system involved in the uptake of labelled sulphate will not survive cellular death, and that a positive result may be regarded as a reliable test of vital activity (Curran and Gibson, 1956). In support of this, the results with specimens of cartilage stored in various ways and at different temperatures (table IV, p. 201) are of the highest significance, in that they are in close agreement with the times of survival of mammalian skin as determined by a grafting technique (Pepper, 1954).

The test indicates that human homograft cartilage can survive for at least 7 months in the tissues, while autografts are able to remain alive indefinitely.

The diffusion experiments (A5; fig. 80, p. 202 and fig. 81, p. 203), which show that electrolytes can diffuse through cartilage matrix for at least 1 cm., even against gravity, suggest that grafts up to 2 cm. in diameter may survive in toto if bathed in tissue fluids, and apparently even a layer of plasma clot 0.5 cm. thick would not have a deleterious effect on their chances of survival.

C. Significance of the cytoplasmic location of sulphate.

The mesenchyme surrounding an area in which cartilage

is developing becomes the perichondrium. In its outer part, the cells differentiate into fibroblasts, but in the inner region the mesenchymal cells form chondroblasts which are quickly surrounded by intercellular substance and then become chondrocytes. Their common origin suggests that the fibroblast and the chondroblast share a number of activities, though in different stages of development. Thus, both may be capable of forming fibres and mucopolysaccharide, but whereas the fibroblast is predominantly fibre-forming, the chondroblast is especially concerned with synthesis of mucopolysaccharide. If a close relationship between the cells is accepted, the striking illustration in human cartilage of the fact that sulphate is metabolised by cells and not directly incorporated by ion exchange in the sulphated mucopolysaccharide of the matrix must be regarded as highly significant. This principle will almost certainly apply to all tissues, and indeed the results in Part IV strongly reinforce the suggestion. Consequently, it may be asserted with more assurance that the positive uptake obtained both in vivo and in vitro with the koniophage means that this cell is secreting sulphated mucopolysaccharide.

The in vitro technique should prove suitable for determining the sites of formation of sulphated

mucopolysaccharide in many human tissues. Its sensitivity is such that failure to show uptake by a cell secreting mucoid material would suggest that the substance formed by the cell lacks sulphate. Pathological deposits of mucopolysaccharide should also be investigated readily, and preliminary positive results have been obtained, for example, with the pleomorphic adenoma of salivary gland origin.

CONCLUSIONS

Human cartilage incorporates labelled sulphate into chondroitin sulphate *in vitro*. This activity is exhibited only by the living chondrocyte and the test may serve as a reliable indicator of the viability of cartilage. Sulphate does not appear to undergo direct ion exchange in chondroitin sulphate even in living cartilage.

The test has been used to investigate several aspects of human cartilage metabolism. It shows that, though cartilage cells are invariably killed by freezing, they remain viable up to 40 days at 4°C. At 37°C. or in the body post-mortem, however, they die within a few days. Applied to graft material, the test indicates that human autograft cartilage can survive indefinitely and homograft material for at least 7 months. Moreover, a modified technique suggests that it is possible for electrolytes

to diffuse through cartilage matrix for at least 1 cm., in quantities sufficient to maintain the viability of chondrocytes.

It would appear that adult human chondrocytes continue to form chondroitin sulphate at a considerable rate.

Further applications of the method to other human tissues, normal and pathological, open a wide field of investigation into the behaviour of connective tissues.

SUMMARY

Collagen formation was studied in the silicotic focus in the peritoneal cavity and subcutaneous tissues of the mouse. The cells which ingested the finely-divided quartz were stimulated to form not only reticulin and collagen fibres but also abundant acid mucopolysaccharide. This substance was liberated from the cytoplasm of the fibroblasts and persisted throughout the evolution of the silicotic focus.

Though it was not possible to determine what relationship the mucopolysaccharide had to the argyrophilia of the reticulin, it was clearly not responsible for an intensely metachromatic reaction present in the quartz focus. This was probably caused by a layer of colloidal silicic acid, and a gel of this type might play a role in the development of the silicotic focus similar to that postulated for endogenous mucopolysaccharide in other sites of collagen formation. The presence of a layer of colloidal silica on quartz particles would render the solubility theory of silicosis more feasible, and the metachromatic reaction should be very useful for studying silicosis in man.

Administration of cortisone reduced considerably the rate of formation of acid mucopolysaccharide

and connective-tissue fibres in the quartz lesion, but inhibition was not complete, and the times of first appearance of mucopolysaccharide and fibres were not delayed under its influence. The hormone effected a quantitative rather than a qualitative reduction in the inflammatory response of the tissues to the dust, and the activities of the individual fibroblast were apparently unaffected. However, cortisone was capable of altering completely the form of the large foci which form on the serosal surface of the abdominal wall; these were replaced by a specific, flat, fibrous plaque.

To devise a new technique for studying the acid mucopolysaccharides of the tissues, the distribution of sulphate labelled with S35 was determined by autoradiography in almost all of the tissues of the mouse, and in every case the radioactive material was closely associated with stainable mucopolysaccharide. Even more significant was the high concentration of the S35 within cells which apparently incorporated it in sulphated mucopolysaccharide synthesised by them. This idea was strongly reinforced by the finding that when human cartilage was kept alive by tissue culture, the ion was located only in the cytoplasm of the chondrocyte. The fibroblasts in the quartz focus also took up sulphate, and so it may be inferred that they

also form sulphated mucopolysaccharide which they subsequently release into their environment. This conclusion is in accord with that derived previously from use of staining methods.

The uptake of sulphate ion by the chondrocyte depended upon the survival of the cell, and a test of viability based on this observation provided much information with regard to the behaviour of human cartilage in grafts and in storage and to the permeability of cartilage matrix. The *in vitro* method used to show uptake of sulphate by cartilage cells will almost certainly permit investigation of mucopolysaccharide formation in a wide range of human tissue.

APPENDIX

TECHNICAL METHODS

FIXATION

All tissues for fixation were cut in very thin slices, usually about 1 mm. thick, and a period of 18-24 hours in fixative solution was then ample. Bouin's fluid, neutral 10 per cent. aqueous formalin, alcohol-formol-acetic, absolute alcohol, Carnoy's solution, and a mixture of equal parts of absolute alcohol and acetone were used. Though they produced markedly different histological appearances, all fixed acid mucopolysaccharide. In this respect, the aqueous fixatives, particularly Bouin's fluid, served as well as alcohol or Carnoy's solution. Bouin's fluid was employed on many occasions, since it was particularly good for reticulin and collagen fibres, and tissues fixed by it readily yielded thin sections. When the vascularity of the quartz focus was being studied, however, it was replaced by neutral aqueous formalin. Alcohol and alcohol-acetone mixture were used at 4°C. when parallel enzyme studies were in progress, and these fixatives were specifically chosen for the earlier autoradiographic experiments, in view of the possibility that the others might occasionally inhibit the development of the photographic emulsion. This difficulty

was not encountered, and aqueous fixatives were used in later experiments of this type.

PROCESSING PROCEDURE

Paraffin sections were used routinely, and all tissues were taken through celloidin solution before clearing in benzol and embedding in paraffin in the vacuum oven for 90 minutes.

Several blocks of tissue in the experiments described in Part I of this thesis were prepared by the freeze-drying technique. The sections thus obtained were briefly fixed in absolute alcohol before staining.

STAINING METHODS

Haematoxylin. Several preparations were employed. Harris's (Lee, 1950, p.145) was in most frequent use, and Mayer's (Lee, p.145) also was satisfactory, but Ehrlich's acid haematoxylin (Lee, p.148) proved to be the method of choice for demonstrating acid mucopolysaccharide of both epithelial and connective tissue origin. The counter stain in each case was 1 per cent. alcoholic eosin.

A rapid iron haematoxylin method was used in conjunction with Van Gieson's stain. A haematoxylin solution ready for immediate use (Lee, p.152) was very reliable and constant in its results, which were superior to those obtained with Weigert's iron haematoxylin mixture.

1. Bring paraffin sections to water.
2. Mordant for 30 minutes in 2.5 per cent. ferric alum solution.
3. Place in the 1 per cent. haematoxylin solution for 30 minutes.
4. Differentiate in the ferric alum solution till only the nuclei are stained.
5. Wash for 30 minutes.
6. Stain by Van Gieson's method.

Van Gieson's stain. This proved to be the most satisfactory method for demonstrating collagen fibrils. It is practically a "histochemical" technique when used with care, and gave very consistent results. It is important to add the correct amount of acid fuchsin, and not more than 5 parts of a 1 per cent. solution of this dye were combined with 100 parts of a saturated solution of picric acid in water. The mixture was tested on carefully selected sections.

1. Fix, preferably in Bouin's fluid.
2. Process to paraffin.
3. Bring sections to water and stain by the rapid iron-haematoxylin method.
4. Wash the sections thoroughly before immersion in the Van Gieson's stain for 5 minutes.
5. Rinse briefly in water, dehydrate, clear and mount in D.P.X.

Reticulin stain. The method was based on that of Gordon and Sweets (1936).

1. Fix in Bouin's solution or 10 per cent. formalin.
2. Process to paraffin.
3. Bring sections to water and oxidise for 1-2 minutes in acidified permanganate solution: 47.5 ml. of 0.5 per cent. aqueous potassium permanganate plus 2.5 ml. of 3 per cent. sulphuric acid.
4. Wash in water and bleach until white in 1 per cent. oxalic acid.
5. Rinse in distilled water and mordant for 30 minutes in 2.5 per cent. aqueous iron alum.
6. Wash thoroughly in water.
7. Impregnate for 10-15 seconds in silver bath (Lee, p.431).
8. Rinse quickly in water and place in 10 per cent. formalin solution for 3-5 minutes.
9. Tone in 0.2 per cent. gold chloride solution for 1-3 minutes.
10. Wash and fix in 5 per cent. sodium thiosulphate for 5 minutes.
11. Wash well.
12. Dehydrate, clear and mount in D.P.X.

METHODS FOR MUCOPOLYSACCHARIDES

Metachromasia. Thionin, toluidine blue and cresyl fast violet (basic dye) were used. Selection of a suitable sample of dye is very important.

1. Bring paraffin sections to water.

2. Stain for 30 minutes in 0.05 per cent. solution of the stain in citrate buffer pH4. The pH was adjusted on occasion as high as 7 or as low as 2.

3. Dehydrate very rapidly, clear and mount in D.P.X.

If the metachromatic reaction is weak, the section should not be dehydrated; it may be mounted in glycerine jelly.

Sulphation. This process of esterifying carbohydrates (Kramer and Windrum, 1953) to render them metachromatic was occasionally employed. Sometimes the sections thus treated were stained by the Hale or Alcian-blue method instead of metachromatically, to determine whether sulphation had any effect on the results with these stains.

1. Bring paraffin sections to water.

2. Dry thoroughly in air. This is important.

3. Immerse in concentrated sulphuric acid for 60-75 seconds. Sections tend to be lost at this stage, and less destructive is a sulphuric acid-acetic anhydride-ether mixture. This contains fuming sulphuric acid 50 ml., acetic anhydride 35 ml., and di-ethyl ether 15 ml. The sulphuric acid is added slowly, drop by drop, to a mixture of the other two reagents.

4. Wash thoroughly in running tap water.

5. Stain for metachromasia with cresyl fast violet or thionin.

Colloidal iron method. Hale (1946) recommended fixation in Carnoy's solution, but all the fixatives listed (p. 215)

proved suitable.

1. Fix thin tissue slices for 18-24 hours.

2. Dehydrate, clear and embed in paraffin. Mount sections without albumen.

3. Bring sections to water and flood with a mixture of dialysed iron (B.D.H.) 1 vol. and acetic acid (2M) 1 vol. for 10 minutes. The colloidal iron solution described by Rinehart and Abul-Haj (1951) may be used in place of this mixture, but it is in no way superior, in my experience.

4. Wash in running water for 10 minutes. This helps to eliminate non-specific staining.

5. Flood with a potassium ferrocyanide (0.02 M) - hydrochloric acid (0.14 M) solution for 10 minutes.

6. Wash and counterstain with neutral red or safranin.

7. Differentiate the counterstain during dehydration till only the nuclei are red.

8. Mount in D.P.X.

Hyaluronidase may be introduced at Stage 3. After its use, counterstaining should be avoided, since it tends to obscure the effect of the enzyme on the section. Acid mucopolysaccharides stain deep blue by Hale's method.

Alcian blue.

1. Bring paraffin sections to water.

2. Stain in a freshly-prepared and filtered 1 per cent aqueous solution of Alcian blue for 30-45 seconds.

3. Rinse in distilled water.

4. Counterstain with neutral red for 1-2 minutes.

5. Dehydrate rapidly, clear, and mount in D.P.X.

Sections may be placed in 1 per cent. alkaline alcohol (pH9) for 2 hours after Stage 3. Thereafter, they may be stained with haematoxylin and differentiated in acid alcohol with no effect on the Alcian blue.

Periodic acid-Schiff technique. The following solutions are required:-

1. Periodic acid, 0.5 per cent. solution in distilled water.

2. Schiff's reagent. 1 g. of basic fuchsin is dissolved in 200 ml. of boiling distilled water, and when this has cooled to 50° C., 1 ml. of concentrated hydrochloric acid and 2 g. of sodium bisulphite are added. The flask is shaken, tightly stoppered, and left for 24 hours at room temperature. It should be straw-coloured. Adsorbent charcoal (0.5 g.) is added, and, after shaking, the solution is filtered. It should now be colourless, and is stored at 4°C.

3. Reducing bath. A mixture of 5 ml. of N hydrochloric acid and 5 ml. of 10 per cent. potassium metabisulphite is diluted to 100 ml. with distilled water.

4. Mayer's haemalum solution.

Method:

(1) Bring paraffin sections to water.

(2) Immerse in periodic acid solution for 5-10 minutes.

(3) Rinse in water.

(4) Pour on Schiff's reagent and leave for 20 minutes.

(5) Rinse in water.

(6) Place in reducing bath for 1-2 minutes.

(7) Wash in running water for 5-10 minutes.

(8) Counterstain with haematoxylin for 1-2 minutes. Blue with Scott's tap-water substitute.

(9) Dehydrate, clear and mount in D.P.X.

Control sections were always used, to determine the activity of the Schiff's reagent.

Hyaluronidase.

1. Bring paraffin sections to water.

2. Place in a Coplin jar containing 0.025 per cent. enzyme solution (1 ampoule of Benger's Hyalase in 4 ml. distilled water or phosphate buffer pH6.8). An equal number of control sections are placed in boiled enzyme solution.

   The filtrate from a 24-hour culture of Cl.welchii or a 1 in 500 solution of the venom of Bothrops jararaca may replace the testicular enzyme.

3. Incubate for ½ - 4 hours at 37°C. If long incubation periods are used, 1 drop of toluol should be added to the medium to inhibit growth of micro-organisms. When only one or two sections are required, they should be incubated in Petri dishes to prevent evaporation.

4. Stain by the colloidal-iron or Alcian-blue methods. Sections treated with a solution of hyaluronidase stain less intensely with all stains, and allowance should be made for this non-specific action when estimating the specific effect of the enzyme on mucopolysaccharide.

OTHER HISTOCHEMICAL PROCEDURES

Ribonuclease. This should be prepared by the McDonald (1948) modification of the Kunitz (1940) method.

1. Bring paraffin sections to water.

2. Incubate for 1 hour at 37°C. in 0.1 per cent. solution of the enzyme in distilled water.

3. Wash in running water.

4. Stain sections and controls with thionin or cresyl fast violet.

5. Wash, dehydrate quickly, clear, and mount in D.P.X.

Diastase.

1. Bring paraffin sections to water.

2. Cover with 1 per cent. solution of malt diastase in distilled water for 30 minutes at room temperature.

3. Stain sections and controls by the P.A.S. routine.

Romieu reaction.

1. Bring paraffin sections to water.

2. Cover with syrupy phosphoric acid, and incubate for ½ - 1 hour at 56°C.

The method is destructive to sections. Proteins containing tryptophane give a purple-red colour.

Millon reaction.

1. Bring paraffin sections to water.

2. Incubate for 10 minutes at 37°C. in a solution containing 5 per cent. mercuric acetate and 15 per cent. trichloracetic acid.

3. Add about one-tenth volume of a 1 per cent. solution of sodium nitrite, and incubate for a further 25 minutes.

4. Rinse sections in 70 per cent. alcohol.

5. Dehydrate, clear and mount in D.P.X.

Proteins containing tyrosine are coloured pink or brick-red.

AUTORADIOGRAPHY

The technique is described on pages 134 , 135 , and 136 . The method given (p. 135) for staining the tissue section through the photographic emulsion was evolved only after trial of a number of dyes, including haematoxylin and eosin, neutral red, metanil yellow, iron haematoxylin, lithium carmine, picric acid alcohol, alum cochineal, and celestin blue. Mayer's haemalum often provides an excellent histological picture, but it tends to mask a weakly-positive result.

An attempt to stain the sections at 60°C. with a neutral red-carbol fuchsin mixture before application of the stripping film failed, for the film overlying the section later resisted development. Pre-staining in this way was therefore abandoned.

Many different developers and fixers were tried and found to be satisfactory. The time of development may be greatly increased with no ill-effects. A stop bath of 1 per cent. acetic acid is advisable between developing and fixing, and it is important to allow autoradiographs stored at 4°C. to warm to room temperature in the dark before

immersion in the developer.

TISSUE CULTURE

A roller-tube machine was designed and built for these experiments.

The medium in use at first was based on that described by Layton (1950b), but it was replaced by the following solution:-

NaCl	8.00 g.
KCl	0.20 g.
$CaCl_2$ anhydrous	0.20 g.
$MgCl_2$	0.10 g.
NaH_2PO_4	0.05 g.
$NaHCO_3$	0.7 g.
Glucose	1.00 g.
Redistilled water.	

Measure 950 ml. of redistilled water into a large beaker and add 4 drops of 1 per cent. solution of phenol red. Weigh and add each of the first five salts to the water separately and in the order listed, stirring the solution until each is completely dissolved before adding the next. Now add the glucose and pour the mixture into a 2-litre volumetric flask. The sodium bicarbonate solution is prepared separately, by measuring 150 ml. of redistilled water into a 250 ml. flask and adding 0.7 g. of sodium bicarbonate. Mark the fluid level in the two flasks with a diamond pencil. Stopper each with a cellophane-covered cork. Autoclave as soon as possible.

The volumes, when the flasks have cooled, should closely approximate those before autoclaving, but sterile distilled water may be added to compensate for a small loss by evaporation. The solution in the large flask is now yellow in colour. Add enough sodium bicarbonate to change its colour to pink (pH 7.4). The solution is stored at 4°C., the flask being sealed with a tightly fitting sterile rubber stopper covered with a sterile paper-cap.

When required, a small quantity of the medium is taken and penicillin (100 units/ml.) and streptomycin (100 µg./ml.) added to it. The pH is again adjusted to 7.4, if necessary, with bicarbonate solution, and 2 ml. quantities measured into sterile $\frac{5}{8}$" Pyrex test tubes, which are closed with vaccine-type rubber caps.

The tissue tested should be in the form of a slice not more than 1 mm. in thickness and 1 cm. in diameter. This sample should be taken with sterile precautions, as far as possible. After incubation in the roller-tube machine for 48 hours at 37°C., the tissue is fixed and processed to paraffin, when sections may be prepared for autoradiography.

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