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An enzyme histochemical study of mesothelial cells in rodents

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INTRODUCTION

Few studies on the enzyme histochemistry of peritoneal mesothelium have been published. Savinovskaya (1957, 1963) has demonstrated the presence of acid phosphatase and alkaline phosphatase in the peritoneal mesothelial cells of the rabbit, and Shanthaveerappa & Bourne (1965) have reported a positive reaction for a variety of enzymes in the mesothelial cells of the mesentery of the cat. The problem involved in the study of mesothelial cells is that too little cytoplasmic detail of such flattened cells is apparent in cross-section. This may be overcome by studying whole mounts of mesentery or by using Häutchen preparations (Eskeland, 1966; Raftery, 1973), in which a single layer of mesothelial cells is obtained on a celloidin film which can subsequently be viewed *en face*. The preparation involved in the latter method, however, renders the cells unsatisfactory for the techniques of enzyme histochemistry. Cheng & Berry (1972) have devised a rapid method for obtaining a monolayer of mesothelial cells which can be viewed *en face* in the same way as Häutchen preparations, thus allowing more adequate inspection of cytoplasmic detail. Their method involves coating glass slides with gelatin, which is allowed to set; a peritoneal-covered viscus is then applied to the slide and subsequently peeled off, leaving behind a single layer of mesothelial cells on the gelatin film. The authors described a number of techniques which could be used in conjunction with this method, but the method is not suitable for enzyme histochemistry, since when the slides are incubated at 37 °C the gelatin softens and the cells become detached. This paper describes a method of obtaining a single layer of mesothelial cells on an agar film (agar–Häutchen preparation), which can subsequently be submitted to the techniques of enzyme histochemistry, and reports the observations obtained by the application of this method to the study of parietal and visceral mesothelium in rodents.

MATERIALS AND METHODS

Adult male rats, guinea-pigs, mice and rabbits were used, and the animals were killed by exsanguination under ether anaesthesia. The procedure for obtaining a monolayer of mesothelial cells was as follows. A 3 % solution of agar was prepared by dissolving 3 g of agar in 100 ml of distilled water in a beaker in a boiling water bath. A few drops of the solution were then pipetted on to warmed slides on a hot plate at 50 °C and allowed to spread evenly. Any excess agar was drained off and the slides were dried overnight, leaving them coated with a thin film of agar. To test the ability of the film to remove a single layer of cells, the slides covered with the

film were applied to areas of peritoneum. Areas of parietal and visceral peritoneum were excised, rinsed briefly in 5 % glucose to remove any adherent cells derived from the peritoneal fluid, dipped in 0·25 % silver nitrate for 30 seconds, and rinsed again in 5 % glucose; the excess glucose was allowed to drain off the peritoneal surface. An agar-coated slide was then gently applied to the peritoneal surface and maintained in contact for about 10 seconds, after which it was gently removed. The preparations were then fixed either in absolute methanol for 10 minutes or in neutral 4 % formaldehyde-saline for ½–12 hours. After this they were stained with Harris's haematoxylin, and either dehydrated and mounted in Canada balsam or mounted directly in glycerin jelly. Fig. 1 is a photomicrograph of such a preparation. It shows a single layer of mesothelial cells with the intercellular substance demonstrated by silver lines; in this respect the appearance does not differ from a normal Häutchen preparation using a celloidin film. Nuclear staining, however, is not so sharp as that obtained in a normal Häutchen preparation, and under high power magnification (× 40 objective) the preparations appear slightly cloudy, because of some opacity in the moist agar film. This was more marked in specimens mounted in glycerin jelly. Having verified that a single layer of mesothelial cells could be obtained by this method it was subsequently used in conjunction with enzyme histochemistry. The specimens obtained by this method will be referred to in the text as agar–Häutchen preparations.

For enzyme histochemistry monolayers of mesothelial cells were obtained from the following; the parietal peritoneum of the abdominal wall and diaphragm; the visceral peritoneum of the stomach, liver and caecum; and the mesentery. The peritoneal surface was rinsed in ice-cold (4 °C) 5 % glucose prior to the application of the agar-coated slides. The agar–Häutchen preparations were then fixed for ½–12 hours in neutral 4 % formaldehyde-saline at 4 °C. Specimens of parietal peritoneum of the anterior abdominal wall and visceral peritoneum of the liver together with a piece of underlying tissue were removed and fixed in neutral 4 % formaldehyde-saline at 4 °C for 12–18 hours. Subsequently sections 12–20 μm thick were cut perpendicular to the peritoneal surface in a cryostat at a temperature of −25 °C.

Fig. 1. Parietal mesothelium. Anterior abdominal wall. Rat. Note the single layer of mesothelial cells with the intercellular substance impregnated with silver. Haematoxylin. Agar–Häutchen.

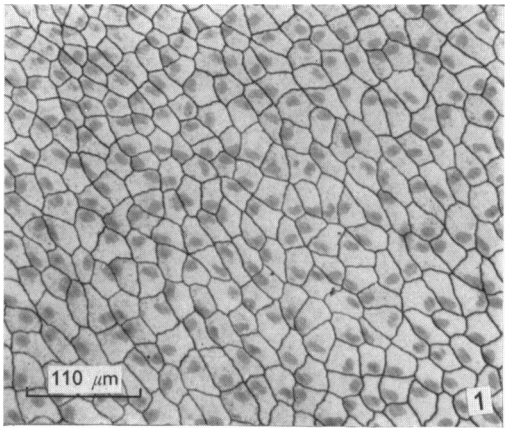
Fig. 2. Parietal mesothelium. Anterior abdominal wall. Rat. Acid phosphatase. Note the punctate deposits of enzyme activity chiefly in the perinuclear area of cytoplasm. Note also the positive reaction in the nuclei. Agar–Häutchen. Incubation time 1 hour.

Fig. 3. Visceral mesothelium. Liver capsule. Rat. Acid phosphatase. No cytoplasmic detail is seen in the mesothelial cells. 15 μm cryostat section. Incubation time 1 hour.

Fig. 4. Visceral mesothelium. Stomach. Rabbit. Acid phosphatase. Note the strongly positive reaction in the perinuclear area of cytoplasm. Agar–Häutchen. Incubation time 1 hour.

Fig. 5. Visceral mesothelium. Liver. Rat. ATPase. Note the weak positive reaction in most cells. Enzyme activity is strongest in the perinuclear cytoplasm. Agar–Häutchen. Incubation time 1½ hours.

Fig. 6. Parietal mesothelium. Anterior abdominal wall. Rat. ATPase. In the majority of cells the reaction is strongest at the junctional region between adjacent cells. Agar–Häutchen. Incubation time 1½ hours.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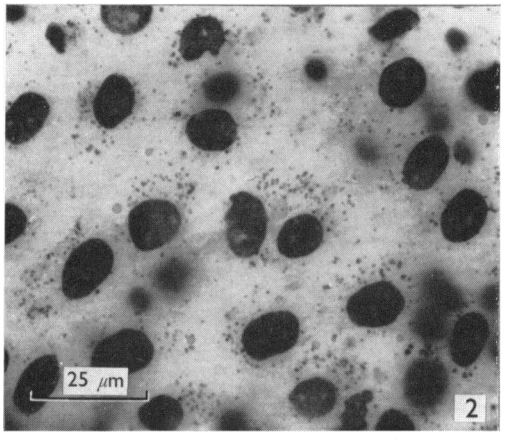
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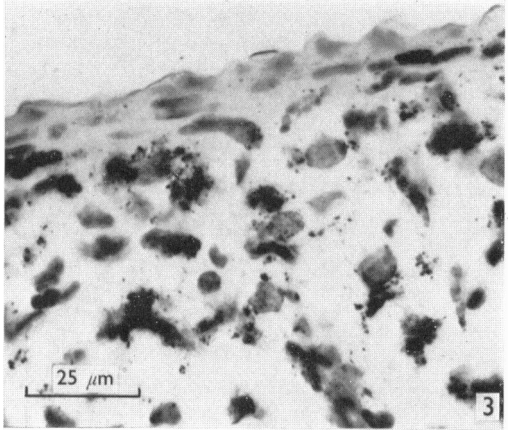
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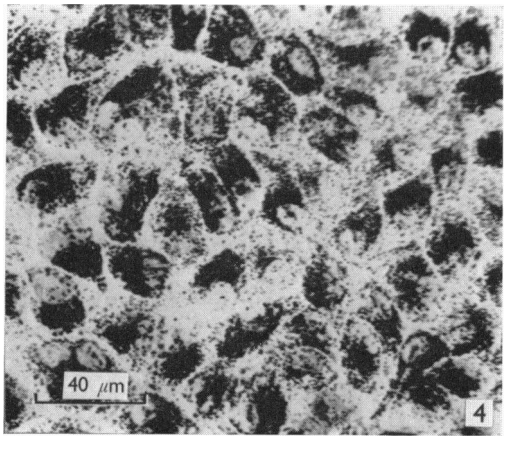
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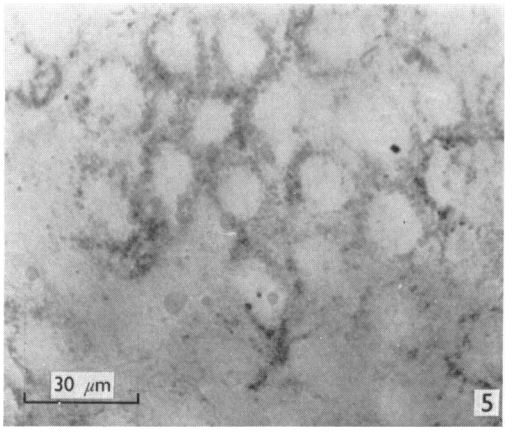
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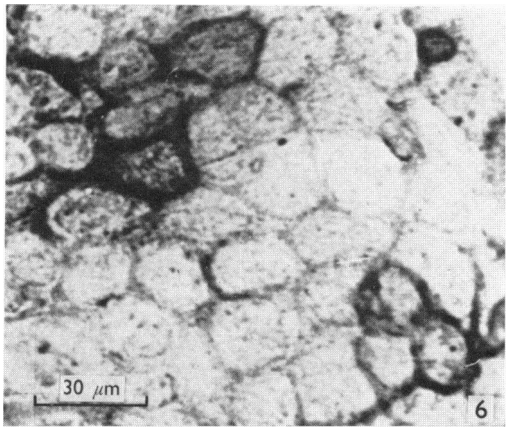
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The following enzymes were investigated:

Acid phosphatase: by the method of Gomori (Pearse, 1960). Controls were incubated in media lacking the substrate and in media containing 0·01 M sodium fluoride as inhibitor.

Alkaline phosphatase: by a coupling azo-dye method, using the stable diazotate of 5-chloro-*o*-toluidine (Pearse, 1960). Controls were incubated in media lacking the substrate.

Adenosine triphosphatase (ATPase): by the method of Wachstein & Meisel (1957). Controls were incubated in media lacking the substrate.

Non-specific esterase (NSE): using α-naphthyl acetate and *O*-acetyl-5-bromoindoxyl as substrates. Controls were incubated in media lacking the substrate. Some preparations were incubated in the presence of 10^{-5} M eserine to inhibit cholinesterase.

Cytochrome oxidase: by the *N*-phenyl paraphenylene diamine method of Burstone (Pearse, 1960). Some specimens were incubated in the presence of 0·0005 M sodium azide as inhibitor.

Succinate dehydrogenase: by the method of Nachlas *et al.* (1957). Controls were incubated in media lacking the substrate.

RESULTS

The findings will be described collectively, since for the most part there was little difference between the enzyme histochemical characteristics of mesothelial cells obtained from different sites, whether parietal or visceral, in the same species of animal.

Acid phosphatase

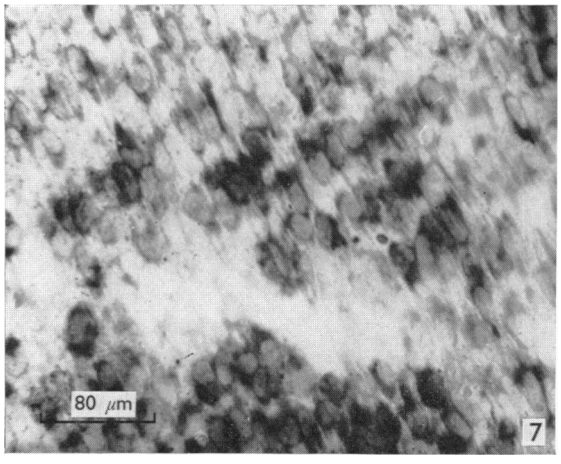
The mesothelial cells of all four species examined gave a positive reaction for acid phosphatase. This was seen as punctate deposits of enzyme activity, mainly in the perinuclear area of the cytoplasm (Fig. 2). Occasionally marked nuclear enzyme activity was seen. In sections cut perpendicular to the peritoneal surface it was not possible to detect any detail in the cytoplasm of the mesothelial cells (Fig. 3). The reaction was more strongly positive in some cells than in others, and this was particularly apparent in the mesothelial cells covering the stomach of the rabbit (Fig. 4). Control specimens incubated in media lacking the substrate were negative, and those incubated in media containing 0·01 M sodium fluoride were either negative or showed a greatly reduced enzyme activity.

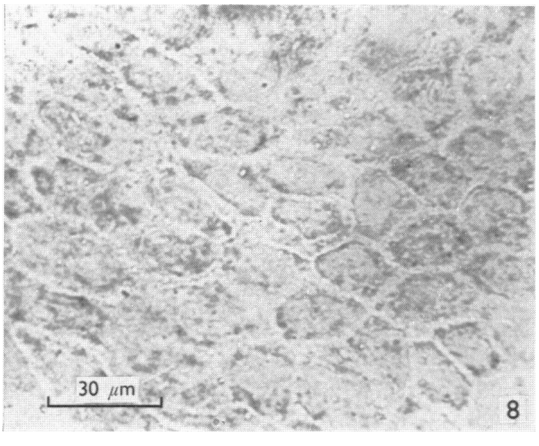
Alkaline phosphatase

Specimens incubated in media for demonstrating alkaline phosphatase were negative in all four species examined.

Adenosine triphosphatase

The majority of mesothelial cells gave a weak positive reaction (Fig. 5), but in some it was stronger. In some cells enzyme activity was most prominent in the perinuclear region of the cell, while in others a strong reaction was seen at the





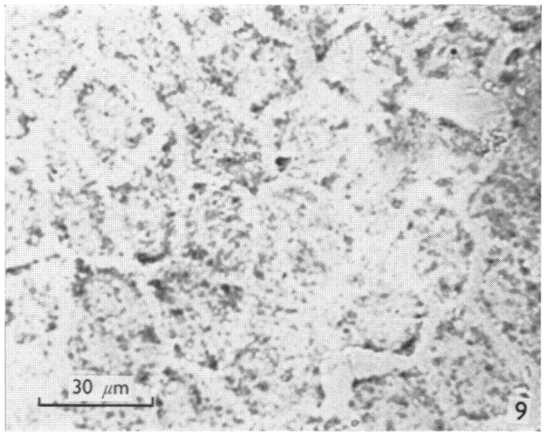


Fig. 7. Parietal mesothelium. Anterior abdominal wall. Mouse. NSE (substrate – α-naphthyl acetate). Some cells are strongly positive, others weakly so. Agar–Häutchen. Incubation time 10 min.

Fig. 8. Parietal mesothelium. Anterior abdominal wall. Rat. Cytochrome oxidase. Note the weak reaction in all cells. Agar–Häutchen. Incubation time 1½ hours.

Fig. 9. Parietal mesothelium. Anterior abdominal wall. Rat. Succinate dehydrogenase. Note the weak reaction in all cells. Agar–Häutchen. Incubation time ½ hour.

junctional region between adjacent cells (Fig. 6). The pattern was similar in all four species examined. Sections incubated in the absence of substrate were negative.

Non-specific esterase

There were species differences in the distribution of this enzyme. The mesothelial cells of the mouse and rabbit gave a positive reaction, but the degree of enzyme activity varied from cell to cell (Fig. 7), being strong in some but weak in others. Sections incubated in media lacking the substrate were negative. Sections incubated in the presence of 10^{-5} M eserine showed no reduction in enzyme activity, demonstrating

that NSE, and not cholinesterase, was responsible for the positive reaction. The mesothelial cells of the rat and guinea-pig failed to give a positive reaction.

Cytochrome oxidase

A weak positive reaction occurred in the cytoplasm of the mesothelial cells of all four species examined (Fig. 8). Sections incubated in the presence of 0·005 M sodium azide were negative.

Succinate dehydrogenase

A weak positive reaction occurred in the cytoplasm of the mesothelial cells of all four species examined (Fig. 9). Sections incubated in the absence of substrate were negative.

Examination of agar–Häutchen preparations, in which the mesothelial cells were viewed *en face*, was found to be the only adequate method of gaining information on the distribution of enzymes in mesothelial cells. Examination of cryostat sections cut perpendicular to the peritoneal surface yielded no useful information, since cytoplasmic detail could not be seen in the flattened cells.

DISCUSSION

The findings reported in the present study are in some respects at variance with the findings of other investigators. Savinovskaya (1957) reported that very little acid phosphatase was present in the cytoplasm of the mesothelial cells of the parietal peritoneum of the rabbit. Mohr, Beneke, Paulini & Krutzsch (1972) reported a negative reaction for acid phosphatase in the mesothelial cells of the parietal peritoneum of rats, but showed that, following intraperitoneal injection of phytohaemagglutinin, acid phosphatase activity could then be demonstrated in these cells. They concluded that the findings may indicate the participation of mesothelial cells in phagocytosis. In the present study acid phosphatase activity was found in the cytoplasm of the mesothelial cells of all species examined. The sites of activity were demonstrated as punctate deposits, chiefly in the perinuclear cytoplasm. Enzyme activity was more strongly positive in some cells than in others and the reaction was particularly strong in the visceral mesothelium covering the stomach of the rabbit. The finding of acid phosphatase in mesothelial cells correlates well with the electron microscopical findings of lysosomes and multivesicular bodies in such cells (Baradi & Hope, 1964; Kluge & Hovig, 1967; Cotran & Karnovsky, 1968; Raftery, unpublished observations) since these organelles are known to be associated with acid phosphatase (Novikoff, 1961; Gordon, Miller & Bensch, 1965; de Duve & Wattiaux, 1966). It is interesting that, following exposure of peritoneal mesothelium to horseradish peroxidase, the peroxidase was seen in large vacuoles and multivesicular bodies by Cotran & Karnovsky (1968). They pointed out that this uptake of horseradish peroxidase was similar to that occurring in other protein-absorbing cells such as renal tubular epithelium, and, since dense bodies, vacuoles and multivesicular bodies were seen in normal mesothelium, they suggested that uptake of protein by mesothelial cells may occur constantly under normal conditions. The finding of acid phosphatase in mesothelial cells is probably associated with a phagocytic function.

The absence of alkaline phosphatase from the cytoplasm of the mesothelial cells

is in agreement with the finding of Savinovskaya (1963), who reported the absence of alkaline phosphatase in the mesothelial cells of the parietal peritoneum of the rabbit. Shanthaveerappa & Bourne (1965) reported a positive reaction for alkaline phosphatase in the mesothelial cells of the mesentery of the cat. This probably reflects species differences.

ATPase activity has been implicated in transport mechanisms, and may be concerned with the active transport of materials across the mesothelium. The presence of a strong reaction at the junctional region between adjacent cells is in agreement with the finding reported for the mesothelial cells of the mesentery of the cat (Shanthaveerappa & Bourne, 1965). ATPase activity has been found to be associated with the plasma membrane of a variety of cell types (see North, 1966). The concentration of ATPase activity in the area of the junctional regions between adjacent cells is interesting. It has been shown that junctions between mesothelial cells are often tortuous and cytoplasmic processes have been shown to project into the intercellular spaces, some processes having the typical appearance of microvilli (Baradi & Hope, 1964; Kluge & Hovig, 1967; Cotran & Karnovsky, 1968). If ATPase activity is associated with the plasma membrane of the numerous cytoplasmic processes of mesothelial cells at these junctions it might explain why the ATPase activity is strongest at the junctional region between cells. It is also worth noting that transport across the mesothelial membrane may occur, in part, through intercellular clefts (Cotran & Karnovsky, 1968). It is possible that ATPase activity associated with the junctional region between cells is responsible for active transport of materials past foci of constriction, such as tight junctions, along the intercellular cleft by a process of focal vesicular transport around the constriction. Such a mechanism has been demonstrated in corneal endothelium (Kaye & Pappas, 1962; Kaye, Pappas, Donn & Mallett, 1962), and it is possible that the energy released by the enzymatic breakdown of ATP is responsible for the process.

The function of non-specific esterases is obscure, but it has been suggested that they play a role in protein metabolism (Myers, Tol & de Jonge, 1957). This may be related either to addition of protein to, or to removal of protein from, peritoneal fluid. The absence of non-specific esterase from the mesothelium of the rat and guinea-pig may be a reflexion of differences in the protein composition of peritoneal fluid in different species.

The positive reaction for the mitochondria-associated enzymes, succinate dehydrogenase and cytochrome oxidase indicates an oxidative metabolism within the cells, and this may be related to the furnishing of energy for active transport of materials across the mesothelial cell. The previously held view that the mesothelium is a passive membrane is now open to some dispute. Cascarano, Rubin, Chick & Zweifach (1964) have shown that mesothelium exerts an appreciable control on the passage of certain solutes across it, and their findings further suggest that oxidative metabolism and ATP formation are intimately linked with the permeability of the mesothelial membrane. The differential permeability of mesothelium to rubidium and phosphate in response to certain pharmacological agents (Berndt & Gosselin, 1962) and the effect of vasopressin on sodium flux (Sheer, Harvey & Barry, 1966), also suggest a more complex transport system. Certainly the enzyme content of mesothelium reported here is an indication that it does not play merely a passive role.

SUMMARY

The enzyme histochemistry of parietal and visceral mesothelium has been investigated using monolayers of mesothelial cells on an agar film (agar–Häutchen preparation). The mesothelial cells of four species (rats, guinea-pigs, mice and rabbits) gave a positive reaction for acid phosphatase, adenosine triphosphatase, cytochrome oxidase and succinate dehydrogenase. Specimens incubated in media for demonstrating alkaline phosphatase were negative in all four species. The mesothelial cells of the mouse and rabbit gave a positive reaction for non-specific esterase, but those of the guinea-pig and rat failed to do so. The findings are discussed in relation to the facts known of the ultrastructure and physiology of mesothelium, and it is concluded that the enzyme content of mesothelium suggests that it is not merely an inert, passive membrane.

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