Histochemical changes in organs and tissues of scorbutic guinea-pigs

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The histological changes accompanying scurvy have been known for many years and have often been described in detail. Thus Wolbach & Howe (1926) say: 'We characterize the condition of scorbutus as inability of the supporting tissues to produce and maintain intercellular substances.' In the same paper they make a remark that remains pertinent today, 30 years later: 'The mechanism by which ascorbic acid promotes the formation of collagenous intercellular substances is not known.' They go on to say that ascorbic acid 'may be involved in the chemical mechanisms (enzymes) of the cells responsible for the synthesis of this protein product (collagen)'. It is true that, in spite of extensive biochemical work on the problem, little is yet known of the exact part played by ascorbic acid in the metabolism of the cell and of how it prevents scurvy. Does it in some way control the activity of enzymes involved in the synthesis of collagen? Is it, by virtue of its oxidation–reduction potential, associated particularly with enzymes possessing a sulphydryl group, as suggested by some authors?

Little histochemical work has been performed on scorbutic tissues, especially the soft tissues. We thought that such a field should not be left unexplored, and that, in the course of studying it, some observations might possibly give a clue to the role of ascorbic acid in cell metabolism.

EXPERIMENTAL

Materials

Since guinea-pigs and primates are the only animals known to be unable to synthesize vitamin C, the guinea-pig was chosen as the experimental species. In all experiments the animals were pair-fed as a control of changes due to inanition.

The eighty-nine pairs of male guinea-pigs used, with weights ranging from 200 to 220 g, were allotted to the different experimental groups as shown below:

Measurement of:	No. of pairs	Measurement of:	No. of pairs
Esterase	13	Periodic Acid Schiff	13
Lipase	5	Metachromasia	12
Acid phosphatase	12	Sulphydryl	8
Succinic dehydrogenase	12	Autoradiography	9
Cytochrome oxidase	5		

The diet of rat cake, which was supplemented with a few drops daily of vitamins A and D in oil (Adexolin, Glaxo Laboratories Ltd), was supplied by the North-Eastern

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Agricultural Co-operative Society Ltd of Aberdeen and had the percentage composition shown below:

Fine bran	17·4	Meat-and-bone meal	9·6
Ground wheat	17·4	Dried skim milk	14·0
Sussex-ground oats	17·4	Dried yeast	1·2
Ground maize	8·7	Salt	0·4
Ground barley	8·7	Cod-liver oil	0·4
White-fish meal	4·8		

The control animal of each pair was also given 10 mg ascorbic acid daily by mouth. After about $2\frac{1}{2}$ weeks the experimental animals began to lose weight rapidly, became inactive and sat for most of the day hunched up in a corner of the cage. At 3 weeks both control and experimental animals were killed, always by breaking the neck, and the tissues were removed immediately after death. Control and scorbutic tissues were subjected to the same procedures; when slides bearing tissue sections were stained or used for histochemical procedures they were placed in pairs, a test and a control slide back-to-back, and put through all procedures together.

Methods

Histochemical techniques for enzymes

Simple esterases. For simple esterases (chilled acetone-fixed material, Gomori, 1952*b*) the following techniques were used: (*a*) α-naphthyl-acetate technique (Gomori, 1952*b*), (*b*) naphthol AS acetate technique (Gomori, 1952*b*), (*c*) β-naphthyl-acetate technique (Nachlas & Seligman, 1949).

Lipase. For lipase, the Tween technique (chilled acetone-fixed material, Gomori, 1945) was used.

Acid phosphatase. The histochemical technique for acid phosphatase on chilled acetone-fixed material developed by Gomori (1950) is unfortunately most capricious, often producing inconsistent results for no obvious reasons. Sections cut from the same block and under identical conditions one day give positive results and the next day negative. In a ribbon of sections, some may give a strong reaction, others only a weak or no reaction. When a section is not perfectly flattened, the folds often give a positive, though the rest of the section gives a negative, reaction. These inconsistencies can be reduced to a minimum by (*a*) fixing thin pieces of tissues, not more than 1 cm thick, (*b*) using a water-bath at not more than 40° for floating out sections, and (*c*) floating out sections within $\frac{1}{2}$ min. Goetsch & Reynolds (1951) also showed that in sections not freed from wax before incubation in the substrate mixture the reactions were stronger and more consistent and localization was more precise. Our sections were therefore not freed from wax before incubation. Tissues known to have a high acid-phosphatase content, such as liver, spleen, prostate and kidney, were incubated for only 6 h. All other tissues were incubated overnight for 17 h.

Succinic dehydrogenase. It was measured on fresh frozen sections by Shelton & Schneider's (1952) modification of Seligman's technique.

Dehydrogenases are enzymes that catalyse the transfer of hydrogen to immediate acceptors other than oxygen and peroxides, although the ultimate acceptor may be oxygen. In the histochemical technique for succinic dehydrogenase, hydrogen split off

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from succinate present in the substrate reduces a tetrazolium salt (neo-tetrazolium chloride, in this instance) to an insoluble brightly coloured formazan. The resultant staining varies from a pale pinkish background to a deep-purple granular reaction, and is interpreted as follows: partial reduction to a pink monoformazan indicates low succinic-dehydrogenase activity; complete reduction to purple granules of diformazan indicates moderate to high enzyme activity; where diformazan granules are dissolved in lipid globules in the tissue, the globules appear pink.

Cytochrome oxidase. It was demonstrated in fresh frozen sections by the 'Nadi' reaction (Moog, 1943).

The 'Nadi' reaction for cytochrome oxidase is not a particularly trustworthy histochemical technique, but it was nevertheless considered to be worth using. Both cytochrome oxidase and succinic dehydrogenase are involved in processes of aerobic respiration and can therefore serve to indicate cellular metabolic activity. As the final reaction product of this technique is not permanent, sections were examined immediately after mounting.

Sulphydryl groups

Barnett & Seligman's (1952) 2:2-dihydroxy-6:6-dinaphthyl disulphide (DDD) technique was used on material fixed with alcohol and trichloroacetic acid to demonstrate protein-bound SH groups. The dihydroxydinaphthyl disulphide is believed to be specific for SH groups. Though the authors of this technique have adapted the method to demonstrate SS groups also, by first reducing them with sodium thioglycolate to SH groups, the modification proved in our hands to be unreliable and its use was discontinued. Only SH groups were studied.

Sulphydryl groups, which are invariably attached to amino-acids, are of great biological importance. The activity of succinic dehydrogenase depends upon the presence of its SH groups: intracellular proteolytic enzymes of the cathepsin type require the presence of SH groups for their activation. Such enzymes are inactivated by agents that can oxidize adjacent pairs of sulphydryl groups to form disulphide linkages, but these disulphide linkages are easily reduced again to sulphydryl groups in the presence of reduced glutathione. It is possible that ascorbic acid, being a strong reducing agent, can also do this, controlling the relation between SH and SS groups. Because of this possibility and of the many and varied biological activities in which SH groups play an important part, it was thought desirable to apply to normal and scorbutic tissues the Barnett & Seligman DDD technique.

The various colours of the reaction product are interpreted as follows: a pink or red colour indicates sparse, widely separated SH groups; a reddish-blue colour indicates a denser concentration of SH groups; a blue colour indicates a very high concentration of SH groups.

Polysaccharides

(*a*) To demonstrate polysaccharides the Periodic Acid Schiff (PAS) technique (McManus, 1948) was used on Bouin-fixed tissue. With this technique a pinkish to purple colour indicates a positive reaction.

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Other anionic groups besides sulphate also give a metachromatic reaction: the precise mechanism of the reaction is still in doubt. Sulphated mucopolysaccharides are demonstrated by this technique; this is not the place to discuss the many complications of its interpretation, but those interested may consult the review by Hale (1958).

Though there has been a good deal of controversy over the years as to the chemical nature of metachromatic substances, histochemical techniques that allow metachromasia to appear may be useful, especially when used in conjunction with other methods. Such techniques will demonstrate with certainty the presence of chondroitin sulphuric acid in cartilage, heparin in mast cells and many types of mucin; they will often show up the acid mucopolysaccharides of connective-tissue ground substance. As any metachromatic staining of connective-tissue ground substance is greatly weakened by dehydration, all sections were first examined in water before being dehydrated and mounted permanently in balsam.

(*b*) Acid mucopolysaccharides were demonstrated by their metachromatic reaction with a 0·02–0·05% solution of toluidine blue in a citrate buffer of pH 4 (Gomori, 1952*b*). Material was previously fixed either in 10% neutral formalin or in a mixture consisting of equal volumes of 4% (w/v) lead subacetate and 10% neutral formalin.

Autoradiography

Several workers have shown by biochemical methods that the radioactive isotope ^{35}S when injected or administered by mouth as $Na_2{}^{35}SO_4$ is taken up and retained, chiefly as ester-bound sulphate and slightly as free sulphate; there is little or no significant uptake by the amino-acids methionine, cysteine and taurine (Tarver & Schmidt, 1939; Odeblad & Bostrom, 1952). Further, Dziewiatkowski (1949) has demonstrated, by means of biochemical methods and the Geiger counter, that ^{35}S-labelled Na_2SO_4, when injected intraperitoneally into rats, is incorporated chiefly in the chondroitin sulphate of cartilage. Using similar methods Reddi & Nörstrom (1954) have shown that the incorporation of ^{35}S into the chondroitin sulphate of scorbutic costal cartilage is only about one-third as marked as the incorporation into normal costal cartilage; Friberg & Ringertz (1954) have shown that the uptake of ^{35}S by various tissues and organs of scorbutic guinea-pigs is also only about one-third of that by normal guinea-pigs' tissues. In this study autoradiography was employed to confirm, if possible, the biochemical findings and also to try to discover the sites of variation in uptakes of the isotope in the different organs.

The stripping-film technique (Pelc, 1947) was used to demonstrate the uptake of ^{35}S as labelled $Na_2{}^{35}SO_4$. Both control and scorbutic animals were given an intraperitoneal injection of 15μc/g body-weight of ^{35}S-labelled sodium sulphate in 0·9% saline with 0·02% sodium sulphate as carrier. They were killed 4 h after injection; pieces of tissue were removed, fixed in absolute alcohol, dehydrated, cleared and embedded in paraffin in the usual way. Sections 10μ thick were cut and mounted on 'subbed' (i.e. gelatinized) slides, hydrated and covered with Kodak autoradiographic stripping film. After exposure on the slides for a period sufficient to give a strong autoradiograph (i.e. cartilage, kidney and intestine 2–3 weeks, skin 3–4 weeks, testis and salivary glands

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5 weeks and all other tissues 9–11 weeks) the slides were developed in Kodak D 19*b* developer, fixed and washed. Some sections were then stained for 3 min in a 0·5% solution of celestin blue in 5% iron alum, rinsed, and dried in front of a hair dryer. Other sections were left unstained. All were mounted in Canada balsam.

Cytology

A few organs were examined for Golgi material and for mitochondria. The methods used were Aoyoma's silver-nitrate and Kolatchev's osmic-acid Golgi techniques. Those used for the mitochondria were Mallory's phosphotungstic acid—haematoxylin method on freeze-dried liver and kidney and Pritchard's silver-reduction method on fixed submandibular gland and pancreas.

RESULTS

Only when differences in reaction were obtained between control and scorbutic tissues are results reported in detail. When reactions in normal and scorbutic tissues were the same, the localization of the substance under investigation is not described fully unless it seemed to us of particular interest or has not previously been observed by other workers.

Enzymes

Simple esterases and lipases

The enzyme catalysing the hydrolysis of α-naphthyl acetate is referred to as 'α-esterase', of β-naphthyl acetate as 'β-esterase' and of naphthol AS acetate as 'AS-esterase'.

In normal tissues of the guinea-pig there is, compared with those of other species, little esterase or lipase activity. Strongest esterase activity was found in the liver, pancreas, tracheal epithelium and epithelium of the bronchioles in the lung. The kidney, strongly reactive in many other animals, was not active in the guinea-pig. There was slight esterase activity at the distal border of epithelial cells in the bladder, ureter, vas deferens, epididymis and prostate. Only liver, pancreas, and the striated border of the intestinal epithelial cells gave an unequivocal reaction for lipase.

All scorbutic tissues examined gave the same results as control tissues, except for pancreas and liver, which showed some differences.

Pancreas. In the normal pancreas there was a strong reaction in the distal cytoplasm of the acinar cells, and the islet cells were negative (Pl. 1, 1, 3, 5). Scorbutic pancreas gave a stronger and more uniform reaction than the control pancreas for α-esterase (Pl. 1, 2), β-esterase (Pl. 1, 3, 4) and AS-esterase (Pl. 1, 5, 6). The reaction for lipase tended to increase, but not consistently, and it was difficult to judge owing to the inevitable diffusion and the presence of extracellular granules occurring with this technique. In the control pancreas, many acini showed no sign of esterase activity; in the scorbutic pancreas nearly all acinar cells gave a reaction, in most specimens stronger than any in the control.

Liver. Whereas the α-esterase reaction in control liver was strong and uniform, granules being present in all hepatic cells (Pl. 1, 7), the reaction in scorbutic liver was

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patchy. It was still strong in the hepatic cells at the centre, but much weaker in the cells at the periphery of the lobules (Pl. 1, 8). This change was not so obvious with β-esterase, AS-esterase and lipase activities.

Acid phosphatase

In spite of the care taken to minimize loss or inactivation of the enzyme, inconsistencies appeared even amongst normal tissues, and we therefore report only observations on tissues in which consistent reactions were obtained. These include small intestine, stomach, liver, pancreas, submandibular gland, spleen, epididymis and adrenal. In the normal guinea-pig strong acid-phosphatase activity was noted in the Golgi region of several tissues, for instance the columnar epithelium cells of the small intestine and the epithelial cells of epididymis, vas deferens and prostate. This finding is in agreement with observations of earlier workers.

All scorbutic tissues gave the same results as control tissues, except the liver, which showed some differences.

Liver. Preparations of control liver showed a little difference in results depending on whether or not the sections were freed from wax before incubation in the substrate. In sections so freed, all nuclei were strongly positive and the cytoplasm of the hepatic cells was only faintly positive. In sections treated after incubation the nuclear reaction was only faint, but the cytoplasmic reaction was stronger and seemed to be localized in the Golgi region. In both types of preparation the Kupffer cells gave a strong reaction (Pl. 2, 1). In scorbutic liver the reaction was stronger in both hepatic-cell nuclei and cytoplasm and was always uneven, cells near the centre giving a much stronger reaction than cells at the periphery of the lobules (Pl. 2, 2).

Succinic dehydrogenase

The tissues examined were always cut in the order shown, the last five tissues being kept on solid CO_2 until the first four had been cut and incubated in the substrate. They comprised: liver, cardiac muscle, kidney, skeletal muscle, adrenal, small intestine, seminal vesicles, skin and epididymis.

All scorbutic tissues examined gave the same results as control tissues, except for the kidney and seminal vesicles and occasionally skeletal muscle.

Kidney. Normal kidney gave the reactions for succinic dehydrogenase already described by several authors. Macroscopically the cortex was strongly reactive, but the inner part of the medulla was negative (Pl. 2, 3). Microscopically the glomeruli were negative. All parts of the nephron gave a purple granular reaction, except the lower part of the collecting tubules in the inner half of the medulla, which was negative or only very weakly reactive. The nuclei of all cells were negative. There was an intense reaction in either the proximal or the distal convoluted tubules. There was also an intense reaction in tubules in the mid-region of the medulla; these were the ascending limbs of Henle's loop (Pl. 2, 3). The transitional epithelium of the pelvis also gave a slight purple granular reaction.

The localization of succinic dehydrogenase in the scorbutic kidney was the same as

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in the control kidney, but the intensity of the reaction was considerably less, though still granular (Pl. 2, 4).

Seminal vesicles. The epithelial cells of normal seminal vesicles showed a pale-pink background and a moderate granular reaction (Pl. 2, 5). In scorbutic seminal vesicles the reaction was much less intense, the epithelial cells containing few or no purple granules (Pl. 2, 6).

Skeletal muscle. Normal skeletal-muscle fibres gave a strong reaction in the form of purple granules packed closely in parallel rows between the myofibrils, but some fibres contained only a few granules or appeared completely negative.

The reaction in scorbutic muscle was never stronger than in control muscle, but was often of the same intensity, though there was frequently a clear reduction in the intensity of the reaction and many more fibres were negative.

Cytochrome oxidase

Only four tissues were examined, liver, cardiac muscle, kidney, and skeletal muscle. The livers and kidneys of scorbutic guinea-pigs differed neither in intensity nor in localization of reaction from those of normal guinea-pigs.

Cardiac muscle. Control cardiac muscle gave a fairly strong reaction in the form of blue granules arranged in roughly parallel rows between the myofibrils. In scorbutic cardiac muscle there was a clear reduction in intensity of the reaction.

Skeletal muscle. Control muscle fibres gave a moderate reaction in the form of parallel rows of blue granules. In scorbutic muscle fibres the reaction was sometimes of the same intensity as in the control, but sometimes there was a clear decrease in intensity. This result is similar to that obtained for succinic dehydrogenase.

Sulphydryl groups

Sulphydryl groups are widespread in the tissues of the guinea-pig, occurring particularly in epithelial cells of the skin, in all types of muscle fibre and in connective-tissue cells. In general, the results of this study on guinea-pigs are in agreement with those obtained by Barnett & Seligman (1952), who studied the distribution of SH groups in the rat. The cytoplasm of cells was either negative or gave only a weak reaction. Occasionally, however, e.g. in islet cells of pancreas and cartilage cells, the nucleus gave a stronger reaction than the cytoplasm. Even when the nucleoplasm was negative or weak, the nucleoli often gave a strong reaction. The nuclear membrane and the plasma membranes of epithelial cells were often conspicuously reactive. The reaction in the cytoplasm of cells was generally uniform, but sometimes rather granular and 'mottled'. In the tubules of the kidney the mitochondria, and in skeletal-muscle fibres the sarcosomes, appeared to be rich in SH groups. Barnett & Seligman (1952) do not appear to have noted this positive reaction in mitochondria. Tissues that consistently gave a negative reaction for SH groups included intercellular material of connective tissues, cartilage ground substance, stratum granulosum of the skin and the distal parts of the cortex of hair, mucus, and bone matrix.

No characteristic feature could be found in either the distribution or the amount of protein-bound sulphydryl in scorbutic tissues.

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Skin. In skin from control animals all layers of the epidermis gave a moderate reaction for SH groups except the stratum granulosum, which was negative. Sebaceous-gland cells gave a moderate reaction, and the cell borders were strongly reactive. The hair-root sheaths showed a moderate reaction. The cortex of the hair from bulb to 'keratogenous zone' gave an intense reaction, but above it it was negative. Connective-tissue cells and capillary walls gave a moderate reaction (Pl. 2, 7). In scorbutic skin the reactions in the epidermis, hair sheaths and sebaceous glands were considerably weaker (Pl. 2, 8).

Polysaccharides

We know that scurvy is essentially a disease in which the supporting and connective tissues of the body are unable to produce and maintain intercellular substances; accordingly much research, biochemical, histochemical and cytological, has been done on the three components of connective-tissue ground substance (Persson, 1953; Reppert, Donegan & Hines, 1951), fibres (Elster, 1950; Robertson, 1950; Hass & McDonald, 1940) and cells (Wolbach & Howe, 1926; Gersh & Catchpole, 1949) in scorbutic tissues and in particular on the process of wound-healing in scurvy (Bourne, 1944; Penney & Balfour, 1949). Polysaccharides are an important component of connective tissues, yet a review of the literature shows that there has been no systematic investigation by histochemical methods of the distribution of polysaccharides in the various organs and tissues of control and scorbutic guinea-pigs. Such a study therefore seemed desirable.

Periodic Acid Schiff technique

Tissues and organs examined included oesophagus, body of stomach, duodenum, small intestine, rectum, tongue, pancreas, submandibular gland, liver, trachea, lung, ureter, bladder, kidney, testis, epididymis, vas deferens, seminal vesicles, prostate, striated and cardiac muscle, skin, adrenal, cerebellum, cerebrum, lymph node, spleen, thymus, eye and costochondral junction. The following organs in scorbutic animals showed in their reactions consistent differences from the normal.

Liver. In normal liver the hepatic-cell cytoplasm contained varying numbers of PAS-positive granules which were shown by digestion with saliva to be glycogen granules. Kupffer-cell cytoplasm gave a slight-to-moderate reaction (Pl. 3, 1). In scorbutic liver there was a reduction in number, and often a complete absence, of glycogen granules (Pl. 3, 2) in the hepatic cells.

Seminal vesicles. The epithelial cells of normal seminal vesicles often contained strongly PAS-positive granules which were shown by salivary digestion to be glycogen granules. The basement membrane of the epithelium gave a strong positive reaction. The connective tissue and edges of the muscle fibres were slightly positive (Pl. 3, 5). In scorbutic seminal vesicles there was a reduction in the number, often complete absence, of glycogen granules in the epithelial cells (Pl. 3, 6).

Costochondral junction. The cartilage matrix of the normal costochondral junction gave a moderate positive reaction which was stronger at the edges of the cell capsules than in the rest of the tissue. The matrix of the calcified cartilage at the region of ossifi-

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cation gave a stronger reaction than that of the non-calcified cartilage and was a little more intense at the edges of the framework. Proliferating cartilage cells contained strongly positive granules (Pl. 3, 3). Bone matrix was moderately reactive, periosteum and perichondrium faintly so.

In the scorbutic costochondral junction the reaction in the central part of the calcified cartilage framework was weaker than in the control, but the reaction at the edges was conspicuously more intense than in the control (Pl. 3, 4). There was also a strong PAS-positive reaction in the fibrous unorganized material at the junction.

Spleen. The connective tissue of the capsule and trabeculae of the normal spleen gave a fairly strong reaction, some reticular fibres a slight one. A few cells, probably reticulo-endothelial cells, in the red pulp, contained PAS-positive granules and droplets (Pl. 3, 7).

In the scorbutic spleen there was a striking and very consistent increase in the number of cells containing PAS-positive droplets (Pl. 3, 8). Further tests were used in an attempt to establish the nature of these droplets. They still gave a positive PAS reaction after treatment with saliva, and also after treatment with hyaluronidase, which indicates that these cells contained neither glycogen nor hyaluronic acid. They showed no metachromasia with toluidine blue and therefore probably contained no acid mucopolysaccharide. They gave no reaction with Sudan black and so contained no glycolipid. The conclusion is that the droplets contain some form of mucoprotein.

As the spleen is the chief site of erythrocyte breakdown, Perl's method for ferric iron was applied to sections of control and of scorbutic spleens; a deep Prussian-blue colour reveals the presence of ferric iron. There was a positive reaction in both control and scorbutic spleens in what appeared to be the PAS-positive droplets. This positive reaction for ferric iron indicated that these droplets may also have contained haemosiderin, a pigment derived from the breakdown products of red blood corpuscles.

Lymph node. The capsule and some reticular fibres within the normal lymph node gave a slight-to-moderate reaction. A few cells in the medulla, probably reticulo-endothelial cells, contained orange-brown droplets (Pl. 4, 1).

In the scorbutic lymph node many more cells in the medulla contained orange-brown droplets (Pl. 4, 2). These droplets, though similar in appearance to those seen in the spleen, did not give a reaction for ferric iron.

Metachromatic staining

The tissues examined were: oesophagus, small intestine, body of stomach, rectum, submandibular gland, pancreas, liver, trachea, lung, kidney, ureter, vas deferens, seminal vesicles, testis, epididymis, spleen, adrenal, skin, cerebrum, cerebellum, nasal cartilages, pinna and costochondral junction.

In normal kidney there was a variable metachromatic reaction of the connective tissue between the collecting tubules of the medulla. In some animals there was found only a moderate and in others a fairly strong purplish reaction. Also there was often a faint metachromatic reaction at the free border of the cells of the collecting tubules in the medulla.

Differences in reaction were observed as described below.

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Seminal vesicles. In normal seminal vesicles there was slight metachromasia in the subepithelial connective tissue and sometimes in the basement membrane. When mast cells were present in the connective tissue, they were strongly metachromatic.

In scorbutic seminal vesicles there was a tendency for metachromasia to be reduced in, or absent from, the basement membrane and connective tissue.

Costochondral junction. In normal costochondral junctions the cartilage matrix was found to be intensely metachromatic. The cytoplasm of chondrocytes and of osteocytes was often slightly metachromatic, as was that of the cytoplasm in the canaliculi.

In scorbutic costochondral junctions there was no change in the metachromatic reaction, but there was additional moderate-to-strong metachromasia in the connective tissue of the periosteum and in the intermuscular connective tissue, which appeared to have increased in amount in this region just outside the periosteum.

Liver. There was no metachromasia in control or scorbutic livers. However, the basophilia of the hepatic cells was considerably deeper in scorbutic than in control livers. This increase in basophilia was due mainly to an increase in cytoplasmic rather than in nuclear basophilia, which could have been due to an increase in ribonucleic acid, though it was not checked with ribonuclease treatment (Pl. 4, 3, 4).

Autoradiographic studies

Since ^{35}S, when injected into an animal as $Na_2{}^{35}SO_4$, is taken up and retained in the body in ester-bound sulphates, one would expect to get a strong autoradiograph after such injection from tissues known to contain chondroitin sulphate or mucoitin sulphate, as in fact one does. In tissues of the normal guinea-pig, therefore, ^{35}S was found particularly in cartilage, in connective-tissue ground substance and in mucus-secreting cells of many types.

There was always a strong autoradiograph from the goblet cells of the intestine and from the free mucus in the intestinal lumen, from the goblet cells of the bronchiolar and tracheal epithelium and from the cells of the subepithelial mucus glands of the trachea. It is curious to note that, whereas many of the cells of Brunner's glands in the guinea-pig produce a fairly strong autoradiograph, Belanger (1954) observed no uptake of ^{35}S at this site in either rats or hamsters.

There was a variable uptake of ^{35}S in subepithelial connective-tissue layers, the autoradiograph being weak from some organs but fairly strong from others.

Belanger (1954) noted also in rats and hamsters that the intensity of the autoradiographs from the subepithelial connective tissue increased progressively from the epididymis, where the reaction was moderate, to the first turns of the vas deferens, where it became considerably stronger.

The basement membranes of the testis and ureter gave a positive autoradiograph and in many other tissues there were indications of a localization of activity in this site. The epithelial cells of some organs also appeared to accumulate ^{35}S .The autoradiograph indicated that the isotope was fairly uniformly distributed throughout the cell. In the oesophagus the basal layers of the stratified epithelium took up ^{35}S. The hepatic cells of the liver, after a long exposure, produced an autoradiograph, but the cells of the

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branches of the bile duct gave one much earlier indicating that they had a higher concentration of the isotope. Belanger described an uptake of ^{35}S by the wall of the extrahepatic ducts and pancreatic ducts in rats and hamsters, but made no mention of an autoradiograph from the bile-duct branches within the liver.

It is known that in the testis there is an uptake of ^{35}S by the spermatogonia (Glucksman, Howard & Pelc, 1955). It appeared after injection of ^{35}S-labelled methionine, when it could be assumed that the autoradiographs were due to ^{35}S incorporated into the methionine or cysteine of protein. In our experiments, however, autoradiographs were also obtained from spermatogonia, which must have been due to an uptake of ^{35}S into an ester-bound sulphate. In none of the organs described above was there a significant reduction in the autoradiograph given by the same tissues in scorbutic animals.

In the kidney there was an intense autoradiograph from the distal border of the cytoplasm of the cells in the collecting tubules, and also from the lumen. The reaction in the lumen may represent an excretion of free sulphate, and the reaction in the distal cytoplasm of the cell might indicate either excretion or re-absorption.

Sections of skin showed a strong autoradiograph from the papilla and outer root sheath of the hairs. The uptake of ^{35}S in these sites has been studied in detail (Hill & Montagna, 1957).

The most striking and consistent differences in uptake of ^{35}S by scorbutic animals were found in cartilage-containing structures.

Trachea. In the trachea of normal guinea-pigs there was a strong autoradiograph from the cartilage cells (Pl. 4, 5), the goblet cells, subepithelial mucus-gland cells, and free mucus of the epithelial surface. There was a moderate autoradiograph from the cartilage matrix and a slight-to-moderate one from the subepithelial connective tissue.

Even in preliminary experiments, with the less sensitive 'contact method' of autoradiography, the autoradiograph from scorbutic tracheal cartilage was always considerably weaker than that from the normal animal (Pl. 4, 6). With the stripping-film technique the result was even more definite. Occasionally the autoradiograph from scorbutic cartilage was as strong as that from normal cartilage, but the granules were usually confined to the area of the film immediately above the cells, few being present above the matrix. The uptake of ^{35}S in other parts of the trachea was the same as in that of the normal animal.

Lung. As in the trachea, the autoradiograph from the bronchiolar cartilage in the scorbutic animal was usually weaker than that from the normal animal, though the difference was not so striking as in the tracheal cartilages.

Kidney. The uptake of ^{35}S was the same in the scorbutic as in the normal kidney, except in the connective tissue underlying the transitional epithelium of the pelvis, which often showed a stronger, and never a weaker, reaction than the same tissue in the normal animal.

Costochondral junction (*undecalcified*). In the normal costochondral junction there was a strong uptake in the proliferating and hypertrophying cartilage cells at the region of ossification and in the cartilaginous matrix of this region. The resting cartilage cells also gave a fairly strong autoradiograph (Pl. 4, 7). A slight autoradiograph was given

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by the bone, except the edges of the spicules of newly formed bone where it appeared as a fairly sharp black line indicating appreciable uptake of ^{35}S. The connective tissue between muscle fibres of the attached muscle gave a slight reaction.

After a long exposure an autoradiograph was obtained from the cells of the bone marrow, some cells producing a conspicuously stronger reaction than the others. It was not possible to identify these cells even after Leishman or celestine-blue staining.

The costochondral junction of the scorbutic animals showed the most striking differences of all the tissues. In preliminary experiments with the contact method, in the control animal a strong autoradiograph from the junction of the cartilage with the bone (i.e. at the region of ossification) was always observed as a dense black line; in the scorbutic animals the autoradiograph from this region was much fainter, appearing as a grey rather than a black line. The use of the stripping-film technique confirmed these observations. By this technique it could be seen that in most instances there was a reduction in uptake of ^{35}S by the proliferating and hypertrophying cartilage cells and the cartilage matrix at the zone of ossification (Pl. 4, 8). Occasionally the autoradiograph from this region was as strong as from the control tissue, but in that event it was confined to the cartilage cells, and there were few black granules in the matrix, whereas, in the control tissue, the reaction was distributed over both cells and matrix (Pl. 4, 9, 10).

Knee joint (*decalcified*). In the normal knee joint there was a strong autoradiograph from the proliferating and hypertrophying cartilage cells and in the matrix at the zone of ossification. The cells of the articular cartilage also gave a fairly strong reaction, the cartilage matrix a weak one.

In scorbutic knee joints there was a tendency for a reduction in the uptake of ^{35}S by the cells of both ossifying and articular cartilage, but the differences from the normal were not as striking or as consistent as in the costochondral junction.

Digit (*decalcified*). In digits from the control guinea-pigs a strong autoradiograph from the cartilage cells was given at regions of ossification and articulation, the reaction always being stronger in the cells of the ossifying cartilage than in those of the articulating cartilages. In both regions the cartilaginous matrix gave a slight, uniform autoradiograph.

In scorbutic digits the results were similar to those found in knee joints, that is a tendency for a reduction in the uptake of ^{35}S by cells of both ossifying and articular cartilage.

Golgi material and mitochondria

Classical Golgi and mitochondria pictures were obtained in all the normal tissues. No changes in localization, form or amount could be seen in scorbutic organs. There appeared to be neither clumping nor fragmentation of mitochondria.

DISCUSSION

Alkaline-phosphatase activity in scorbutic tissues has been studied by earlier workers (Russell, Rouse & Read, 1944; Zorzoli & Nadel, 1950; King & Delory, 1936; Gould & Schwachman, 1941–2; Bourne, 1943; Danielli, Fell & Kodicek, 1945), but there is no record of any previous investigation of acid-phosphatase activity. Though many organs

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and tissues were studied, the only consistent change was observed in the liver, where absence of ascorbic acid appears to have had an activating effect upon this enzyme. The effect was not uniformly distributed, however, the activity being strongest in the hepatic cells near the central vein of the hepatic lobules and not so strong, though stronger than normal, in the peripheral cells of the lobules.

Some biochemical and cytochemical work has already been done on esterase and lipase activity in scorbutic tissues of the guinea-pig. Harrer & King (1941) and Palladin (1915) showed by biochemical methods that liver esterase (probably lipase) diminishes in scurvy. Kraut, Pantchenko & Jurawicz (1935) believed that there is a relation between vitamin C and liver esterase, vitamin C being either a coenzyme or an activator, and Gajdos & Hochwald (1948) induced an increase in esterase activity in rabbit serum by administration of vitamin C. The only cytochemical studies made previously were by the French workers, Verne, Hebert & Barbarin (1953), who studied lipase activity with Gomori's Tween technique and found reduced activity in scorbutic liver and lung, but no change in activity in the pancreas.

Our histochemical results are not exactly in agreement with these earlier findings. Deficiency of vitamin C is accompanied by a consistent increase in esterase activity of the pancreas. This was evident with all three substrates used to demonstrate 'simple' esterase activity, i.e. α- and β-naphthyl acetates and naphthol AS acetate. There also appeared to be an increase in lipase activity.

In the scorbutic liver there was only a slight decrease in esterase activity (in particular of α-esterase activity) and this was from the hepatic cells at the periphery of the hepatic lobules.

A histochemical study of succinic-dehydrogenase and cytochrome-oxidase activity in scorbutic tissues has not previously been undertaken. Both enzymes are associated directly or indirectly with the tricarboxylic Krebs cycle. Harrer & King (1941) have shown by biochemical methods a pronounced drop in succinic-dehydrogenase activity in both skeletal and cardiac muscle of scorbutic guinea-pigs. Histochemical methods revealed no change of activity in cardiac muscle, which in both normal and scorbutic animals gave an intense reaction. There was a tendency towards decreases in activity in skeletal muscle, but this was not consistent. Both kidney and seminal vesicles showed consistently reduced succinic-dehydrogenase activity in scurvy.

Biochemical work, again by Harrer & King (1941), showed decreased cytochrome-oxidase activity in both cardiac and skeletal muscle of scorbutic guinea-pigs. Our histochemical work also revealed a consistently reduced activity in cardiac muscle, though the decrease of activity in skeletal muscle was not very consistent, resembling in this respect the results for succinic dehydrogenase.

The study of protein-bound sulphydryl groups produced no results of interest except possibly for the skin.

The use of the PAS technique revealed in scurvy a great and consistent increase in the number of cells in the red pulp of the spleen containing PAS-positive droplets. These droplets are probably haemosiderin and, if so, indicate an increased breakdown of erythrocytes in scurvy. It was at first thought that the orange-brown droplets seen in some cells of the lymph node (probably reticulo-endothelial cells) which are in-

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creased in number in scurvy, are of the same nature as those observed in the spleen. They gave no reaction for ferric iron and so could not be composed of haemosiderin. The increase in intensity of the PAS reaction at the edges of the calcified cartilage framework in ossifying zones of scorbutic animals is not easy to interpret. It might represent a secretion of abnormal mucopolysaccharides by the osteoblasts, which in scurvy cannot lay down normal bone at this site. On the other hand, it might represent a movement of chondroitin sulphate from the central to the peripheral part of the calcified cartilage matrix.

The technique of autoradiography with $Na_2{}^{35}SO_4$ produced the most interesting results, in both normal and scorbutic tissues. Although Friberg & Ringertz (1954) observed by biochemical methods a reduced uptake of ^{35}S by several tissues and organs of a scorbutic guinea-pig, this decrease was not detectable by autoradiography, so that it was not possible, as had been hoped, to discover the sites of decrease in the various organs. However, a decrease in uptake of ^{35}S was observed in the cartilage of some structures of scorbutic guinea-pigs, in particular the costochondral junction. The autoradiograph from cartilage is due to the incorporation of ^{35}S into chondroitin sulphate. The injected ^{35}S-labelled sulphate is taken up first by the cartilage cells and is then slowly released into the matrix as chondroitin sulphate. This sequence of events has been demonstrated in detail in various types of cartilage in the mouse by Pelc & Glucksman (1955). The observations reported here appear of interest in view of the fact that in scurvy the synthesis of ground-substance material by certain cells is believed to be inhibited, perhaps with formation of abnormal products that cannot be converted into the necessary intercellular material. Our autoradiographs showed that in scurvy there is a reduced uptake of labelled sulphate by many cartilage cells and a weaker autoradiograph from the cartilaginous matrix, particularly at the zone of ossification in the costochondral junction and in the tracheal cartilage. This finding may mean that the cartilage cells in these regions, having taken up some labelled sulphate, are unable to use it for synthesis of the normal intercellular material, chondroitin sulphate.

SUMMARY

1. Histochemical techniques for demonstrating a number of enzymes, for sulphydryl groups and polysaccharides, and the autoradiographic method for demonstration of uptake of ^{35}S, together with mitochondrial and Golgi techniques, have been applied to tissues of normal and scorbutic guinea-pigs. In all, eighty-nine pairs of male guinea-pigs were used.

2. A diminution was found in succinic dehydrogenase in skeletal muscle, in kidney and in seminal vesicles; cytochrome-oxidase activity in cardiac and skeletal muscle was also diminished.

3. There was an increase of simple esterase and lipase in the pancreatic acini and a decrease of α-naphthol esterase in the periphery of the liver lobules of scorbutic animals.

4. There was a considerable increase in the acid-phosphatase reaction from the liver of scorbutic animals.

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5. Skin from scorbutic guinea-pigs showed a decreased sulphydryl reaction.

6. There was a decrease of glycogen in scorbutic liver and seminal vesicles.

7. In the scorbutic spleen and lymph glands there was an increase in Periodic Acid Schiff reacting cells, which gave a strong Prussian-blue reaction for ferric iron.

8. Metachromasia (which indicates the presence of sulphated mucopolysaccharides, such as chondroitin and mucoitin sulphuric acid) was diminished in the scorbutic seminal vesicles, unchanged in the scorbutic costochondral junction, but increased in the fibrous parts of the periosteum of the latter organ and in the nearby intermuscular connective tissue.

9. The cytoplasm of the liver cells showed increased basophilia in scorbutic animals, which may indicate an increase in ribonucleic acid.

10. In trachea and in costal cartilage and cartilage of knee joints and digits of scorbutic animals there was a reduced uptake of ^{35}S. Where there was little difference in total uptake, most of the reaction in some specimens was given by the cells and not by the cartilage, indicating a breakdown in the cellular synthetic mechanisms for producing cartilage matrix.

11. Little change could be seen in the mitochondria and Golgi material of the various organs of scorbutic animals.

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EXPLANATION OF PLATES

PLATE 1

Esterases

1. 'α-Esterase' in pancreas of normal guinea-pig. Reaction in distal cytoplasm of acinar cells.
2. 'α-Esterase' in pancreas of scorbutic guinea-pig. More acinar cells give a positive reaction and the reaction is increased in intensity.
3. 'β-Esterase' in pancreas of normal guinea-pig. Reaction in distal cytoplasm of acinar cells.
4. 'β-Esterase' in pancreas of scorbutic guinea-pig. Note great increase in intensity of reaction.
5. 'AS-Esterase' in pancreas of normal guinea-pig. Reaction in distal cytoplasm of acinar cells.
6. 'AS-Esterase' in pancreas of scorbutic guinea-pig. More acinar cells give a positive reaction and reaction is increased in intensity. Islet cells present are mostly negative as in control sections.
7. 'α-Esterase' in liver of normal guinea-pig. There is a fairly strong, uniform reaction in all hepatic cells.
8. 'α-Esterase' in liver of scorbutic guinea-pig. The reaction is patchy. Esterase appears to have been lost from hepatic cells at the periphery of the lobules.

PLATE 2

Acid phosphatase

1. Acid phosphatase in liver of normal guinea-pig. The section was de-waxed after incubation in the Gomori substrate. Nuclei of hepatic cells give a very faint reaction but there is a strong reaction in the Golgi region of the cytoplasm. Kupffer cells strongly positive.
2. Acid phosphatase in liver of scorbutic guinea-pig. Note increase in intensity of reaction. This increase is not uniform, cells near the centre of lobules giving stronger reaction than those at periphery.

Succinic dehydrogenase

3. Succinic dehydrogenase in kidney of normal guinea-pig. Glomeruli and lower part of collecting tubules in medulla are negative. Strong, purple, granular reaction in other parts of nephron, particularly in proximal convoluted tubules.
4. Succinic dehydrogenase in kidney of scorbutic guinea-pig. Marked decrease in intensity of reaction in all parts.
5. Succinic dehydrogenase in seminal vesicles of normal guinea-pig. Moderate granular reaction in epithelial cells.
6. Succinic dehydrogenase in seminal vesicles of control guinea-pig. Marked decrease in intensity of reaction. Very few granules in the epithelial cells.

SH groups

7. Protein-bound sulphydryl groups in skin from back of normal guinea-pig. Cortex of hair from bulb to 'keratogenic zone' gives intense reaction. Moderate reaction in hair-root sheaths, sebaceous-gland cells, and all layers of epidermis except stratum granulosum which is negative. Moderate reaction in connective-tissue cells and capillary walls.
8. Protein-bound SH groups in skin from back of scorbutic guinea-pig. Marked decrease in intensity of reaction in epidermis, hair-root sheaths and sebaceous glands.

PLATE 3

Periodic Acid Schiff technique

1. PAS reaction in liver of normal guinea-pig. Hepatic-cell cytoplasm contains many glycogen granules. Kupffer-cell cytoplasm gives a slight-to-moderate PAS reaction. Positive nuclei here and in 2 are due to a nuclear counterstain.
2. PAS reaction in liver of scorbutic guinea-pig. Hepatic-cell cytoplasm contains little or no glycogen. Kupffer cells still give a positive PAS reaction.

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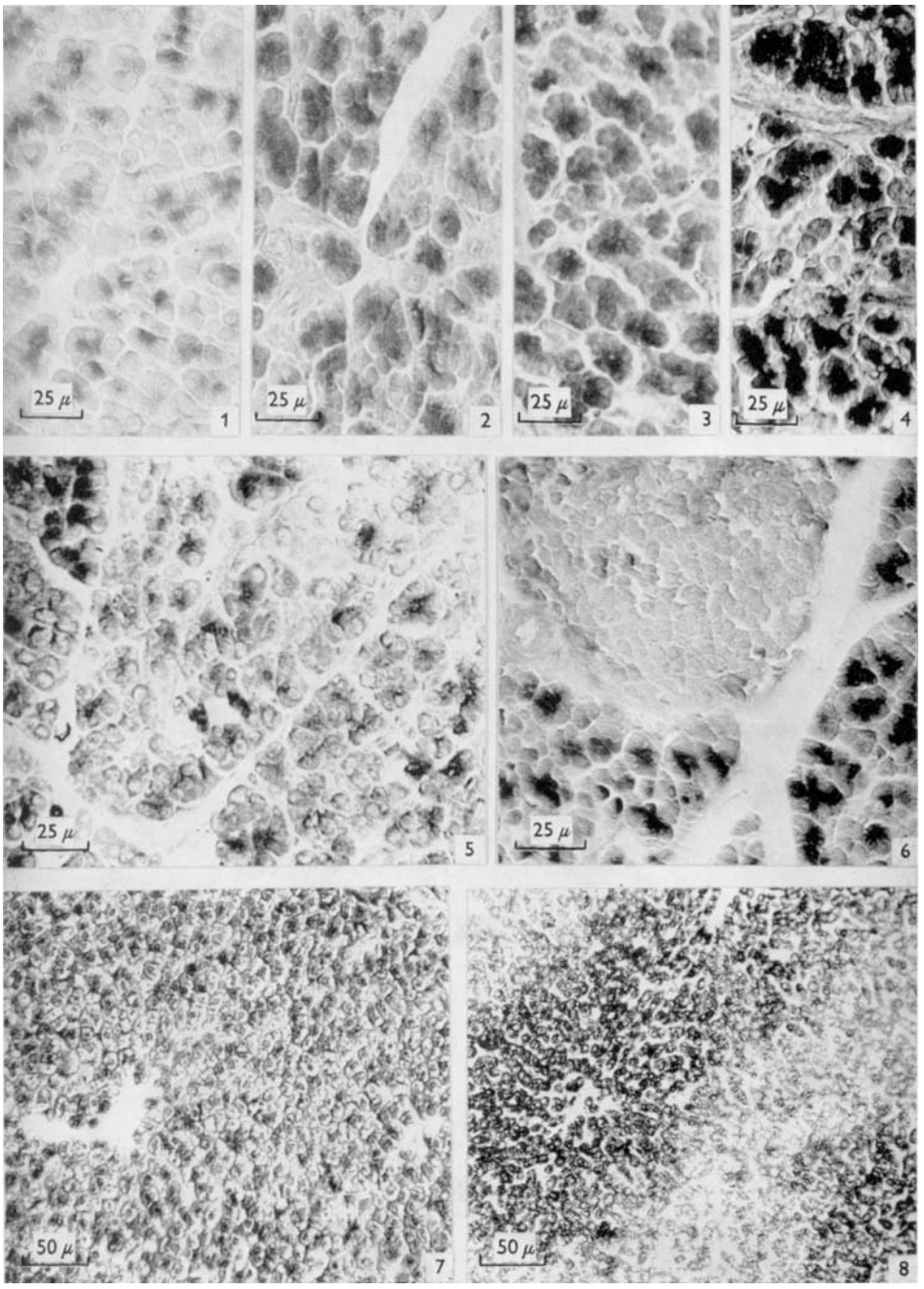

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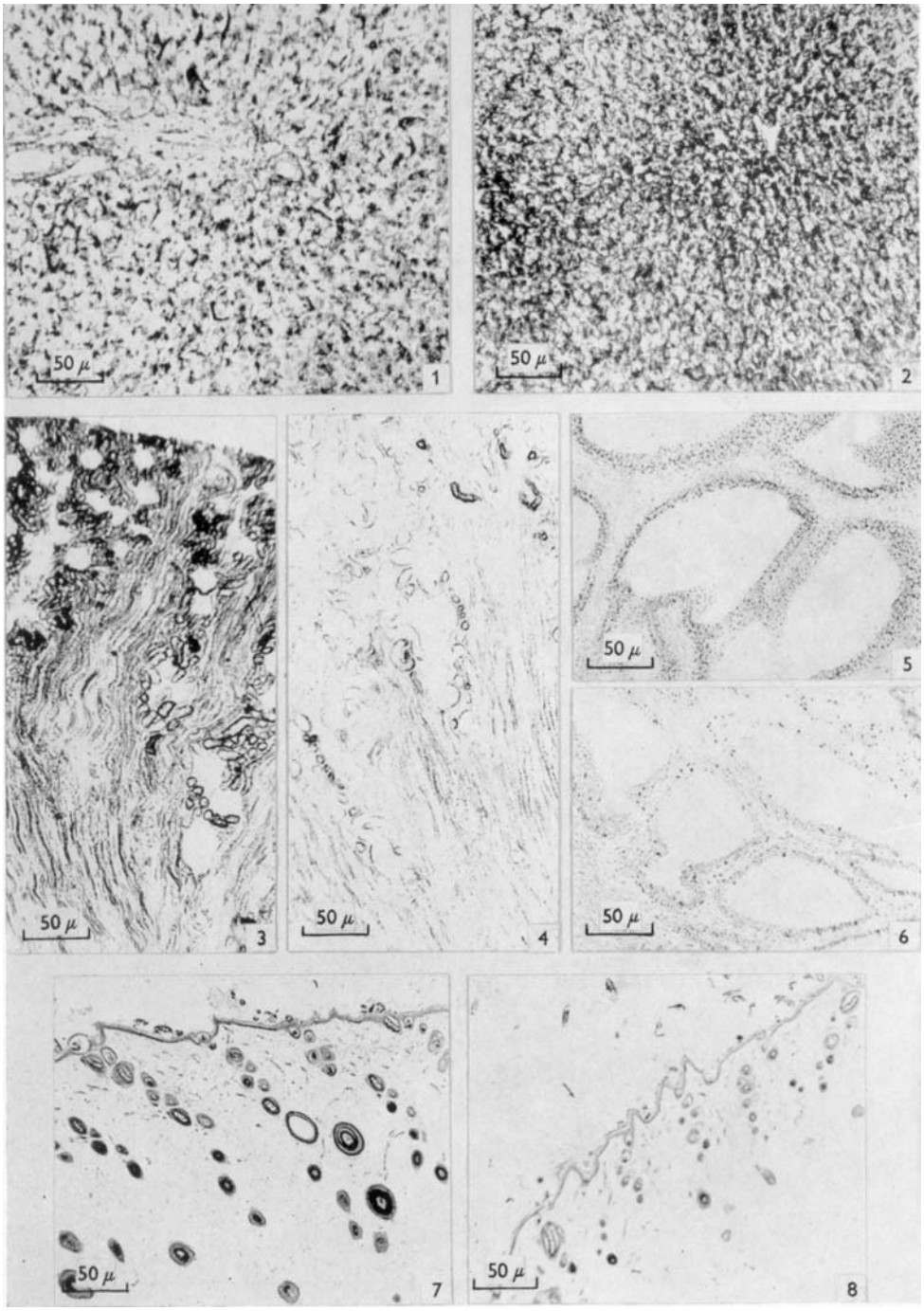

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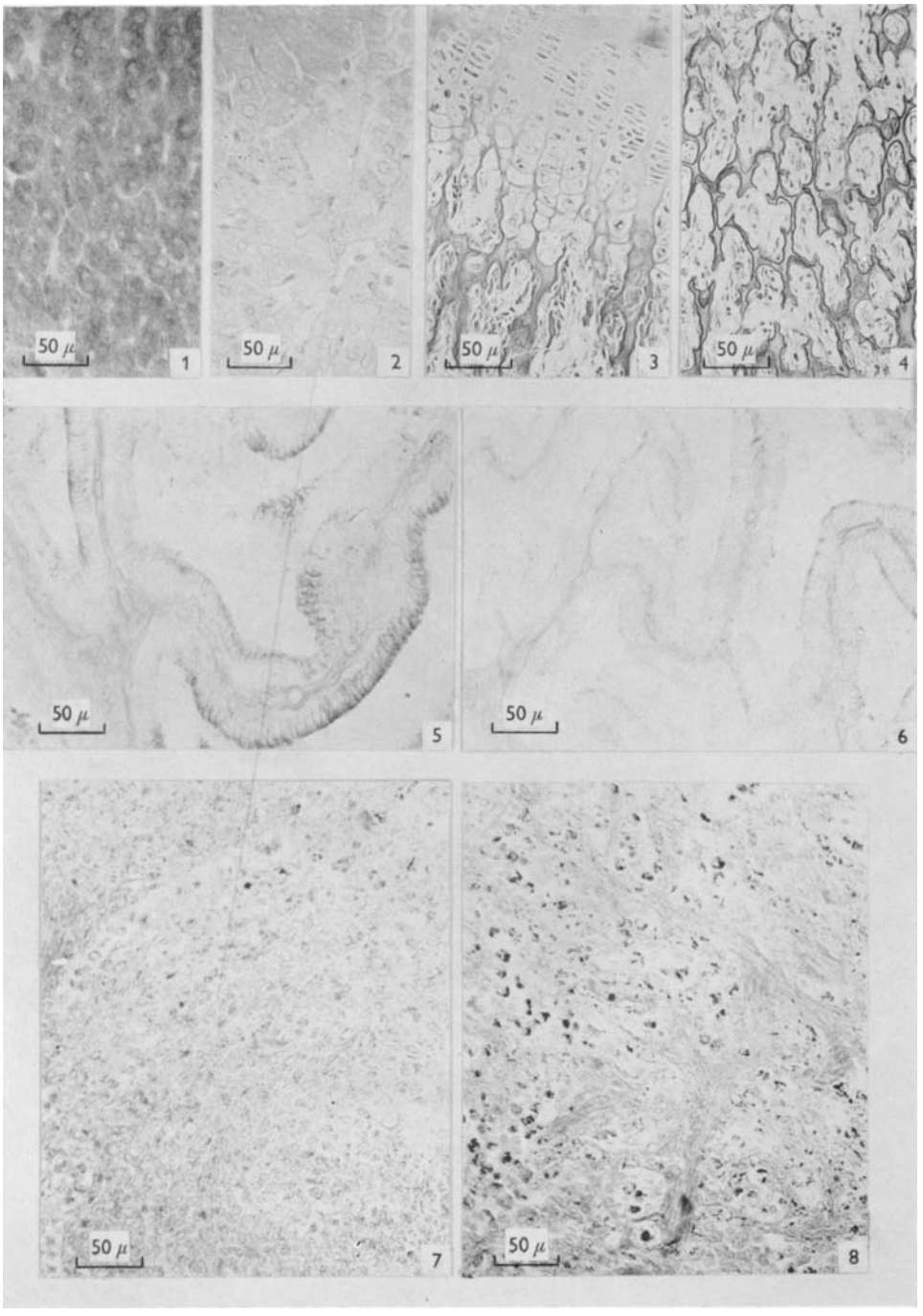

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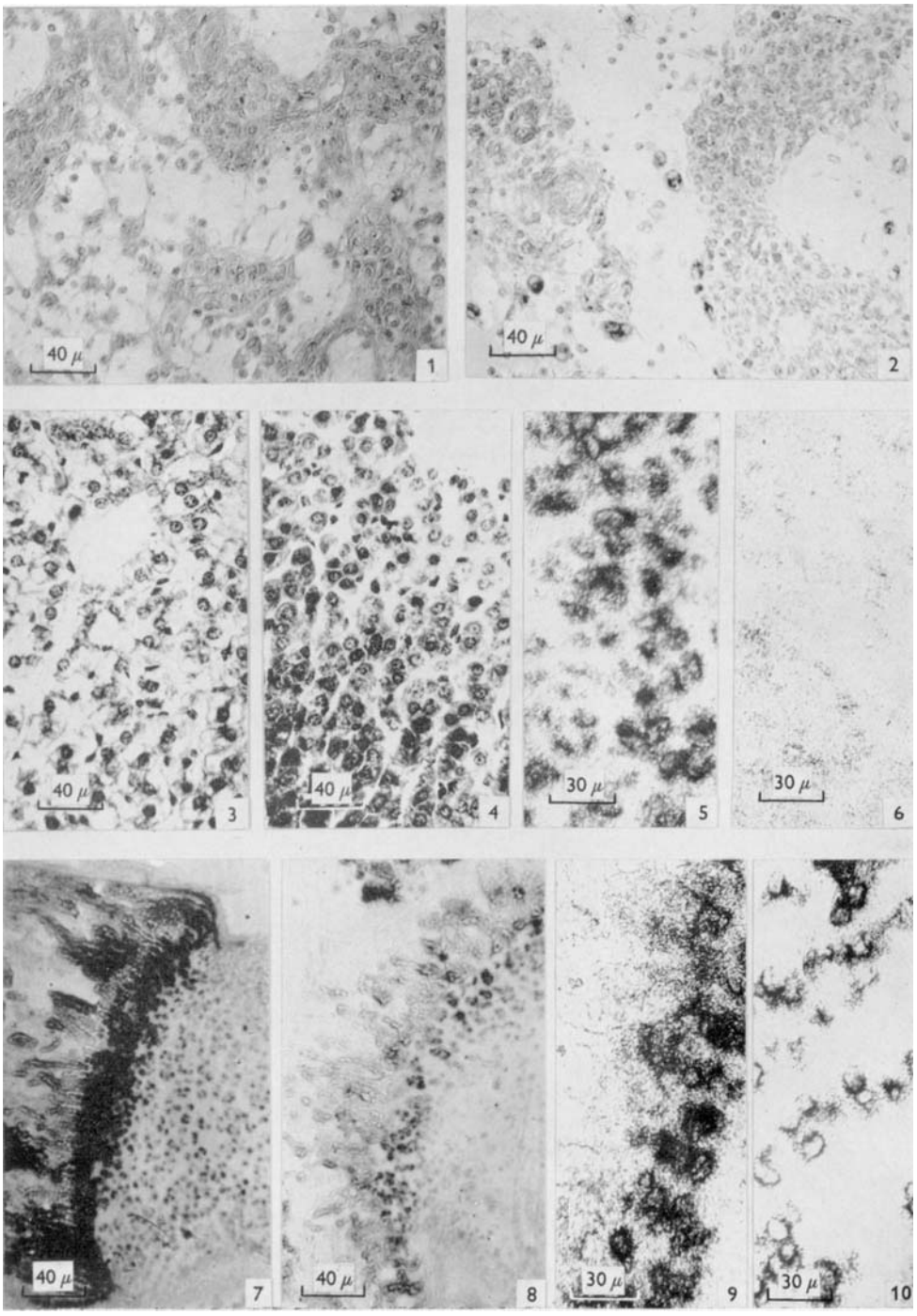

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3. PAS reaction in costochondral junction of normal guinea-pig. Cartilage matrix gives moderate reaction. Calcified cartilage matrix gives a strong, evenly distributed reaction. Proliferating cartilage cells contain strongly positive granules. Positive nuclei here and in 6 are due to a nuclear counterstain.
4. PAS reaction in costochondral junction of scorbutic guinea-pig. Reaction in the central part of the calcified cartilage framework is weaker than in the normal, but is conspicuously more intense at the edges of the framework.
5. PAS reaction in seminal vesicles of normal guinea-pig. Basement membrane of the epithelium gives quite a strong reaction. Distal cytoplasm of epithelial cells contains many glycogen granules.
6. PAS reaction in seminal vesicles of scorbutic guinea-pig. Basement membrane still gives quite a strong reaction. Glycogen is almost completely lost from the epithelial cells. A few scattered granules can be seen in the distal edge of some cells.
7. PAS reaction in spleen of normal guinea-pig. A few cells, chiefly in the red pulp, contain PAS-positive granules and droplets. Some reticular fibres give a slight reaction.
8. PAS reaction in spleen of scorbutic guinea-pig. Striking increase in the number of cells in the red pulp containing PAS-positive granules and droplets.

Plate 4

Periodic Acid Schiff technique

1. PAS reaction in lymph node of normal guinea-pig. A few cells in the medulla contain orange-brown droplets.
2. PAS reaction in lymph node of scorbutic guinea-pig. Increase in the number of cells in the medulla containing orange-brown droplets.
3. Basophilia in liver of normal guinea-pig with 0·02–0·05% toluidine blue. Hepatic-cell nuclei show fairly strong basophilia.
4. Basophilia in liver of scorbutic guinea-pig. Basophilia is stronger, particularly at the periphery of lobules (bottom of photomicrograph) owing mainly to an increase in cytoplasmic rather than in nuclear basophilia.

Autoradiography (AR)

5. Stripping-film AR of high-power view of tracheal cartilage of control guinea-pig injected 4 h previously with $Na_2{}^{35}SO_4$, showing heavy uptake of ^{35}S by cartilage cells.
6. High-power view of stripping-film AR of tracheal cartilage of scorbutic guinea-pig showing marked decrease in uptake of ^{35}S by cartilage cells.
7. Low-power view of stripping-film AR of costochondral junction of normal guinea-pig injected 4 h previously with $Na_2{}^{35}SO_4$, showing intense uptake of ^{35}S in proliferating and hypertrophying cartilage cells and in cartilage matrix at region of ossification. Resting cartilage cells also give a fairly strong AR. Though some large fragments of bone present in the picture appear black, these are not autoradiographs but artifacts.
8. Low-power view of stripping-film AR of costochondral junction of scorbutic guinea-pig. There is a marked reduction in uptake of ^{35}S by proliferating and hypertrophying cartilage cells and in the cartilage matrix at the region of ossification.
9. High-power view of stripping-film AR of costochondral junction of normal guinea-pig showing presence of ^{35}S in both hypertrophying cartilage cells and cartilage matrix.
10. High-power view of stripping-film AR of costochondral junction of scorbutic guinea-pig showing presence of ^{35}S in hypertrophying cartilage cells but very few black granules in the matrix. The capsules give quite a conspicuous AR.

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