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Pathological Consequences of Copper Deficiency and Cobalt Deficiency [and Discussion]

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Pathological consequences of copper deficiency and cobalt deficiency

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[Plate 1]

Aspects of the pathology of copper deficiency in several species, and cobalt deficiency in sheep, are summarized. An attempt is made to interpret morphological changes in copper-deficient animals in terms of biochemical defects. The common denominator may be mitochondrial lesions, with a generalized effect on energy-dependent synthetic functions of the cell.

In copper deficiency, such defects can be attributed to depletion of copper-dependent enzymes, while deficiency of cobalt in ruminants is, in effect, deficiency of vitamin B_{12}. The pathological consequences of vitamin B_{12} deficiency form a syndrome, notable features of which are neurological and muscular lesions, in which the metabolic consequences of hepatic damage may play a significant role.

Introduction

Disease, often in the unspectacular and non-specific form of unthriftiness and malaise, accompanies many states of trace element deficiency, whether these are of nutritional origin or are attributable to genetically mediated disorders of absorption.

Grazing animals in particular are liable to become deficient in copper or cobalt. Not only may undeveloped land be deficient in these elements, but modern pastures, which often consist of well fertilized and highly productive grass of a single species, commonly contain less than 10 mg copper per kilogram dry matter, the availability of which is reduced by their molybdenum content (Epidemiology Unit 1980). Furthermore, under conditions of modern economic stringency, hay and silage of low copper content, supplemented with urea, may be fed to animals seasonally, while imported concentrates with a high content of copper are used less frequently.

This introduction paragraph contains an indication of several of the causes of copper deficiency categorized by Underwood (1977*a*). The problems are not only that soils and plants may be deficient in copper but that the situation is constantly changed by new methods of pasture management, by the use of fertilizers, and by the fact that copper absorption and storage are strongly influenced by other dietary components. Many of the grosser clinical manifestations of copper deficiency can now be recognized as such, and can be compared with certain more absolute and spectacular heritable forms of copper deficiency. Homologous examples of the latter are found in the mottled mutant mouse (Hunt 1974) and in children suffering from Menkes's syndrome (Danks 1980).

Less is known about subclinical effects of copper deficiency, even though these are of great economic importance because of insidious effects on growth and production. The aim of this paper is to consider a few selected examples of known lesions and to seek possible common denominators, guided more by concepts of cell physiology than by a biochemical approach to metabolic pathways.

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This approach derives from a recent observation that in copper-deficient rats, not otherwise grossly affected, there was severe atrophy of the exocrine tissue of the pancreas, whereas the islet tissue, vasculature and ducts appeared relatively normal (B. F. Fell, T. P. King & N. T. Davies, unpublished). This observation, together with the finding that pancreatic acinar cells, which have secretory functions, show a rapid loss of labelled protein (Lipkin *et al.* 1961) focused attention on the long-standing concept that the nutrient requirements of an animal depend not only on growth, i.e. cell proliferation and hypertrophy, but on 'turnover', the implications of which have been stated by Neuberger & Richards (1964). Within a tissue, rates of protein synthesis and degradation are influenced by cell multiplication and death. At the cellular level, the life-span of a protein may be a function of an organelle in which the protein is relatively inert. When the structure is broken down, its proteins are degraded and utilized again. In addition to 'turnover' attributable to the life cycles of cells and organelles, virtually all proteins including enzymes, are in a state of flux.

In tissues such as the intestinal mucosa, that have a high rate of cell renewal, there is rapid protein synthesis associated with mitosis, followed by degradation when the cell is expended. During the active life of the cell, however, both protein formation and breakdown occur (Holmes & Crane 1967). While the proteins of proliferating cells appear to be relatively stable (Lipkin *et al.* 1961; Lipkin & Quastler 1962), there is extensive turnover in resting cells (Buchanan 1961).

The liver of the adult animal does not have a high rate of cell division; in rats the life-span of the hepatocyte is at least 150 days (Swick *et al.* 1956; Buchanan 1961; MacDonald 1961); nevertheless in liver, protein turnover attributable to metabolic processes is very rapid (Buchanan 1961; Schimke 1964), the majority of protein being replaced within 20 days (Schimke *et al.* 1968). The proteins of the liver membrane fractions – smooth and rough endoplasmic reticulum and plasma membrane – are in the greatest state of flux, with a mean half-life of about 2 days (Schimke *et al.* 1968). Such observations led Schimke and his collaborators to conclude that the dynamic state of the body constituents is not only extensive and continuous but markedly heterogeneous. While membrane structures have a stable pattern and a continuous existence throughout the life of a differentiated cell, it is clear that the molecular components do not; the membrane lipids of rat liver, as a whole, turn over even faster than the membrane proteins (Omura *et al.* 1967). Superimposed on this concept of continuous molecular turnover, there is some evidence that, in rat liver, whole mitochondria are broken down as a unit with a half-life of about 8–10 days, and are reconstituted *de novo* (Fletcher & Sanadi 1961; Beattie *et al.* 1967). But these are not permanent arrangements: it appears that in response to an increase in physiological load, mitochondria can proliferate and hypertrophy, and that their life expectancy may be correspondingly reduced. Meerson *et al.* (1964) found that in rabbit myocardial cells undergoing hypertrophy, there was a significant augmentation of myocardial mass due to an increase in number, size and complexity of mitochondria. These changes preceded an increase in mass of myofibrillae and other structures, and was accompanied by electron microscopical evidence of an increased rate of mitochondrial destruction.

In contrast to such highly labile tissues, and in keeping with its role as an insulating membrane system, myelin is relatively stable and relatively impermeable to transmembrane ion movement (Rumsby 1978). Rumsby has suggested that the stability generated by lateral networks of hydrogen bonds within the plane of the membrane might be important in maintaining the integrity of the lipid-rich system, and that the extra stability provided in this way might

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account for the slow turnovers observed for both lipid and protein components in myelin. Lipid is not readily transferred to myelin by phospholipid-exchange proteins (Miller & Dawson 1972; Carey & Foster 1977). Nevertheless, the fatty acids of certain lipid components of myelin are metabolized at a significant rate (Smith 1968). Such activity would seem to render the membrane lipids liable to the incorporation of abnormal fatty acids, which might affect physiological function. Such a biochemical basis for neurological disease is discussed later in the context of cobalt deficiency. In the same paper, Smith (1968) estimated that the half-life of the total lipid in myelin was more than 6 months, while that in the subcellular fraction containing crude mitochondria, from the same rat brains, was 17 days. This finding reinforces the view that, regardless of differential effects between the lipid components of myelin, it is the more labile organelles and membranes that may be most at risk.

Clinical pathology of copper deficiency

It is useful to view the pathology of copper deficiency from the angle indicated in the Introduction. The salient lesions vary with the species, and some, such as defects in the synthesis of elastin and collagen, can be attributed to a specific effect of copper deficiency on a copper-dependent enzyme (Harris *et al.* 1980), while defective melanin production can be attributed to a depression in tyrosinase activity. Comprehensive descriptions of the clinical manifestations of copper deficiency are available in several recent discussion papers and reviews (Mills *et al.* 1976; Underwood 1977*a*; Mills 1979; Danks 1980; Hunt 1980; McMurray 1980). Conspicuous features in several species are decreased rates of growth, neurological lesions, bone lesions, and defects in the structure of hair. In spite of much progress, the biochemical basis for the lesions is not understood in every instance; uncertainty remains about the role of copper in the maintenance of normal neurological function (Hunt 1980), and in the promotion of normal skeletal development (Suttle & Angus 1978). Suttle & Angus suggested that, in calves suffering from copper deficiency, defective bone formation might be attributable to an effect on the development of bone collagen, which might have affected the mineralization process, or to an inhibiting effect of copper deficiency on bone matrix formation. Many other investigators have noticed that the osteoporosis of copper deficiency is frequently associated with a decrease in the number and size of osteoblasts, while osteoclasts are unaffected or increased in number (Mills *et al.* 1976). This raises the point that to look specifically for a copper-dependent enzyme within a tissue, without reference to other aspects of cell metabolism, may achieve no more than the satisfaction of a preconceived idea. The biological role of lysyl oxidase depends on its synthesis by cells *in situ*, in regions of connective tissue formation. Lysyl oxidase production by cartilage cells (Stassen 1976), by fibroblasts (Starcher *et al.* 1977) and by the cells of the hen oviduct (Harris *et al.* 1980) are representative examples. But collagen can be a substrate for lysyl oxidase only after fibre formation has occurred; furthermore, lysyl oxidase would not be produced at normal levels if there were a general impairment of the synthetic capacity of the cell.

The pancreatic lesion in copper-deficient rats

In a recent experiment at the Rowett Research Institute, it was observed that when weanling rats were depleted of copper by dietary means, there was a very striking loss of cytochrome oxidase from the exocrine tissue. This was demonstrated histochemically; the control sections

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were from pair-fed, copper-supplemented rats. Cytochrome oxidase activity in the islet tissue appeared to be unaffected. These histochemical changes were accompanied by severe disorganization of acini, loss of exocrine cells, and severe depletion of zymogen granules, while the endocrine cells, blood vessels and ducts appeared normal (figure 1, plate 1). There were marked mitochondrial changes in the exocrine cells. The electron microscopical picture was dominated by greatly hypertrophied, cup-shaped mitochondria, many of which were interlocked. Some of the mitochondria were swollen, and showed degenerative changes comparable with those reported by Fell *et al.* (1975) in the gut of copper-deficient cattle but, for the most part, there was little evidence that loss of cytochrome oxidase was accompanied by changes within the inner mitochondrial membrane. The presence of so many large mitochondria in complex form suggested that a type of adaptation or compensatory change might have occurred. These changes appear to be of fundamental interest; atrophy of the pancreas occurs in protein malnutrition in man (Tandon *et al.* 1969), but since the rats referred to above were pair-fed, the changes are probably attributable to copper deficiency. This observation may have an application to human medicine since kwashiorkor may well be accompanied by a dietary deficiency of copper.

With regard to the fundamental aspect, Palade (1975) has commented on the stringent energy requirements for secretory discharge in the exocrine pancreatic cell. Analysis of the secretory process of the cell reveals the following steps, several of which require energy supplied by oxidative phosphorylation:

(1) synthesis on polysomes attached to the membrane of the rough endoplasmic reticulum;
(2) segregation within the cisternal space of the rough endoplasmic reticulum;
(3) intracellular transport to the Golgi complex;
(4) concentration of secretory proteins in condensing vacuoles of the Golgi cisternae (at this level the secretory product is transferred from the highly permeable membrane of the endoplasmic reticulum to a membrane of low permeability resembling in lipid composition the plasmalemma);
(5) discharge by exocytosis.

In view of the gross mitochondrial abnormalities, and with the knowledge that zymogen synthesis in the pancreas involves several energy-dependent steps, including a considerable demand for membrane synthesis, it seems possible that the cytological lesion may be attributable to a defect in the synthesis of ATP, or to its localization within membranes. Whatever the cause of the lesion may be, and although the exocrine cell of the pancreas is a highly specialized unit, it seems possible that other secretory cells may be affected, to some degree, in the same fashion. Other cells noted for their secretory capacity are neurons, hepatocytes, fibroblasts, osteoblasts, melanoblasts, granulocytes, parotid acinar cells and secretory epithelial cells such as those of the prostate, uterus and gut. It has been shown that in such cells (and in osteoblasts) the Golgi apparatus is also involved in the synthesis of glycoproteins (see review by Beams & Kessel 1968). Beams & Kessel comment that 'the problem of maintaining the steady state of the Golgi apparatus, with its consistent and rapid loss of membranes through pinching off of vesicles at its surface in rapidly secreting cells, seems to resolve itself basically into one of membrane synthesis'. There is a heavy demand for membrane synthesis in the neurons of both the central nervous system and autonomic ganglia. Not only are neurons committed to considerable protein synthesis (Holtzman 1971), as demonstrated by axoplasmic flow, but such cells constantly synthesize neurosecretory vesicles whose membranes contribute to the main-

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tenance of the synaptic membrane (Peters *et al.* 1976; Holtzman *et al.* 1977). Indeed, Peters *et al.* (1976) have commented that the nerve terminal can be regarded as a distant outpost of the Golgi apparatus.

In addition to such dramatic examples of cell specialization, Palade (1975) has drawn attention to the generality of the secretory process and has suggested that, with the evidence at hand, all eukaryotic cells can produce secretory proteins. He concludes that the production of highly specialized proteins exported by a variety of differentiated cell types is superimposed on a basic activity common to all cells.

The mitochondrial lesion in copper deficiency

Mitochondrial lesions such as those found in the enterocytes of copper-deficient cattle (Fell *et al.* 1975) appeared to be essentially degenerative. The loss of cytochrome oxidase activity, associated with defects in inner mitochondrial membrane and swelling of the organelle, seemed compatible with the changes described by Gallagher *et al.* (1956*a*, *b*) in hepatic mitochondria of rats deficient in copper. The major biochemical dysfunction can be attributed to loss of the terminal respiratory enzyme, cytochrome oxidase, which leads to a depression of phospholipid synthesis by interfering with the provision of endogenous ATP (Gallagher & Reeve 1971*a*, *b*). Gallagher *et al.* (1956*a*, *b*) found little evidence of a compensatory change or adaptation in mitochondrial function, except that in extreme copper deficiency there was an increase in the activity of isocitrate dehydrogenase.

The response of myocardial mitochondria to copper deficiency differs from that seen in the liver and in the ephemeral cells of the gut mucosa. Both in rats (Dallman & Goodman 1970) and in cattle (Leigh 1975) there is a considerable enlargement of the mitochondrial component of the heart attributable to an increase in mitochondrial size, and possibly also in number. A marked increase in mitochondrial size and complexity has also been noted in the pancreas of copper-deficient rats (Fell, King & Davies, unpublished). Non-degenerative hypertrophy of this type is suggestive of adaptive change. Although cardiac hypertrophy in copper-deficient rats was accompanied by a marked depression of cytochrome oxidase activity, Goodman *et al.* (1970) found that both cardiac and hepatic mitochondria functioned normally with regard to respiration and phosphorylation. It is possible, however, as those authors commented, that a serious loss of mitochondria had occurred either because of increased fragility or because the enlarged mitochondria passed into different subcellular fractions during sedimentation. More recently, Kelly *et al.* (1974) found that while cytochrome oxidase activity was reduced in the myocardium of rats severely depleted of copper, the activity of several other mitochondrial oxidative enzymes was distinctly increased. Lactate dehydrogenase and monoamine oxidase activities were similarly increased. As Kelly *et al.* pointed out, these data indicate that a pronounced biochemical change had taken place in the metabolism of the myocardium. The changes appeared to include the possibility that in the (hypertrophied) myocardium the oxidative mechanisms for energy production are augmented through a utilization of the less efficient glycolytic pathways. These changes in cell metabolism appear to be similar to those that occur in the brain of copper-deficient rats. Electron microscopical examination of the cerebral cortex of severely depleted rats (Prohaska & Wells 1975) revealed that about half of the mitochondria were enlarged, were of abnormal shape, and showed a reduction in electron density. Mitochondria isolated from the brain of deficient rats showed a 30% reduction in the

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rate of both succinate and glutamate oxidation, decreases in the A components of cytochrome, and a marked decrease in cytochrome oxidase activity. There were, however, slight increases in the activity of succinate dehydrogenase (located in the inner mitochondrial membrane) and fumarase (matrix). There were decreases in the outer membrane enzymes hexokinase and monoamine oxidase. Determination of the ratios of metabolites in fast-frozen tissues suggested that the copper-deficient brain was in a relatively reduced state.

In the same investigation, in spite of earlier evidence of a decrease in superoxide dismutase activity, Prohaska & Wells (1975) obtained no evidence for an increased peroxidation of brain lipids. Prohaska & Wells commented that lipid peroxidation does not appear to provide an explanation for the mitochondrial enlargement, or for the enhanced 'ageing' of rat liver mitochondria *in vitro* (Gallagher *et al.* 1956).

There appear to be considerable differences between tissues in the effect of copper deficiency on monoamine oxidase activity, which is located in the outer mitochondrial membrane (see Ghadially 1975). Although O'Dell *et al.* (1976) reported significant increases in monoamine oxidase activity in brain cortical and cerebellar tissue from swayback lambs, and Kelly *et al.* (1974) found a marked increase in the heart of severely copper-deficient rats, Prohaska & Wells (1975) found a slight decrease in the rat brain. This finding conforms with the observation that monoamine oxidase activity is unchanged in the brain of the mottled mutant mouse (Hunt & Johnson 1972).

Since the exocrine cells of the pancreas of the copper-deficient rats examined by Fell, King & Davies (unpublished) contained large numbers of enlarged, complex mitochondria, the area of the outer mitochondrial membranes was relatively large. Histochemical examination with the method of Glenner *et al.* (1957) revealed that, in the pancreas of the pair-fed control rats, monoamine oxidase activity was weak and diffuse. In the deficient animals, the reaction was strong and punctate, the foci of intense activity having a distribution similar to that of the giant mitochondria. Monoamine oxidase activity was also increased in the heart of the deficient rats (figures 2 and 3). These observations may well relate to the findings of Smith & Reid (1978) that monoamine oxidase activity in rat liver mitochondria, isolated *in vitro*, is influenced by their respiratory state, lowered monoamine oxidase activity being maintained throughout phosphorylating respiration, while in certain other respiratory states, oxidative deamination of tyramine may increase tenfold. The same authors discussed possible ways in which monoamine oxidase activity in the outer mitochondrial membrane is influenced by the redox state of components of the inner membrane.

Monoamine oxidase may also be involved in the regulation of the concentration of noradrenalin within the tissues. This aspect of mitochondrial metabolism impinges on another consequence of copper deficiency namely the activity of dopamine β-hydroxylase, a copper-containing monooxygenase that catalyses the conversion of dopamine to noradrenalin (Walker *et al.* 1977).

Several investigators have found that copper deficiency is accompanied by a reduction in the concentration of noradrenalin in the brain (Hunt & Johnson 1972; Prohaska & Wells 1974; O'Dell *et al.* 1976; Morgan & O'Dell 1977; Feller & O'Dell 1980).

In certain instances this may be caused by a depression in the activity of the rate-limiting enzyme, tyrosine 3-monooxygenase (Morgan & O'Dell 1977). This is not a copper-dependent enzyme, however, and in the mottled mutant mouse there appears to be a compensatory increase in its activity (Hunt & Johnson 1972). Certainly, the reduced levels of catecholamines in the brain cannot be attributed to an enhanced catabolism by monoamine oxidase, or to a

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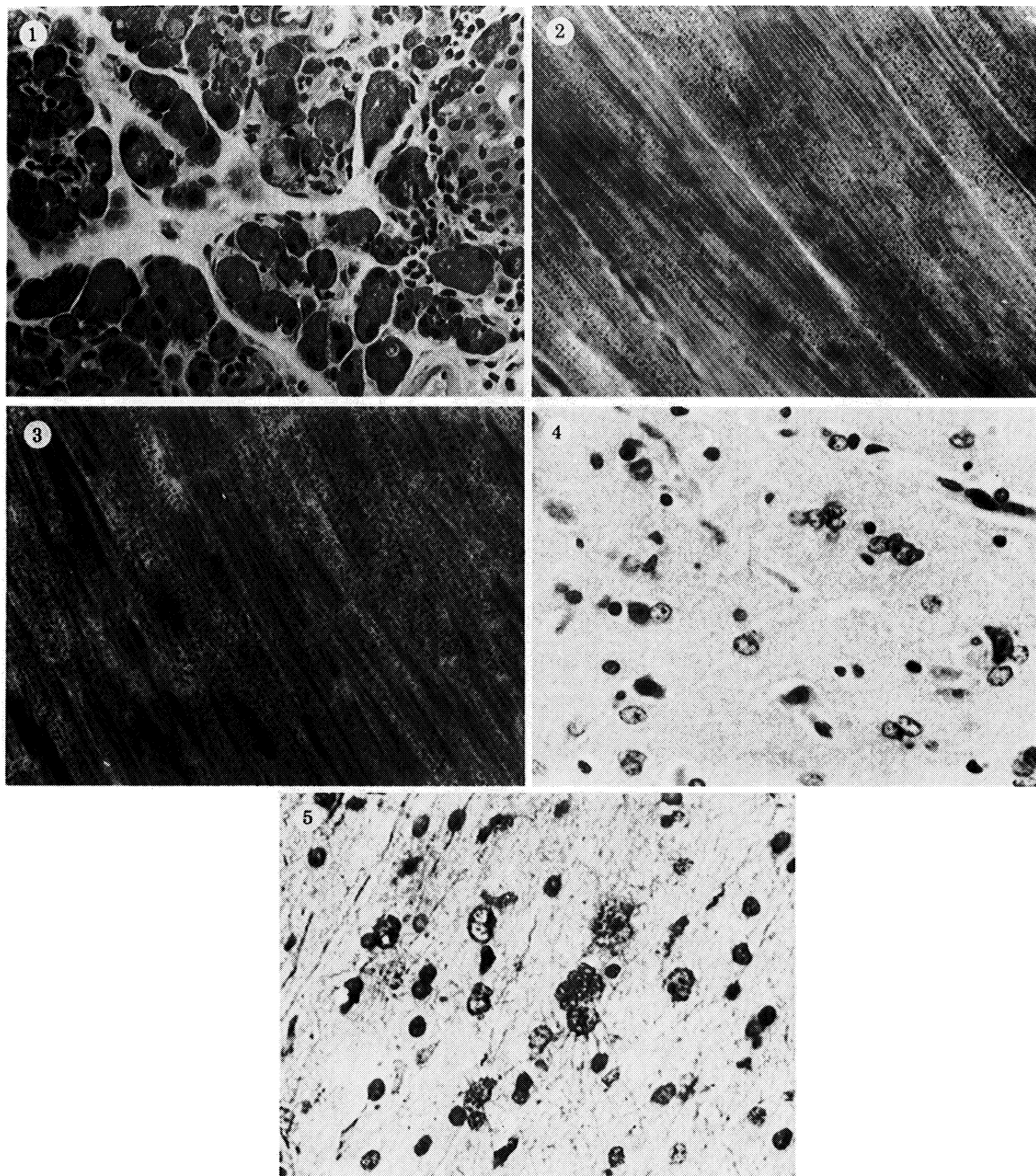

Figure 1. Pancreas of a copper-deficient rat, to show loss of zymogen granules, disorganization, and degenerative changes in exocrine cells while the islet tissue (upper right) is relatively unaffected. Methacrylate section, 2 μm, stained with haemalum and basic fuchsin. (Magn. × 220.)

Figure 2. Myocardium of a normal (copper-supplemented control) rat. Cryostat section, 20 μm, histochemical monoamine oxidase reaction of Glenner *et al.* (1957), with tryptamine as substrate. (Magn. × 420.)

Figure 3. Monoamine oxidase reaction in the myocardium of a copper-deficient rat. Note enhanced reaction in copper deficiency. Cryostat section, 20 μm. The reaction was inhibited by preincubation in iproniazid phosphate (isonicotinic acid 2-isopropylhydrazide phosphate). (Magn. × 420.)

Figures 4 and 5. Neuronal atrophy and Alzheimer type II astrocytes in a cerebral gyrus of a cobalt-deficient sheep suffering from severe fatty and lipofuscin infiltration of the liver. Stains: figure 4, haematoxylin and eosin; figure 5, Holzer's method. (Both magn. × 420.)

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failure of dopamine uptake by synaptic membranes (Hunt & Johnson 1972). A reduction in dopamine β-hydroxylase activity may possibly be involved; Feller & O'Dell (1980) have suggested, however, that in the severely copper-deficient rat the cause of lowered concentrations of catecholamines in the brain may be a reduction in the number of functional neurons.

The situation in the peripheral tissues seems less clear: Hesketh (1980, 1981) found that in both cattle and rats depleted of copper by dietary deprivation, there was a reduction in the amount of noradrenalin, but not adrenalin, in the adrenal medulla. In cattle, the reduction in concentration of noradrenalin was accompanied by a small depression of dopamine β-hydroxylase activity that was not statistically significant in comparison with the controls. In copper-deficient rats, the activity of dopamine β-hydroxylase was higher than that in the control tissues. Hesketh (1981) suggested that this increase in activity was probably a compensatory change attributable to a feedback control mechanism; there was no evidence that copper deficiency had interfered with the synthetic pathway. Possible causes of the lowered concentration of noradrenalin within the tissues appear to be either a failure of synaptic membranes to recapture and recycle this neurotransmitter or its precursors, or enhanced catabolism, possibly by an increase in monoamine oxidase activity in several tissues.

While it may seem plausible to attribute the diversity of lesions in copper deficiency to a particular susceptibility of tissues with high rates of metabolism and synthesis, it remains difficult to explain why there is a selective depletion of cytochrome oxidase activity. Examples of selective depletion are the large motor neurons of the brain stem in copper-deficient lambs, which contrast strongly with the surrounding neuropil (Fell *et al.* 1965), and the exocrine cells of the pancreas of rats deficient in copper.

Possibly the mitochondria of such cells have a shorter life-span than those in less active tissues. The half-life of whole mitochondria in rat liver is about 8.5 days, while that in the brain is about 26 days (Beattie *et al.* 1967); however, there is some evidence that the short half-life of liver mitochondria, which appear to turn over as a unit, conceals the fact that mitochondrial proteins have unique half-lives, and turn over at different rates. In tissues with a slower turnover it becomes clear that the proteins are synthesized in extramitochondrial sites. Possibly there is an initial synthesis of membrane structure, including lipid components, followed by an appropriate integration of proteins (Beattie *et al.* 1967). In this situation it appears that copper deficiency may well either impede the synthesis of proteins or affect their integration into mitochondrial membranes.

Cobalt deficiency

There is much evidence, reviewed by Underwood (1977*b*) and by Young (1979) that the addition of small quantities of cobalt to the feed of many animals and birds has improved growth and development. It appears that in most instances the mode of action is through a stimulation of bacterial vitamin B_{12} synthesis in the gut; vitamin B_{12}, which is an essential factor in the nutrition of many animal species, contains significant amounts of cobalt (Smith 1948; Rickes *et al.* 1948). It is because of such intestinal biosynthesis that cobalt is of nutritional importance for chicks fed on diets lacking in choline and vitamin B_{12} (Burns & Salmon 1956). Other beneficial effects of dietary cobalt in simple-stomached animals can be traced to the ingestion of vitamin B_{12} by coprophagy, and by the consumption of litter enriched by the growth of faecal bacteria.

Although birds and simple-stomached animals are much less efficient than ruminants in the

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utilization of cobalt and the synthesis of vitamin B_{12} (Burns & Salmon 1956), it is ruminants that are notoriously susceptible to lack of dietary cobalt.

Cobalt is apparently not necessary for the metabolism of higher plants but is taken up by them; when the content of cobalt in the herbage is less than 0.07 mg/kg dry matter, deficiency is likely to develop in sheep and cattle grazing that pasture. This condition occurs in many parts of the world (Young 1979), but disorders of ruminants attributable to nutritional cobalt deficiency are of greatest economic importance in Australia and New Zealand. Most of the pioneering investigations that led to the recognition of cobalt as an essential trace element for ruminants were carried out in those lands (Underwood 1977*b*).

It was shown by Marston & Lee (1949) that sheep suffering from cobalt deficiency obtained no benefit from cobalt administered by parenteral routes but responded rapidly when cobalt was ingested. Since cobalt injected intravenously enters the intestines below the level of the duodenum, and since marsupials, rabbits and horses flourished on pastures that would not support ruminants, Marston & Lee (1949) suggested that an important function of cobalt in ruminants concerned its effects on the ruminal flora. This concept was quickly followed by evidence that the deficiency syndrome shown by cobalt-deficient sheep was in fact that of vitamin B_{12} deficiency, an observation that led to the conclusion that cobalt favours the proliferation of microorganisms responsible for the production of accessory food factors essential for the animal (Marston & Lee 1952; Marston & Smith 1952). Thus, it is only in ruminants that a lack of dietary cobalt is manifested as a deficiency disease. Most of the vitamin B_{12} passing from the sheep rumen is bound to bacteria; it appears that much of it is liberated during passage through the acid conditions of the abomasum (Smith & Marston 1970). As Rickard & Elliot (1978) have commented, the possible role of intrinsic factor is largely unresolved in the ruminant; nevertheless, as in other species (Taylor *et al.* 1958; Simnet & Spray 1965; Adams *et al.* 1971), the efficiency of absorption is increased when less vitamin B_{12} is passing through the gut.

This observation is consistent with the hypothesis that an intrinsic factor mechanism is operative in sheep (Rickard & Elliot 1978). In normal sheep, when the cobalt content of the fodder imposes no limit (Marston 1959), ruminal vitamin B_{12} production is about 700 μg/day (Smith & Marston 1970), but in comparison with simple-stomached species the efficiency of absorption is relatively low (Smith & Marston 1970; Rickard & Elliot 1978). This is one of the reasons why the oral requirement for vitamin B_{12} of calves, or of lambs grazing cobalt-deficient pastures, is much higher than that of other species. The vitamin is also partly degraded within the rumen (Smith & Marston 1970). The requirements of ruminants for parenterally administered (or absorbed) vitamin B_{12} are of the same order as those found for the rat, pig and chick but are higher than those for man (Underwood 1977*b*). Presumably, under normal conditions of excess, associated with the development of ruminant function, there has been no evolutionary pressure to develop a particularly efficient conservation mechanism. These circumstances appear to make sheep good subjects for the experimental investigation of the pathological consequences of vitamin B_{12} deficiency, particularly in comparison with laboratory animals and primates, which show extremely small net daily losses of the vitamin and may take several years to manifest clinical signs of deficiency (Siddons *et al.* 1975).

At the Rowett Institute, recent interest in the biochemical and pathological consequences of cobalt deficiency in sheep had a dual origin. On the one hand, colleagues with an interest in ruminal microbiology (see acknowledgements) were anxious to investigate further the reported

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relation between cobalt deficiency and cerebrocortical necrosis (Hartley *et al.* 1962; MacPherson *et al.* 1976, 1977). As Smith (1976) has pointed out, uncomplicated deficiencies are rare; specific deficiencies may become multiple and complicated because of malabsorption or because of destruction in the gut. With regard to the present topic, in addition to a possible deleterious effect of cobalt deficiency on thiamine synthesis in the rumen, there is the possibility of defective storage of thiamine, or some other aspect of liver function affected by liver damage (MacPherson *et al.* 1977). Fatty degeneration of the liver is a consistent lesion in cobalt-deficient cattle and sheep (Filmer 1933; Smith *et al.* 1950; Marston 1952; Underwood 1977*b*; Sutherland *et al.* 1979).

The other approach stemmed from the discovery that when normal sheep are fed on diets rich in readily fermentable carbohydrate, their adipose tissues contain unusually high proportions of odd-numbered fatty acids and branched-chain fatty acids (Garton *et al.* 1972; Duncan *et al.* 1974). Garton and his colleagues attributed this finding to an overloading of the vitamin B_{12}-dependent enzyme system methylmalonyl-CoA mutase, which is involved in the metabolism of propionate to succinate. When swamped by an excessive absorption of propionate, the intermediary metabolite methylmalonate is substituted for malonate during fatty acid synthesis, thereby giving rise to odd-numbered and branched-chain fatty acids.

Very similar consequences, in terms of lipid synthesis, would be expected from deficiency of vitamin B_{12} since it has been established that a specific metabolic defect of vitamin B_{12} deficiency is a depression in the activity of the methylmalonyl-CoA mutase enzyme system. As a result, propionate and other methylmalonyl-CoA precursors cannot be fully metabolized to succinate. Methylmalonyl-CoA does not, however, necessarily accumulate in the tissues; from several tissues it is cleared at normal rates by hydrolysis to methylmalonate (Cardinale *et al* 1969). This metabolite is excreted in the urine in quantities that approximately parallel the adverse physiological effects of the deficiency state (Cox & White 1962).

In this connection, branched-chain and odd-numbered fatty acids have been identified in the nervous system of a child who died from methylmalonic aciduria (Kishimoto *et al.* 1973). This syndrome is attributable to an inborn error of metabolism equivalent to the vitamin B_{12} deficiency lesion (Morrow *et al.* 1969). Kishimoto and his colleagues suggested that the severe neurological abnormalities that occur in methylmalonic aciduria might be attributable to biological malfunction of membranes, and that the cause might be an accumulation of branched-chain fatty acids into membrane lipids. Frenkel (1973), who found an abnormal synthesis of odd-numbered fatty acids in peripheral nerve of patients with pernicious anaemia, has also discussed in detail the possibility that a derangement of fatty acid synthesis could result in abnormal myelin, and so provide a biochemical basis for neurological lesions.

Enhanced proportions of both branched-chain, and odd-numbered straight-chain, fatty acids have also been detected in the liver lipids of vitamin B_{12}-deficient baboons (Garton *et al.* 1975), while unusually high proportions of odd-numbered, straight-chain fatty acids occurred in the liver and adipose tissue of newborn lambs produced by ewes that had been depleted of vitamin B_{12} during pregnancy (Duncan *et al.* 1981). The same trend was observed in experiments by Fehling *et al.* (1978), who found abnormally large amounts of odd-numbered fatty acids in the lipids of the central nervous system, liver and adipose tissue of rats suffering from severe vitamin B_{12} deficiency.

In comparing the effects of vitamin B_{12} deficiency on the methylmalonyl-CoA mutase system in sheep and man, an important difference in degree is immediately apparent. In man, the presursors of methylmalonyl-CoA are mainly certain amino acids, and the urinary excretion

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of methylmalonic acid by patients with pernicious anaemia can be measured in milligram amounts per day. In sheep, however, the main precursor is propionic acid, one of the main steam-volatile fatty acids produced by the ruminal fermentation of carbohydrate. About 60% of the digestible energy intake of sheep is absorbed in the form of short-chain fatty acids. In the absence of vitamin B_{12} the sheep is unable to metabolize propionate (Marston *et al.* 1961); as a result, there is a considerable rise in the concentration of methylmalonate in the liver and blood (Smith *et al.* 1969), and urinary excretion of methylmalonic acid may reach several grams per day. This is clearly a metabolic lesion of major importance.

The site of impaired propionate metabolism is located within mitochondria (Marston & Smith 1961); in sheep, tracer studies with labelled coenzyme (Smith & Marston 1970) showed that the label was firmly retained within liver mitochondria. Distribution studies showed that the liver was the chief site of retention, but relatively high activity was also found in the wall of the rumen. Smith & Marston (1970) commented that this tissue, like liver mitochondria, actively metabolizes propionate.

Many investigators have suggested that the methylmalonate which accumulates in the body in patients with methylmalonyl-CoA mutase deficiency, and in animals suffering from vitamin B_{12} deficiency, may have deleterious effects on cell metabolism. In support of this concept, there is evidence that it may play a role in the development of hypoglycaemia by inhibiting gluconeogenesis from propionate and other precursors. This effect has been demonstrated *in vitro* by using slices of kidney cortex from normal rats (Weidemann *et al.* 1970), and in isolated rat and guinea-pig hepatocytes (Arinze *et al.* 1979). On the other hand, there is evidence that in hepatocyte mitochondria of both rats and man suffering from vitamin B_{12} deficiency there is an increased activity of enzymes involved in fatty acid synthesis (Frenkel *et al.* 1976), and that this is accompanied by an increase in hepatic mitochondrial cristae membranes. These investigators suggested that the enhanced rates of fatty acid biosynthesis might provide an explanation for the long-standing observation that clinical vitamin B_{12} deficiency results in an increased fat deposition in the liver and other tissues.

Returning to the pathology of copper deficiency, there is a tenuous link between the two syndromes in that in both there is a reduction in the concentration of noradrenalin within the brain tissue (Deana *et al.* 1977; Hunt 1980). The connection may be loss of noradrenergic neurons.

As a result of these considerations and although Marston (1959) has stated that there are no neurological consequences of vitamin B_{12} deficiency in sheep, a reappraisal of the central nervous system in cobalt-deficient animals seemed desirable. If lesions were detected in the nervous system, likely aetiological factors appeared to be a deficiency of vitamin B_{12} *per se*, a deficiency of thiamine because of defective rumen synthesis or defective hepatic storage, and a block in thiamine metabolism attributable to absorption from the gut of a thiamine analogue. This could arise from the activity of bacterial thiaminase in the rumen (see review by Markson 1980), a lack of available folate because of depressed activity of the vitamin B_{12}-containing enzyme 5-methyltetrahydrofolate:homocysteine methyltransferase (Underwood 1977*b*), or a primary myelopathy attributable to the incorporation of abnormal fatty acids into lipids of the central nervous system.

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The syndrome of cobalt deficiency disease in sheep

In recent studies at the Rowett Research Institute, cobalt deficiency in sheep was produced by giving them a diet of hay with a cobalt content between 0.03 and 0.05 mg/kg dry matter. This diet was supplemented with ground maize, minerals and trace elements to conform with recommended feeding standards. All the animals were also given vitamin E by injection at intervals of 2 weeks, and injections of vitamins A and D each month. Control animals received the same diet with a supplement of cobalt.

The course of depletion was assessed by feed intake, concentration of vitamin B_{12} in the serum (determined by the method of Spray (1955) with the use of *Lactobacillus leichmannii*), body mass, clinical condition, and by urinary excretion of methylmalonic acid. The bottom of the normal range for serum vitamin B_{12} was taken as 400 pg/ml (MacPherson *et al.* 1976). Thiaminase activity in rumen contents and faecal matter was determined at fortnightly intervals. Assays of urinary excretion of thiamine (vitamin B_1) were also performed at intervals, and when the animals were killed the concentrations of thiamine and vitamin B_{12} in the liver were measured.

The clinical effects of cobalt deficiency followed the course that could be predicted from the observations of others (Smith *et al.* 1950; Gawthorne *et al.* 1966). Generally there was a change in clinical condition long before the animals lost body mass, and before they excreted significant amounts of methylmalonic acid. They became listless; the wool tended to 'lift' on handling; food particles adhered to the mucous membranes; there was reddening of the palpebral margins, and lachrymation. The flow of tears caused maceration of the skin, and loss of hair from the face. Usually, loss of body mass coincided with a low concentration of serum vitamin B_{12}, and a high level of methylmalonic acid excretion. But this was not always so; some animals maintained appetite and body mass while excreting considerable amounts of methylmalonic acid in the urine each day.

In all the animals, including the cobalt-supplemented controls, there were frequent episodes of high thiaminase activity in both rumen contents and faeces. None of them, however, developed clinical manifestations of cerebrocortical necrosis, and when they were killed, as dictated by loss of appetite and bodily condition, there appeared to be adequate amounts of thiamine stored in the liver.

In the event, the emphasis on cerebrocortical necrosis proved unfortunate because sequential studies of brain pathology were not possible, and since the animals were not killed until they were in a moderately advanced state of vitamin B_{12} deficiency, interpretation of the lesions in the central nervous system and other tissues was complicated by the likelihood of secondary changes.

Notes on the histopathology of cobalt deficiency in sheep

Major changes occurred in the liver, central nervous system and skeletal muscle; the lesions found in these tissues appeared to represent an integrated syndrome, with the liver as the organ primarily affected. The liver usually appeared pale or clay-coloured, a condition that was provisionally attributed to simple fatty change. Grosser manifestations, such as those that characterize ovine white liver disease (Sutherland *et al.* 1979), were not seen. Nevertheless, on histological examination, the hepatic lesions appeared similar to those reported by Sutherland and his colleagues except that, in our studies at the Rowett Research Institute, bile duct pro-

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liferation was not a prominent feature. Nevertheless, the three characteristics of fatty change, accumulation of lipofuscin (ceroid) and bile duct proliferation were present. Lipofuscin was present in large amounts, both within hepatocytes and in sinusoidal and portal macrophages, many of which were engorged. Sutherland *et al.* (1979) observed a consistent relation between dietary cobalt deficiency and the development of white liver disease in the field; they also found that the liver degeneration reported by MacPherson *et al.* (1976), in housed sheep fed on a cobalt-deficient diet, had the same histological characteristics. Our experience appears to support the view that ovine white liver disease may be a manifestation of cobalt deficiency; even if the lesions described by Sutherland *et al.* (1979) are not due solely to cobalt deficiency, it seems likely that livers so damaged would have an impaired ability to detoxicate a plant or fungal toxin.

The same concept of hepatic dysfunction can be applied to interpret the lesions found in the central nervous system.

Numerous descriptions are available of the neuropathy, termed subacute combined degeneration of the cord, which may accompany vitamin B_{12} deficiency in man (Pant *et al.* 1968; Smith 1976; Roos 1978). The essential lesion in this disease is an uneven and irregular degeneration of the white matter of the spinal cord. The early lesions start in small foci and consist of swelling of myelin sheaths, often to many times their original diameter, with little change in the axons. The pathways most frequently affected are those that contain numerous thickly myelinated fibres. In the next stage of the disease, the myelin sheaths disintegrate and the axons degenerate, leaving empty spaces that tend to coalesce, giving the tissue a loose, vacuolated appearance (status spongiosus). As a result of the damage to axons at the primary lesion, usually in the thoracic cord, ascending and descending secondary degeneration of a more homogeneous and systematized nature occurs. These changes are accompanied by reparative gliosis. When the brain is involved, the most characteristic lesions are small, ill-defined, often perivascular foci of demyelination within the cerebral white matter (Smith 1976).

There is a lack of suitable animal models for the study of subacute combined degeneration; the rat is highly resistant to changes in the central nervous system attributable to vitamin B_{12} deficiency (Fehling *et al.* 1978). It seemed possible, however, that the sheep, with its susceptibility to vitamin B_{12} deficiency, might prove to be such a model. In fact, considerable lesions were found in the central nervous system but they closely resembled those of acquired (non-Wilsonian) chronic hepatocerebral degeneration (Waggoner & Malamud 1942; Adams & Foley 1953; Smith 1976). They were characterized by atrophy and degeneration of neurons in the cerebral cortex and brain stem, and by a very striking increase in astrocytes of Alzheimer type II variety. Alzheimer type II astrocytes occurred in very large numbers in the cerebral cortex, brain stem and gray matter of the spinal cord (figure 4 and 5). There were also patchy areas of microcavitation in the deeper layers of the cerebral cortex. There was also severe but localized vacuolation of the white matter in the spinal cord, particularly in the vicinity of the gray matter. The spongy areas were caused by demyelination and axonal loss.

In the animals with gross clinical features of cobalt deficiency, namely loss of appetite and bodily condition, a generalized atrophy of skeletal muscle was evident. The atrophy, as demonstrated in the myofibrillar ATPase reaction, was most marked in the type II (fast) fibres, many of which were strikingly angulated. This rather specific fibre type atrophy might indicate that the underlying pathology involved processes of denervation or disuse.

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The anaemia of cobalt deficiency

Marston and his collaborators placed some emphasis on their finding that usually a profound macrocytic anaemia, with marked poikilocytosis and polychromasia, is a feature of the terminal stages of cobalt deficiency (Marston & Lee 1952; Marston 1959). Indeed, it was the similarity of this blood dyscrasia to that of pernicious anaemia of man that led them to investigate the possible therapeutic effects of concentrated liver extracts and, later, crystalline vitamin B_{12}; these investigations helped to elucidate the connection between cobalt and the B_{12} deficiency syndrome. Later investigations, however, have produced findings at variance with Marston's results; Smith *et al.* (1950) and Gawthorne *et al.* (1966), classified the anaemia of cobalt-deficient sheep as simple, normochromic normocytic anaemia. The latter finding is in accordance with results obtained at the Rowett Institute. In our experience and that of Gawthorne *et al.* (1966), the anaemia does not appear until rather late in the course of the disease, i.e. until after the animals have shown a decline in appetite and a significant loss of condition. Our results were complicated by the observation that in the cobalt supplemented controls, plasma volume per kilogram body mass declined as the animals became adult. The cobalt-deficient animals had a mean plasma volume approximately 25% higher than the animals of the control group; this difference between the groups persisted until the investigation was terminated when the animals became emaciated (W. S. Mackie & R. Boyne, unpublished).

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Discussion

A. MacPherson (*The West of Scotland Agricultural College, Auchincruive, Ayr, U.K.*). Dr Fell's sheep appeared to take a very long time to develop cobalt deficiency. I should like to know what was the concentration of cobalt in the diet that the sheep consumed and to what level the plasma vitamin B_{12} concentrations of his sheep decreased. I also wonder whether, in the event of sheep developing cobalt deficiency symptoms in a much shorter time, they might then develop primary lesions in the brain in contrast to the hepatic lesions reported in this study.

B. F. Fell. The concentration of cobalt in the diet was about 0.05 mg/kg dry matter. The concentration of vitamin B_{12} in the serum fluctuated considerably. We accepted 400 pg/ml serum as the lower limit of normality; many of our animals had concentrations of about 200 pg/ml (microbiological assay). In current work, we are using a Radiochemical Centre radioassay kit to determine the concentration of vitamin B_{12} in plasma; most of the animals have concentrations in the range 100–200 pg/ml.

The time taken to deplete the sheep is probably influenced by the amount of vitamin B_{12} stored in the liver at the start of the investigation. This can be depleted by preliminary manoeuvres such as grazing the animals on a cobalt-deficient pasture. The problem of storage may possibly explain the considerable variation that we have experienced, between investigations, in the time taken to deplete the sheep.

With regard to the induction of a primary myelopathy, an alternative approach to a very rapid depletion of vitamin B_{12}, if that is indeed possible, is to use the pre-ruminant animal.

This possibility is under investigation in current work at the Rowett Institute, in a collaborative project with Dr Garton and others. The animals are Blackface ewes that are being fed on a cobalt-deficient diet, and have been depleted of vitamin B_{12}, during pregnancy. The aim is to investigate, by chemical and morphological methods, the effect on myelination in the lambs *in utero*. This approach can possibly be extended by using pre-ruminant lambs or calves fed on a diet deficient not in cobalt but in vitamin B_{12}. The vitamin B_{12} requirement of such animals is relatively high (Underwood 1977*b*).

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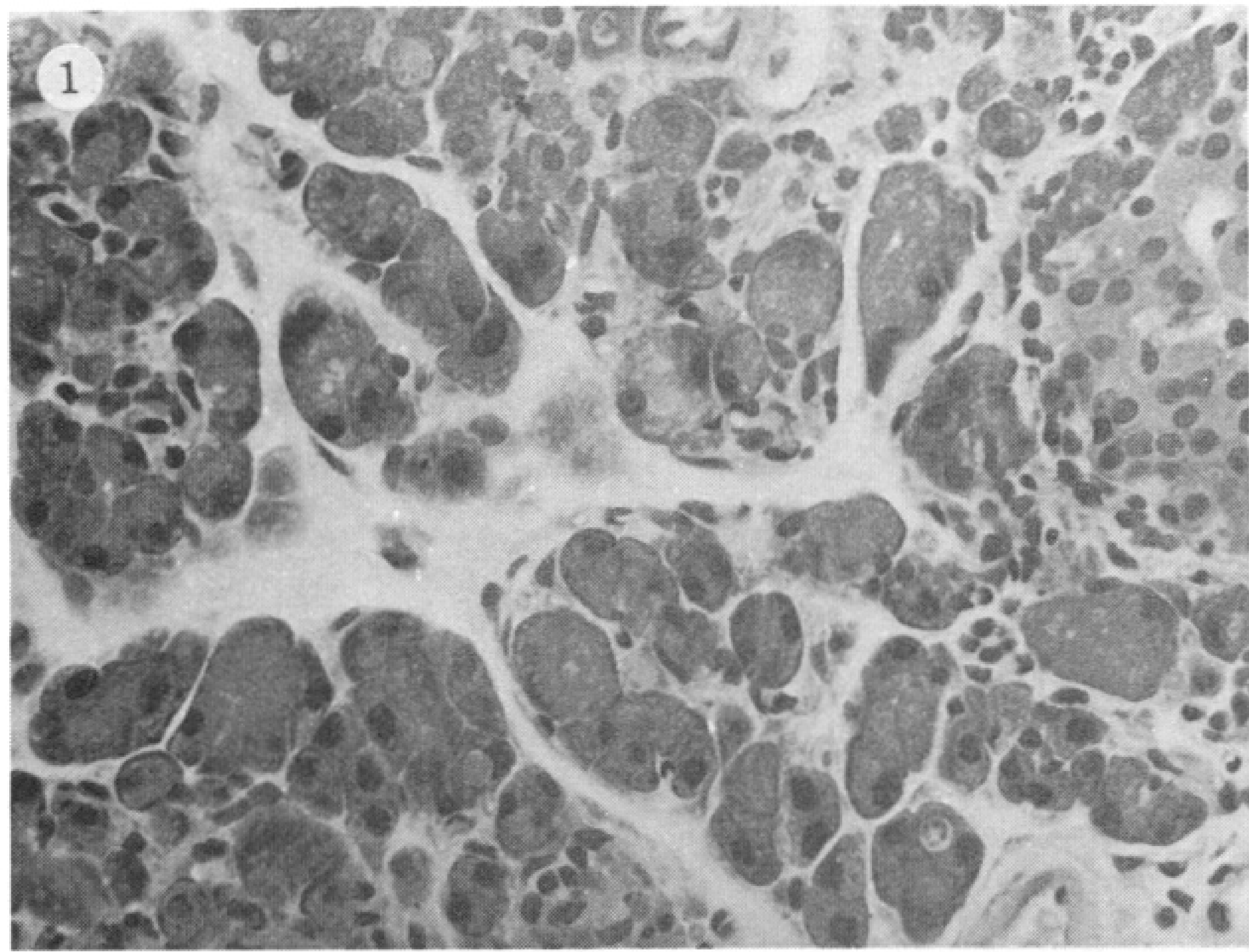

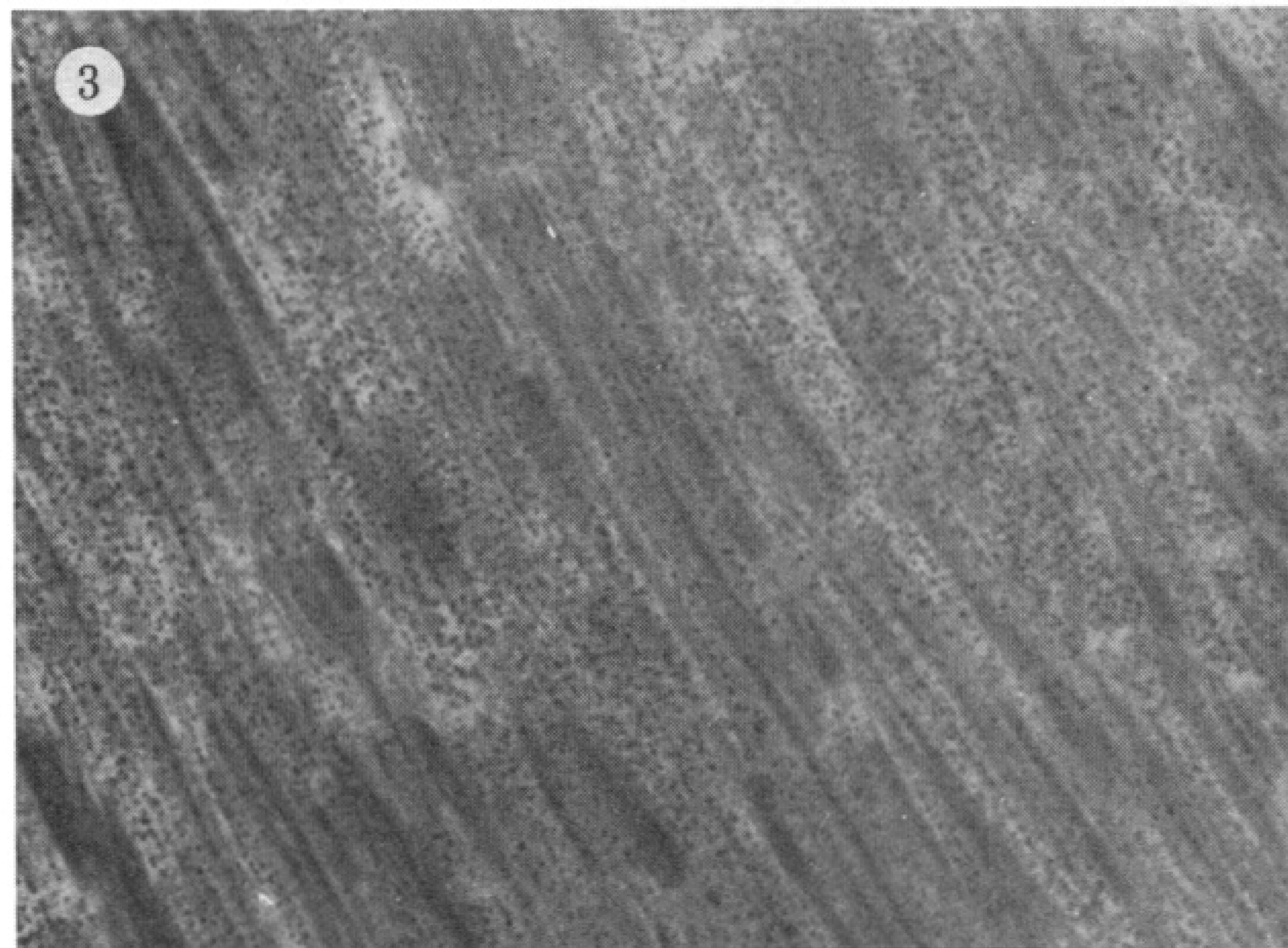

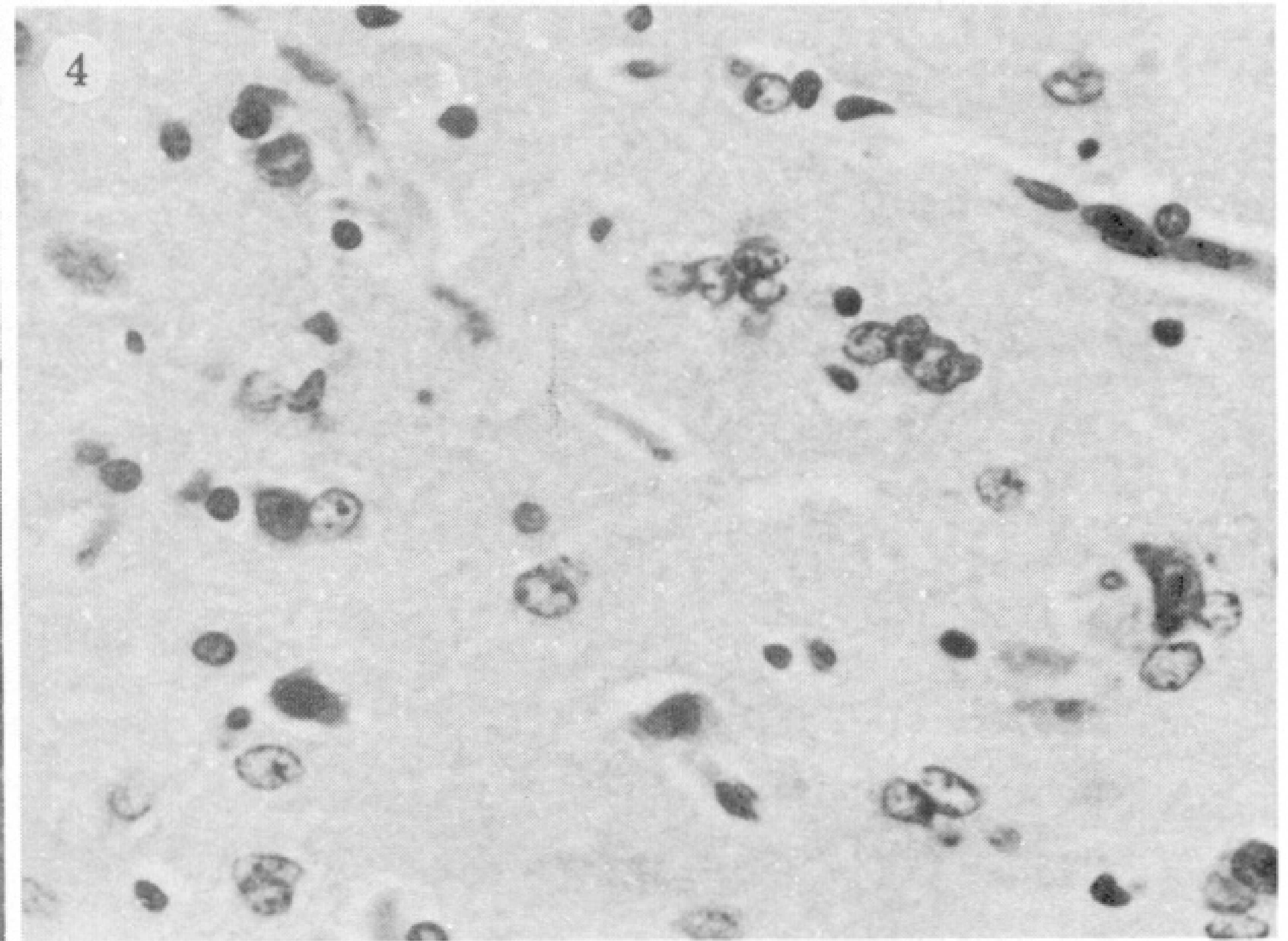

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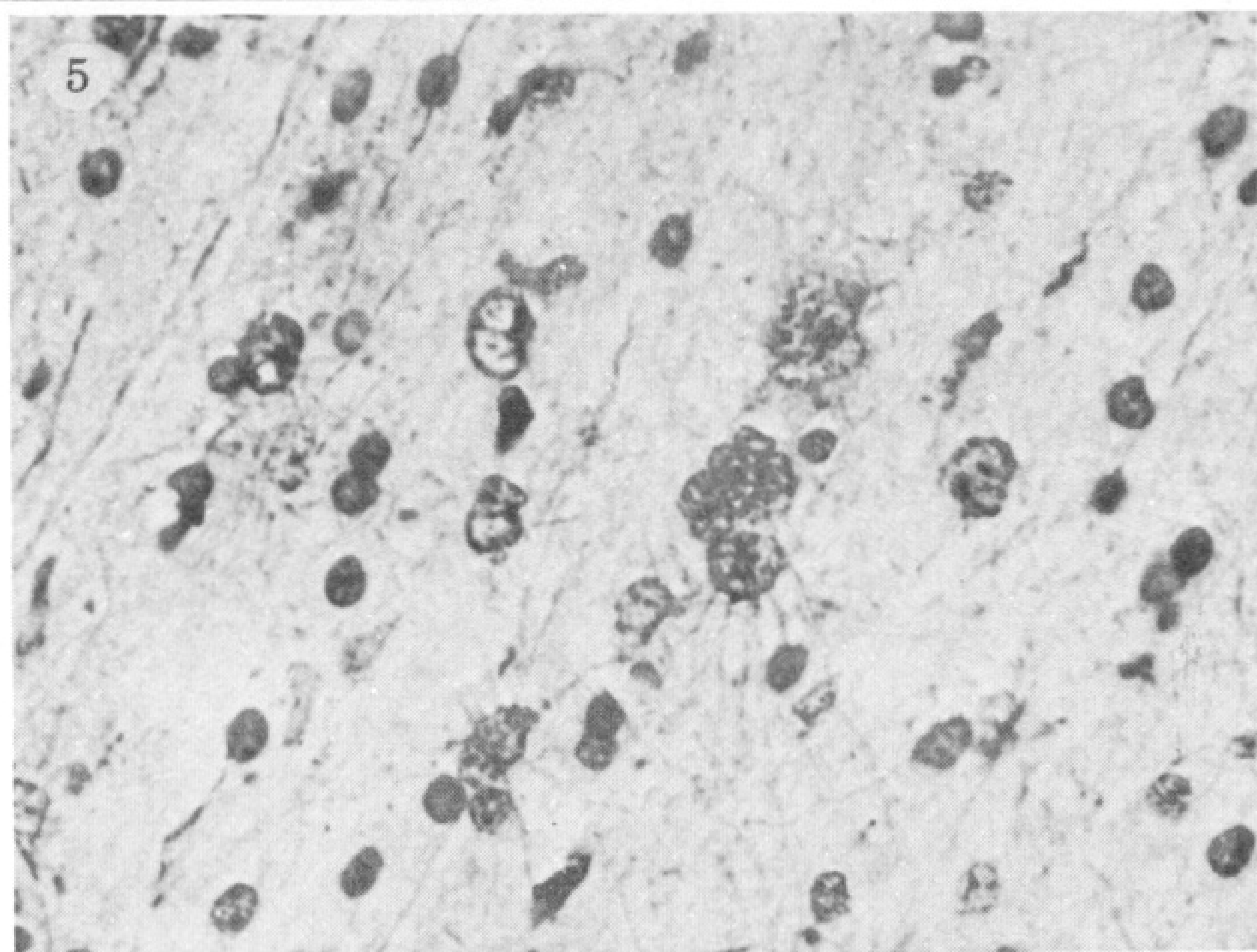

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FIGURE 1. Pancreas of a copper-deficient rat, to show loss of zymogen granules, disorganization, and degenerative changes in exocrine cells while the islet tissue (upper right) is relatively unaffected. Methacrylate section, 2 μm, stained with haemalum and basic fuchsin. (Magn. ×220.)

FIGURE 2. Myocardium of a normal (copper-supplemented control) rat. Cryostat section, 20 μm, histochemical monoamine oxidase reaction of Glenner *et al.* (1957), with tryptamine as substrate. (Magn. ×420.)

FIGURE 3. Monoamine oxidase reaction in the myocardium of a copper-deficient rat. Note enhanced reaction in copper deficiency. Cryostat section, 20 μm. The reaction was inhibited by preincubation in iproniazid phosphate (isonicotinic acid 2-isopropylhydrazide phosphate). (Magn. ×420.)

FIGURES 4 AND 5. Neuronal atrophy and Alzheimer type II astrocytes in a cerebral gyrus of a cobalt-deficient sheep suffering from severe fatty and lipofuscin infiltration of the liver. Stains: figure 4, haematoxylin and eosin; figure 5, Holzer's method. (Both magn. ×420.)