The fine structure produced in cells by primary fixatives.

1. Mercuric chloride

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With one plate (fig. 1)

Summary

The exocrine cells of the mouse pancreas were fixed in mercuric chloride solution, embedded in plexigum, and examined by electron microscopy. The cytoplasm was found to be coagulated as a continuous substance containing innumerable subspherical cavities, mostly between 40 and 200 mμ in diameter and separate from one another. The zymogen granules were preserved, but no trace remained of mitochondria or Golgi apparatus. The nuclear sap was coagulated as a coarse network with thickenings at the nodes. Lumps of electron-dense material (? DNA) were present at the periphery of the nucleus and round the nucleolus.

The proteins of the cell appear to have been fixed by mercuric chloride, but the membranous constituents, which rely for their form on a phospholipid component, are not clearly recognizable. The lipids have presumably been lost during dehydration and embedding.

Introduction

THE work that will be described in this series of papers has been undertaken partly to explain what we are looking at when we examine a fixed preparation by light microscopy, and partly to provide a basis for the rational use of fixatives (including fixative mixtures) in electron microscopy.

There are two reasons why it is necessary to concentrate attention on the action of 'primary' or unmixed fixatives. First, the mode of action of mixtures can only be analysed when that of their constituents is known; secondly, an understanding of the action of mixtures has only a temporary value, since the mixtures themselves change with fashion or new discovery.

The only general study of this kind that has previously been undertaken would appear to be that of Policard, Bessis, and Bricka (1952), who worked with the neutrophil leucocytes and blood-platelets of man, spread out on formvar films. Their investigation differs from ours in certain important respects. They studied the whole cell, while we have studied thin sections. Although their results were most interesting and valuable, it is questionable whether the electron microscope can give a critical view of an intact leucocyte, even when it has spread itself thinly on a formvar film. Further, they studied 'preservation' rather than fixation (Baker, 1958), since they did not investigate the capacity of fixatives to protect the structure of cells against the effects of the media used in the preparation of tissues for sectioning. Beyond this, the object of their study was the single cell. Now it is very frequently

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observed that the great majority of cells in a histological or cytological preparation are quite different in appearance from the superficial ones, which came into immediate contact with the fixative. The latter are often seen to be distorted. In the French authors' work all the cells studied were like the superficial ones in a block of tissue. In our investigation the external part of the piece of tissue was discarded.

The choice of the primary fixative for the first study of the series was necessarily rather arbitrary, but mercuric chloride commends itself since it has been and still is so enormously used in fixation for light microscopy. We need to know what it is—in terms of fine structure—that we dye and examine day after day when we use mercuric chloride as a fixative in routine histological and cytological work. The second paper in the series will deal with fixation by potassium dichromate.

Material and methods

The cells that have been and will be studied throughout this investigation are the exocrine cells of the pancreas of the house-mouse, *Mus musculus*. These cells were chosen partly because they are readily available in every laboratory; partly because they have been much used in studies with the electron microscope, and the appearance given after fixation by osmium tetroxide is therefore well known and available for comparison; partly because the various cell organelles—ergastoplasm, Golgi apparatus, secretory products, mitochondria, nucleoli—are represented in characteristic form; and partly because most of the cells of the pancreas (apart from the islets) are essentially similar, so that nearly every field of view under the electron microscope contains what one wants to examine.

The 5 mice used in the present investigation were kept continuously supplied with food and water. They were killed with chloroform. The pancreas was at once cut out, divided into 2 or 3 pieces, and placed in the fixative at room temperature. Mercuric chloride was sometimes used at 5% w/v and sometimes at saturation (about 7%) in distilled water; no difference was noticed in the results. Fixation lasted for 24 h. The pieces of tissue were transferred to 50% ethanol and then to a 0·5% solution of iodine in 70% ethanol, in which they were left for 2 to 4 h. They were then brought into 80% ethanol. Each piece was sliced, and fragments of the size usual in electron microscopy were cut from the central part of each slice. These fragments were dehydrated as usual in 96% and two lots of absolute ethanol.

At the beginning of the investigation the fragments were embedded in prepolymerized butyl methacrylate, but it was found that tissue fixed in mercuric chloride has a strong tendency to disperse when embedded in this medium, so that the parts of the cell become separated from one another in a manner that is not seen when paraffin is used as an embedding medium for light microscopy. Dispersion of this kind is usually attributed to too violent polymerization ('explosion'), but Parsons and Darden (1961) consider that the opposite is true: failure to polymerize adequately is the cause. It is claimed for the

embedding medium known as 'plexigum' that 'explosion' is avoided (Bayer and Peters, 1959). We had already made a careful study of plexigum before we started work on the fine structure of cells fixed by mercuric chloride, and we think it may be useful if we describe our final procedure with this medium in some detail. The methods recommended by the manufacturers and by Bayer and Peters seem unnecessarily complicated, and in our hands have not given such good results as those described below.

Plexigum is manufactured by Röhm and Haas of Darmstadt and is obtainable from the Cornelius Chemical Co., Ibex House, Minories, London, E.C. 3. Two fluids are available, called P 7469 and M 7466. The former consists largely of butyl methacrylate, the latter largely of methyl methacrylate, both presumably in monomeric form: the exact composition is not stated by the manufacturers. The two fluids may be mixed in any proportions. The more M 7466, the harder the final block will be. It is convenient for most purposes to mix 4·5 volumes of P 7469 with 1 of M 7466, but a 5:1 mixture may be used by those who prefer a softer block. The mixture keeps indefinitely in the refrigerator. In what follows it is referred to simply as 'plexigum'.

The activator is called by the manufacturers the *Härterlösung*. It keeps indefinitely in the refrigerator. Unfortunately its chemical composition has not been divulged. The activation is so intense that plexigum polymerizes readily at the temperature of the refrigerator (about 4° C).

The technique is as follows.

1. Pass dehydrated tissue into absolute ethanol mixed with an equal volume of plexigum. Leave 15 min.
2. Plexigum, 2 lots, 15 min. in each at room temperature (or any longer time in the refrigerator—days, if you like).
3. Mix 1 volume of plexigum activator with 10 volumes of plexigum, by drawing into a pipette and pushing out gently about 15 times. Avoid even momentary formation of air bubbles.
4. Transfer the tissue to plexigum+activator, and leave for 30 min in the refrigerator, swirling occasionally.
5. Transfer the tissue with plexigum+activator into a gelatine capsule.
6. Put the gelatine capsule in the refrigerator and leave for 24 h. (It is permissible to omit this stage.)
7. Leave another 24 h. (or any longer time) at room temperature before cutting.

Dispersion of cell constituents does not occur if this technique is followed. (It must be mentioned that tissues fixed with osmium tetroxide have a tendency to become slightly more opaque to electrons when embedded in plexigum than when embedded in ordinary methacrylate.)

Silver or silver-grey sections were cut on a Huxley ultramicrotome and examined with an Akashi electron microscope.

Since material intended for light microscopy is usually embedded in paraffin, and since this treatment might be supposed to alter the fine structure of cells, the following experiment was performed.

A piece of pancreas was fixed in mercuric chloride solution as usual, washed with 50% ethanol, treated with iodine, and brought into 80% ethanol. Fragments were not cut out at this stage. On the contrary, the whole piece was dehydrated and passed through toluene into melted paraffin wax, which was changed once. The block was cooled and sliced by hand into thick sections. Fragments cut from the central parts of these were left overnight in toluene at 37° C to remove the paraffin. The toluene was then washed away with absolute ethanol, and the fragments were embedded as usual in plexigum. Thin sections were examined with the electron microscope.

Results

Strangely enough, no difference could be detected between material that had gone through the standard procedure described above and that which had been embedded in paraffin before being embedded in plexigum. It seems almost certain that any structural changes produced by embedding in paraffin (apart, perhaps, from slight shrinkage) are altogether outweighed by the effects of fixation with mercuric chloride and subsequent dehydration. The only other possibility is that embedding in plexigum produces the same changes as those produced by embedding in paraffin, but this seems very unlikely.

It is a familiar fact that when osmium tetroxide is used as a fixative, there is considerable variety in the electron-density of the various cell constituents. A first glance at the sections produced by the method described in this paper shows a marked difference, since almost everything that is darkened at all is generally of a nearly uniform pale grey. The degree of contrast shown in fig. 1, A is unusual. The only black objects are small particles of mercury precipitate that resisted extraction because the period in iodine solution was too short (fig. 1, D).

The cytoplasm is coagulated in the form of a nearly homogeneous mass, hollowed out by innumerable subspherical cavities (fig. 1, B, E). Most of these are between 40 and 200 mμ in diameter, but much larger holes are also present, which appear to have been formed by the confluence of smaller ones. In most sections there is no appearance in the cytoplasm of a reticulum with thickenings at the nodes, such as has often been described in material fixed by coagulant fixatives and examined by light microscopy.

FIG. 1 (plate). Electron micrographs of parts of the exocrine cell of the mouse pancreas, fixed with mercuric chloride and treated as described in the text.

A, low-power view of the nucleus and surrounding cytoplasm.

B, cytoplasm; the boundary between two cells passes obliquely across the micrograph.

C, cytoplasm, showing polarized coagulation.

D, zymogen granules.

E, cytoplasm; typical form of coagulation.

F, nucleolus and surrounding part of the nucleus (from same cell as A, at higher magnification).

G, another nucleolus and part of the nucleus.

H, part of a nucleus (with adjacent cytoplasm), to show the nuclear membrane.

cm, cell membrane; *lu1*, lump of electron-dense material deposited round nucleolus; *lu2*, ditto, deposited on a node of coagulated nuclear sap; *lu3*, ditto, deposited at periphery of nucleus; *nl*, nucleolus; *nm*, nuclear membrane; *z*, zymogen granule.

The scale of magnification shown on C applies also to B, D, E, and H; that on G also to F.

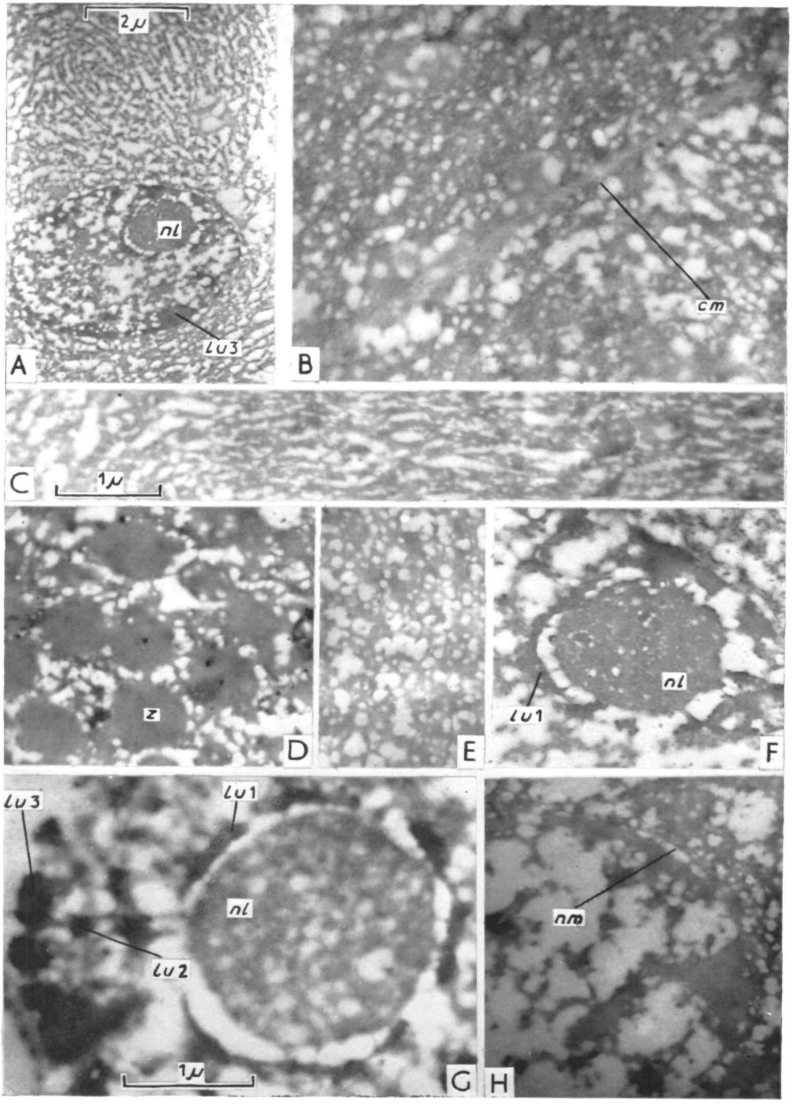

FIG. 1.

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In certain parts of the cell the cavities are often seen to be elongated, all in roughly the same direction (fig. 1, A, C). The appearance is vaguely reminiscent of the ergastoplasm of the basal part of the cell (see especially fig. 1, A). No proof could be obtained that each cavity represents a cisternal cavity of the ergastoplasm, and in the great majority of the cells no particles were seen that could be regarded as ribosomes.

Fully-developed zymogen granules are in many places fairly well fixed, but their membranes have disappeared and their surfaces have been pulled out irregularly by the coagulation of the surrounding ground cytoplasm (fig. 1, D).

Nothing was ever seen that could be interpreted as representing mitochondria or the lamellar-vacuolar system (Golgi apparatus).

The cells have often been pulled slightly apart from one another. A clear cell membrane is not usually seen at their edges. Where the cell membranes have been retained, they are never sharply defined (fig. 1, B).

At low magnifications (fig. 1, A) the nucleus resembles what is seen by light microscopy in cells fixed by mercuric chloride. The nuclear sap has been coagulated in a loose network, and somewhat denser material has been thrown down in large masses round the nucleoli and at the periphery of the nucleus, and also at the nodes of the network (fig. 1, G, left side).

The limits of the nucleus seem often to be represented by the outer edges of the lumps of electron-denser material, but occasionally one seems to see the nuclear membrane itself (fig. 1, H). A clear separation into inner and outer nuclear membranes is, however, never seen.

Nucleoli retain their general form well (fig. 1, A, F, G). They are seen in section as homogeneous grey circles, hollowed out by subspherical cavities; the proportion of substance to cavity is generally much higher than in the cytoplasm. It is very characteristic that strands project radially outwards from each nucleolus, connecting it with the associated electron-denser material. The appearance in section is that of a wheel with a very large hub (the nucleolus), very short, thin spokes, and a somewhat fragmented tread (fig. 1, G).

Discussion

After fixation with mercuric chloride and embedding in plexigum (with or without previous embedding in paraffin), nothing remains of the various intracellular structures except the nucleus, which retains its general form, the nucleolus, and the ripe zymogen granules, which are preserved as homogeneous spheres. The facts are best interpreted by the hypothesis that protein is fixed by coagulation, nucleic acids are precipitated, and lipids have dissolved away (presumably during dehydration and embedding.) Since the cytoplasmic organellae owe their form largely to their phospholipid content, they are not preserved; but the whole of the proteins of the cell remain. The cytoplasmic coagulum consists of a continuous mass of protein, hollowed out by innumerable minute subspherical cavities, mostly not in communication with one another; the nuclear sap, being less rich in protein, is coagulated as

a three-dimensional network. The electron-denser material that is thrown down in masses at the periphery of the nucleus, at the nodes of the network, and round the nucleoli may be presumed to contain DNA, on the evidence of what can be seen by light microscopy.

Large lumps of electron-dense material are not seen in osmium preparations. On the contrary, the nucleus contains a very large number of circles or disks, grey throughout, some 15 to 30 mμ in diameter. There are indications here and there that these circles represent much-convoluted threads, cut in section. They are not distributed uniformly throughout the nucleus, but are most abundant round the nucleoli and in separate aggregations at or near the nuclear membrane. It is probable that the regions that contain many of these minute circles are represented in mercuric chloride preparations by the electron-denser lumps at the periphery of the nucleus, close to the nucleoli, and at the nodes of the coagulated nuclear sap, though no sign of circles or threads can be distinguished in these lumps.

The only part of the cell that has a similar appearance whether the fixative was mercuric chloride or osmium tetroxide is the nucleolus. In both cases it is a rounded, homogeneous body containing small, spherical cavities, rather widely separated as a rule (though not in fig. 1, G).

Dr. S. K. Malhotra kindly permitted us to study his excellent micrographs of the exocrine cell of the mouse pancreas, fixed with osmium tetroxide. The Huxley ultramicrotome was provided by the Royal Society, and the Akashi electron microscope and Edwards vacuum evaporator by the Wellcome Trustees.

References

BAYER, M., and PETERS, D., 1959. J. Ult. Res., **2**, 444.
PARSONS, D. F., and DARDEN, E. B., 1961. Exp. Cell Res., **24**, 466.
POLICARD, A. B., BESSIS, M., and BRICKA, M., 1952. Bull. Micr. appl., **2**, 29.