A Study of Potassium Permanganate 'Fixation' for Electron Microscopy

By S. BRADBURY AND G. A. MEEK

(From the Department of Human Anatomy, South Parks Road, Oxford)

With five plates (figs. 1, 2, 4, 5, and 6)

SUMMARY

The action of buffered potassium permanganate as a fixative for electron microscopy has been investigated. Electron contrast has been shown to be produced by the deposition of granular material in the tissue. The particles are about 50 Å in diameter. Actual fixation of the tissue is performed by the dehydrating alcohol.

Histochemical studies have shown that RNA and histones are removed, whereas phospholipid-protein complexes are 'unmasked'. The reaction of the permanganate with unmasked protein gives rise to high membrane contrast in electron micrographs.

INTRODUCTION

THE preparative techniques at present in use for electron microscopical studies of biological materials involve considerable changes in the structure of the cell (Borysko, 1956). Changes introduced by fixation are obviously important and may represent one of the major sources of error in the interpretation of electron micrographs. Baker (1958) has reviewed the problems of fixation as applied to light microscopy, but relatively little has been published concerning fixation of tissues for the electron microscope. It has become accepted that osmium tetroxide is the most suitable substance for preserving cellular structure for study with this instrument. The researches of Palade (1952) and Bahr (1954) have contributed much to our understanding of the action of this reagent. More recently, however, potassium permanganate has been introduced as an alternative fixative for electron microscopy (Luft, 1956). In general, this reagent reveals cytoplasmic structures which are very similar to those found in osmium-fixed material. There are certain differences, such as the increased contrast of membranous structures, and the 'washed out' appearance of the nuclei after permanganate treatment. It seemed advisable to investigate the effect of permanganate in more detail in an attempt to obtain further information about its action on cells and so help the interpretation of the appearances seen in electron micrographs.

It is the purpose of this paper to describe both the experiments which were carried out in order to test the action of permanganate on various gels (representing crude models of protoplasm), and the results of a combined histochemical and electron microscopical study of permanganate-treated tissue.

MATERIAL AND METHODS

For the studies of the action and rate of penetration of potassium permanganate, experiments were performed on 'models' of protoplasm, consisting [Quarterly Journal of Microscopical Science, Vol. 101, part 3, pp. 241-50, Sept. 1960.]
of gels of gelatin, gelatin and albumin, gelatin and globulin, and gelatin with phospholipid. With the exception of the phospholipid gel, these were prepared as described by Baker (1958). 15 g of gelatin were dissolved in 100 ml of albumin prepared by diluting egg white with twice its volume of water. The preparation was kept at 37°C until all the gelatin had dissolved, when it was strained and cast in 30-grain pessary moulds. The resulting plugs of gel were kept in a refrigerator until required for the experiments. The phospholipid was freshly prepared from homogenized egg-yolk by extraction with an excess of ether. After standing in the cold for 48 h, the supernatant liquid was decanted and the phospholipid precipitated by adding twice the volume of acetone. The resulting white precipitate was washed several times with acetone and stored in the dark under acetone. The gel was prepared by adding to 15% aqueous gelatin enough phospholipid previously dried in a vacuum to give a final concentration of 2.5% lipid in the product. The product was then cast in moulds.

For histochemical and electron microscopical studies, pancreas and liver of CBA mice were chosen as standard experimental material. The tissue was fixed with ice-cold 1% buffered (pH 7.4) osmium tetroxide, or in 10% formaldehyde-saline for purposes of histochemical control. Experimental material was treated with ice-cold 1% buffered (pH 7.4) potassium permanganate (Luft, 1956). Dehydration by passage of the material through graded strengths of alcohol was followed by embedding in butyl methacrylate or in araldite for electron microscopy. The histochemical studies were carried out on material embedded in paraffin or gelatin, and in some cases on 2μm sections cut from the methacrylate blocks. Ultrathin (50 to 100 μm) sections were cut on a Porter-Blum microtome and examined in a modified Siemens Elmiskop I (Meek, 1959), with double condenser (400μ aperture), beam current of 10 μA at 60 kV, and objective aperture of 30μ.

RESULTS

Before attempting to study the effect of permanganate solution on tissues, data were obtained on its action on artificial models of protoplasm. Observations were made on changes in the size and colour of the treated gels; their stability in warm water was also noted.

Volume changes of gels

Fig. 1 shows a series of photographs of plugs of gel, before and after treatment with fixatives. Gelatin-phospholipid gels are shown in fig. 1, A; gelatin-albumin gels in fig. 1, B. It will be observed that in both cases osmium-fixed gels were darkened and shrunken while those treated with permanganate.
Fig. 1

S. BRADBURY and G. A. MEEK
FIG. 2
S. BRADBURY and G. A. MEEK
were darkened and swollen. The formaldehyde-fixed plugs were slightly swollen but not darkened. Similar appearances were obtained with gelatin alone and with gelatin and globulin. Table 1 gives the percentage volume change of gelatin-albumin and gelatin-phospholipid gels; no measurements were made on the other gels.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gelatin-phospholipid</th>
<th>Gelatin-albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMnO₄</td>
<td>+70</td>
<td>+100</td>
</tr>
<tr>
<td>HCHO</td>
<td>+70</td>
<td>+70</td>
</tr>
<tr>
<td>OsO₄</td>
<td>−20</td>
<td>−20</td>
</tr>
</tbody>
</table>

**Stability of gel**

Fig. 1, c shows the stability to warm water of plugs of gelatin-albumin gels after the following treatments: permanganate followed by 70% alcohol; permanganate followed by formaldehyde; permanganate alone; formaldehyde alone. It will be observed that the plug treated with permanganate alone disintegrated completely, whereas further treatment with either alcohol or formaldehyde stabilized the form, although incompletely. Formaldehyde fixation alone stabilized the plug completely. A similar result was obtained with the gelatin gel. With the gelatin-globulin gel, it was found that there was partial stabilization of form towards hot water; again, subsequent treatment with formaldehyde or alcohol increased the stability of form.

When the stability of phospholipid-gelatin plugs to warm water was tested, it was found that the alcohol treatment following permanganate no longer rendered the plug insoluble, and it disintegrated within one hour.

In an attempt to find out whether the dark coloration of the gels was due to the reaction of the permanganate with protein, a 2-6% solution of egg albumin was mixed with an equal volume of permanganate solution. There was an immediate colour change, the purple solution becoming brown and turbid, owing presumably to the formation of a very fine precipitate of manganese dioxide, which remained in suspension for at least 24 h. It had previously been noticed that the addition of alcohol to permanganate-treated tissue caused the brownish colour to darken to black, perhaps by reduction of the excess permanganate in the tissue by the alcohol. In order to find out whether such a reduction could take place, potassium permanganate solution was added to absolute alcohol in a test tube. There was an immediate deposit of a coarse dark-brown precipitate, indicating that these substances do in fact react with each other. Since acetone was found not to reduce permanganate,

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**FIG. 2 (plate)**

A, an electron micrograph of a gelatin-albumin gel. The gel was treated with permanganate and embedded in butyl methacrylate.

B, a light micrograph of a 2µ section of liver embedded in methacrylate after treatment with permanganate; the darkening represents the extent of penetration of the reagent.
the possibility of minimizing such precipitation in the tissues by the use of acetone as a dehydrating agent was considered. When this was tried on gels which had been treated with permanganate and with osmium tetroxide, it was found that they became very hard and much shrunken. A similar result was obtained when the gels were soaked in a solution of permanganate in acetone. There was, however, no gross blackening of the gels, and no reduction of the permanganate remaining in the solution. Tissues treated in the same manner and studied with the electron microscope showed a good preservation of cytoplasmic structure. There appeared to be a little less general precipitation of electron-dense material in the ground cytoplasm, but the effect was not striking. The gross darkening observed with potassium permanganate thus seems to be largely due to the deposition of reduction products of the permanganate, possibly in a granular form. Examination of sections of gels and of tissues treated with permanganate and mounted unstained for examination with the optical microscope failed to reveal any such granular deposit, although their brown colour was very obvious. Pieces of the gelatin-albumin gel treated with permanganate and then prepared for electron microscopy by the standard procedure, when examined at a magnification of ×100,000 were clearly seen to consist of separate granules of electron-dense material, about 50 to 100 Å in diameter (see fig. 2, a). Pieces of a similar gel fixed in osmium tetroxide and examined at the same magnification were found to be structureless.

Penetration of permanganate

Medawar (1941) directed attention to the study of the rate at which fixatives penetrate cells and tissues. Baker (1958) used the coagulation of gels by certain fixatives as an index of penetration and extended Medawar’s work. In our experiments it was found that the limit of penetration of the permanganate could be measured by observing the extent of the darkening which it produced. Observations made at intervals during a period of 64 h were plotted and are shown in fig. 3, together with the rate of penetration of hydrochloric acid for comparison, measured by observing the extent of coagulation. From the results of Medawar, it is apparent that fixatives entering either a tissue or an artificial gel model of protoplasm obey the laws of diffusion; the relationship between distance of penetration and time is given by the expression $d = K\sqrt{t}$, where $d$ is the distance of penetration in a time $t$. $K$ is a constant which has a different value for each fixative fluid. Baker (1958) plotted $d$ against $\sqrt{t}$ and found a linear relationship for most of the fixatives studied. Osmium tetroxide is the only fixative in general use which differs significantly from this; here the fixed gel appears to offer some resistance to the entrance of the fixative, so that although for periods of up to 16 h, $K$ has a value of 1, after this there is a falling off to a value of approximately 0.3.

Our results indicate that the rate of penetration of potassium permanganate is very similar to that of osmium tetroxide. For periods up to 9 h the $K$ value is 1, but after this time the value falls off progressively, to 0.65 at 64 h. It
Electron Microscopy

seems as though a similar barrier exists in the gel to the entrance of fresh permanganate; it may be that deposits of reduction products of the permanganate act as a physical barrier to entrance of the reagent. When a rather homogeneous tissue such as liver is placed in the permanganate, the darken-

![Graph showing the rate of penetration of potassium permanganate and hydrochloric acid into a gelatin-albumin gel.](image)

**Fig. 3.** The rate of penetration of potassium permanganate and hydrochloric acid into a gelatin-albumin gel.

ing produced enables a direct estimate of the distance of penetration to be obtained, as in the case of the gels. Fig. 2, B is a low-power light micrograph of mouse liver soaked in a 1% solution of potassium permanganate for 1 h. It is clear that there has been very even penetration; the tissue subjected to the action of permanganate appears dark, whilst the inside of the block (fixed in effect by the first dehydrating alcohol) appears light in colour. There is a sharp boundary between these zones and it was found that the cells which lie on each side of this ‘penetration boundary’ showed striking variations with histochemical tests to be described. It was also noticed that the cells on the outside of the block were very poorly preserved, showing large cracks and swelling, with gross deformation of the tissue and cell outlines with both light and electron microscopy (fig. 4, B). The optimum fixation was found in the intermediate zone, between the outermost cells and the ‘penetration boundary’.

**Histochemistry**

Before carrying out dehydration for histochemical studies with the light microscope, an attempt was made to remove the excess permanganate from the tissue by leaving the material in gently running water for periods up to
Bradbury and Meek—Permanganate ‘Fixation’ for

12 h. This procedure invariably led to the total disintegration of the tissue, as in the case of gels; passing the tissue directly into the first dehydrating alcohol (70%) preserved the form of the material. It was necessary to change the alcohol at intervals until no further discoloration of the alcohol was seen.

The histochemical tests were directed in the first instance to checking the findings of Luft (1956), who reported that the tissue retains sufficient deoxyribonucleic acid (DNA) after permanganate treatment to give a positive Feulgen reaction, and that the particles of Palade appear to be absent from the membranes of the endoplasmic reticulum, which would imply an absence of cytoplasmic basophilia.

**Nucleus.** The Feulgen reaction was applied to both paraffin sections of permanganate-treated material and also to sections of material embedded in butyl methacrylate which had been soaked for 1 h in ethylene dichloride in order to remove the plastic. Hydrolysis was carried out in N hydrochloric acid at 60° C for a period of 20 min; the sections were then placed in Schiff’s reagent for 90 min. It was found that the nuclei gave a positive reaction; the intensity and coloration were not as great as those in tissues which had been fixed in Zenker’s fluid. It may be that some of the DNA in the nucleus was removed by the permanganate or subsequent treatments, or, if it was preserved, some change had occurred which prevented it from responding so readily to the hydrolysis. Periods of hydrolysis exceeding 20 min were not found to increase coloration. It seems from this that the nuclei do in fact retain a considerable proportion of their DNA after treatment with potassium permanganate. Electron micrographs of permanganate-treated material show decreased electron density in the nuclei compared with material fixed in osmium tetroxide (Barer, Joseph, and Meek, 1959 a, b). It was at first supposed that this difference could be directly attributed to the loss of DNA from the permanganate-treated material, but this does not seem to be borne out by the results of the histochemical studies. Alternatively, permanganate treatment might reduce the basic protein content of the nuclei. The tests for proteins are not very satisfactory; in particular, there do not seem to be any specific tests for the basic proteins—histones and protamines—which form the major protein components of the nucleus. It is well known, however, that the histones are very rich in the amino-acid arginine, for which the Sakaguchi reaction provides a reliable histochemical test (Baker, 1947). When this reaction was applied to osmium-fixed and to permanganate-treated pancreatic exocrine cells, a striking difference was observed in the reactions of the nuclei. In the material fixed in osmium tetroxide, the ground cytoplasm gave a positive reaction, but the nuclear reaction was much stronger. In the permanganate material, on the contrary, only the cytoplasm gave a faint coloration. This suggested that there was in fact some loss of arginine from the permanganate-treated material and that this might account for the observed differences in the electron micrographs. It is necessary to stress the need for caution in interpreting these tests. Since many of these reactions were applied to material fixed in fluids other than those specified by the originators of the
FIG. 4

S. BRADBURY and G. A. MEEK
tests, their value as strict histochemical tests is diminished. Nevertheless, the localization of substances in the exocrine cell of the pancreas corresponds very well in both the osmium-fixed material and in the control tests applied to cells fixed in the correct reagents.

Two other techniques were used in an effort to obtain further evidence concerning the presence or absence of basic proteins after permanganate treatment. The acid ponceau technique (Hyden, 1943), depending upon the ability of the free amino-groups of lysine and arginine to bind this dye in acid solution, gave no clear-cut results when applied either to our permanganate-treated material or to osmium-fixed tissues. The fast green FCF method (Alfert and Geschwind, 1953) was more successful. Although this method does not claim to be a strict histochemical test, it has been shown to colour selectively such compounds as protamine and histone. These authors showed that nucleic acids interfere with selective staining, so that it is necessary to remove them by a preliminary treatment with hot trichloracetic acid before applying the fast green. Fig. 4, a represents a section of pancreas treated with potassium permanganate and stained with fast green FCF. The section passes through the ‘penetration boundary’, so that the tissue at the upper right of the figure was subjected to the action of permanganate whilst that on the left was preserved by the dehydrating alcohol. It can be seen that the only nuclei which react to this stain are in that part of the tissue which was not affected by permanganate. It therefore seems probable that potassium permanganate does not preserve the basic proteins of nuclei; the absence of these compounds from the preparation is almost certainly responsible for the changed appearance of the nuclei in electron micrographs.

The contrast between nucleus and cytoplasm in such preparations is enhanced. This is due first to the reduced electron density of the nuclear contents, which may be explained by the above observations; and secondly to the greatly increased contrast of the bounding membrane which frequently shows well-defined pores.

Cytoplasm. Mitochondria in permanganate-treated material show well-marked cristae, but the mitochondrial matrix, which has considerable electron density when fixed with osmium tetroxide, appears to be almost wholly removed by permanganate. The zymogen granules, which are the most prominent inclusion in osmium-fixed pancreas cells (fig. 5, a), are also not rendered electron dense by permanganate and appear as lighter areas bounded by an imperfect membrane (fig. 6, a). The membranes of the endoplasmic

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**FIG. 4 (plate).**

a, a light micrograph of permanganate-treated pancreas, taken across the ‘penetration boundary’ (dotted line), and stained by Alfert’s method for histones.

b, a low-power electron micrograph of a poorly preserved pancreas cell after permanganate treatment. Swelling and cracking has occurred in the vesicles of the endoplasmic reticulum.

c, a light micrograph of pancreas cells stained with thionine. Cytoplasmic basiphilia appears as dark areas.

d, a light micrograph of pancreas cells treated with permanganate and then stained with thionine; there is no cytoplasmic basiphilia.
reticulum also appear more sharply defined as a consequence of the greater contrast between the paired structures and the ground cytoplasm between them. The distance between the pairs of membranes is significantly greater in permanganate material (figs. 5, B; 6, B) than in osmium-fixed controls.

As reported by Luft, electron micrographs of permanganate-treated material show a complete absence of the ribonucleic acid (RNA) granules, which are such a prominent feature of the endoplasmic reticulum after osmium fixation. Our electron microscope observations confirm this finding (see figs. 5, B; 6, B); as these RNA granules are absent from the cell, the cytoplasm should not show its typical basophilia. In order to check this, sections of both paraffin- and methacrylate-embedded material were coloured with dyes such as basic fuchsine, pyronin, and thionine. After permanganate treatment, there was a total absence of basophilia (fig. 4, D), although pancreas fixed in osmium tetroxide and in formaldehyde as controls showed the typical concentration of basophil substance in the cells (fig. 4, C). The application of the pyronin /methyl green test (Jordan and Baker, 1955), with the ribonuclease control (Bradbury, 1956), shows that this basophilia of the pancreatic exocrine cell may be attributed to the presence of RNA. There was no staining with pyronin/methyl green after permanganate treatment; this result indicates absence of RNA from the cell.

A further characteristic of electron micrographs of permanganate-treated material is the high contrast of the cytoplasmic membrane systems. Since these membranes are generally believed to be composed of phospholipoprotein, it is possible that potassium permanganate acts as an 'unmasking' agent (Bradbury and Clayton, 1958; Clayton, 1959) and reveals both protein (which reduces the permanganate and so increases the electron density) and lipid in those regions of the pancreatic exocrine cell where the endoplasmic reticulum is most highly concentrated. Gelatin sections of pancreas treated with permanganate were tested for lipids by the standard Sudan black technique (Baker, 1949) and for phospholipids by the acid haematein technique (Baker, 1946). The cytoplasm of the basal region of the cell was found to give a strongly positive reaction to both tests; control sections fixed in formaldehyde-calcium showed that this part of the cell does not normally react to either of these tests. Gelatin sections were then made from blocks which had been treated with permanganate and subjected to the solvent action of pyridine at 60° C; this is known to remove all free lipids. After this procedure, the cells no longer gave a positive reaction to these tests.

The disintegration of permanganate-treated tissues on washing with water which has already been described might be caused by the failure of the reagent to stabilize the mucopolysaccharide ground substance of the intercellular

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**Fig. 5 (plate).** Electron micrographs of A, a pancreas cell fixed in osmium tetroxide, embedded in butyl methacrylate. Note the nucleus (n), endoplasmic reticulum (er), mitochondria (m), and zymogen granules (z).

B, the endoplasmic reticulum of the osmium-fixed cell.
FIG. 5
S. BRADBURY and G. A. MEEK
Fig. 6

S. Bradbury and G. A. Meek
connective tissue. Sections were accordingly subjected to the periodic acid/Schiff (PAS) technique, which reveals the sites of mucopolysaccharides. Some sections were treated with sodium iodate as controls in place of the oxidation with periodic acid before the application of Schiff's reagent. When the slides were examined, it was found that there was strong cytoplasmic coloration in both control and test slides. It seems probable that the permanganate oxidizes some component of the cell to aldehydes, which then interfere with the PAS test; it is not therefore possible to form any conclusions as to the presence or absence of carbohydrate material by use of the PAS test after permanganate treatment.

**DISCUSSION**

Several main conclusions emerge from the experiments with artificial gels.

First, treatment with permanganate causes considerable initial swelling, while treatment with osmium tetroxide leads to shrinkage. This may have some bearing on any measurements taken from electron micrographs of materials treated by these techniques.

Secondly, the rate of penetration of permanganate is slow compared with that of other histological fixatives, and the $K$-value falls off with time. The only other common fixative showing a similar behaviour is osmium tetroxide. It is significant that these two substances are the only ones so far discovered which have found widespread use in electron microscopy. The probable reason is that both substances form insoluble electron-dense reduction-products by reaction with tissue constituents, thus giving rise to image contrast by electron scattering. The fall-off in the rate of penetration noticed with these two substances may be directly correlated with the formation of some 'physical barrier' in the form of a particulate deposit in the material, as suggested by Baker (1958). The general granular appearance, so characteristic of material 'fixed' with permanganate and already noted by Luft (1956), is almost certainly due to the deposition of a rather coarse (50 to 100 Å) precipitate in the cell by the reaction of the permanganate solution remaining in the tissues with the first dehydrating alcohol.

Thirdly, none of the gels tested was stabilized in form by permanganate against the action of hot water, although osmium tetroxide treatment both preserves the form of the gel and at the same time renders it insoluble. Since permanganate does not preserve the form of proteins or of phospholipids, it cannot be regarded as a true fixative for these substances. Osmium tetroxide, on the contrary, can justifiably be regarded as a true cytological fixative.

The histochemical studies of tissues 'fixed' with permanganate confirm the

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**Fig. 6** (plate). Electron micrographs of a, a pancreas cell which has been treated with permanganate. Lettering as in fig. 5, a.

b, the endoplasmic reticulum of a permanganate-treated cell, at the same magnification as fig. 5, b. Note that there are no RNA particles adherent to the membranes and that the distance between the membrane pairs is slightly increased.
observations first reported by Luft that the DNA in the nucleus is preserved, whereas the cytoplasmic RNA is absent. Basic proteins in the nucleus are absent and must be presumed to have been leached out by the action of the permanganate solution and the subsequent dehydration and embedding processes. Phospholipoproteins, on the contrary, are 'unmasked' and the phospholipid component may then be revealed in sites which correspond to regions of high content of endoplasmic reticulum as seen in the electron microscope.

It therefore appears that although potassium permanganate is an excellent medium for revealing and studying membrane structures within the cell, it is not in fact a true fixative. The actual fixation of the tissue is performed by the subsequent treatment with alcohol.

We wish to express our sincere appreciation to Dr. R. Barer for invaluable discussions throughout this work. We also wish to acknowledge the generous loan by the Wellcome Trust of a Siemens Elmiskop I electron microscope and accessory equipment.

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