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Endogenous peroxidase in mast cells localized with a semipermeable membrane technique

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Synopsis. Hamster mast cells have been found to give strong peroxidatic reactions at pH 5, 7.5 and 10 when sections of skeletal muscle are incubated for 2.5 h in the dark at room temperature on semipermeable membranes covering a gelled incubation medium consisting of 0.01% hydrogen peroxide, 5.5 mM diaminobenzidine and 1.36% agar dissolved in Universal buffer. The technique is very efficient: with it, all mast cells react in marked contrast to the negative reaction they usually give with conventional techniques.

The peroxidatic reactions are abolished if tissues are perfused beforehand with either aminotriazole or KCN but not if these inhibitors are incorporated in the gelled incubation medium. This and other evidence suggests that the mast cell reactions are not due to either catalase or haemoglobin adsorbed onto mast cell granules from lysed red blood cells.

Skeletal muscle fibres do not exhibit any visible peroxidase activity with the membrane technique.

Introduction

When cells consume oxygen, they often generate potentially toxic hydrogen peroxide. Consequently they usually contain peroxidases of some sort (including catalase) in order to destroy the peroxide. Normally these protective enzymes can be visualized at both the light and electron microscope levels without much difficulty but, except for some recent reports (Hand, 1974; Somer & Waugh, 1976; Christie & Stoward, 1977*a*, *b*), there is little cytochemical evidence for their presence in skeletal muscle fibres. However, the failure of most previous investigators to find peroxidases in muscle cells may have been due to the fact that fixed tissue was always used so that the peroxidases may have been inhibited. Therefore, we thought it would be worthwhile trying to demonstrate peroxidases in unfixed muscle using a semipermeable membrane method similar in principle to the membrane techniques devised by Meijer and his colleagues in Amsterdam and Lojda's group in Prague for the localization of hydrolases and dehydrogenases (see Lojda *et al.*, 1976).

During the development of this technique, we observed that mast cells give a very strong peroxidase reaction – in marked contrast to the variable, usually negative, reaction they give with more conventional techniques (see Selye for references prior to 1965; and Okun *et al.*, 1971, and Desaga, 1972, for more recent work). In this paper, the membrane technique and the perioxidatic reactions of mast cells obtained with it are reported in some detail.

Material and methods

Tissue

Normal adult Syrian hamsters of both sexes were killed by stunning and exsanguination. Their gastrocnemius, soleus and tongue were removed quickly and either fixed or quenched immediately without fixation in melting isopentane cooled with liquid nitrogen. For fixation, pieces of tissue, not exceeding 2 mm in one dimension, were immersed for 2 h at 4°C in either 0.75 or 1.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.3, or in 1 or 2% paraformaldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.3. After fixation, the blocks were rinsed in the appropriate fixative-free buffer for approximately 30 min at 4°C, and then quenched in melting isopentane. The quenched unfixed and fixed tissues were sectioned at 10 μm at −18°C in a cryostat.

Localization of peroxidase activity

Initially attempts were made to localize peroxidase activity in mast cells by incubating fixed and unfixed sections mounted on coverslips in several conventional diaminobenzidine (DAB)–H_2O_2 media such as those used for demonstrating the enzyme in various tissues by, for example, Novikoff *et al.* (1971).

However, it was found that the enzyme could be visualized successfully with the following semipermeable membrane technique. 10 mg DAB–hydrochloride (Sigma, London) was dissolved in 4.9 ml hot (about 70°C) 1.36% agar (Ionagar No. 2 grade from Oxoid Ltd, London) in Britton–Robinson Universal buffer of pH 7.5 (BDH Ltd, Poole, Dorset). 0.1 ml 0.5% H_2O_2 was then added to the hot mixture. Whilst still hot, this medium was poured into Perspex cylinders closed at one end with washed Visking dialysis tubing as described previously by Meijer and Lojda and their colleagues for demonstrating acid phosphatase and other enzymes (Meijer, 1972; Lojda *et al.*, 1976). The gels were allowed to cool at room temperature in the dark and were used within 30 min of preparation. The final concentrations of DAB and H_2O_2 in the gels were 5.5 mM and 0.01% respectively. Gels of pH 5 and 10 were also tried.

Cryostat sections were transferred to the wiped, moisture-free surface of the Visking membrane in the same way that they are collected onto coverslips, and were incubated for 2.5 h at room temperature in the dark. After incubation, the sections with their underlying membrane were removed from the gels, and dehydrated through the ethanols and mounted in DPX. It was not found necessary to fix them after incubation.

Inhibitors

In preliminary experiments, 3-amino-1,2,4-triazole or potassium cyanide were incorporated into the gel media (final concentrations 20 and 5 mM respectively) in an

attempt to inhibit peroxidatic activity. However, as they appeared to have no effect, they were instead perfused through unfixed tissue as follows. 20 mM aminotriazole or 5 mM KCN, made isotonic with NaCl and warmed to 38°C, were perfused through the aorta via the left ventricle of animals lethally anaesthetized with Euthatal, the returning blood and perfusate escaping through the incised right atrium. Perfusion was continued for approximately 6 min, after which the gastrocnemius and the tongue were removed, sliced, and stored (for up to about 20 min) in the appropriate perfusion solution until they could be quenched and sectioned.

Cell counts

In order to assess the proportion of mast cells exhibiting peroxidatic activity, the number of positively reacting cells were counted in 20 consecutive sections of tongue stained alternatively for peroxidase and with 0.05% Thionine.

Peroxidatic activity of haemoglobin

The optimum pH of peroxidation of hamster haemoglobin *in vitro* was determined in order to assess its possible contribution to the mast cell granule peroxidase reaction following lysis of red blood cells on the membrane. Samples of hamster blood were spun in a micro-haematocrit and the buffy coats removed. The red cells were washed in several changes of normal saline, lysed in a few drops of distilled water and spun down to remove their membranes. The supernatants were decanted, diluted (1:200) with distilled water, and assayed as described by Herzog & Fahimi (1973) using Britton–Robinson Universal buffer (BDH) over the pH range 3.8–10.0.

Results

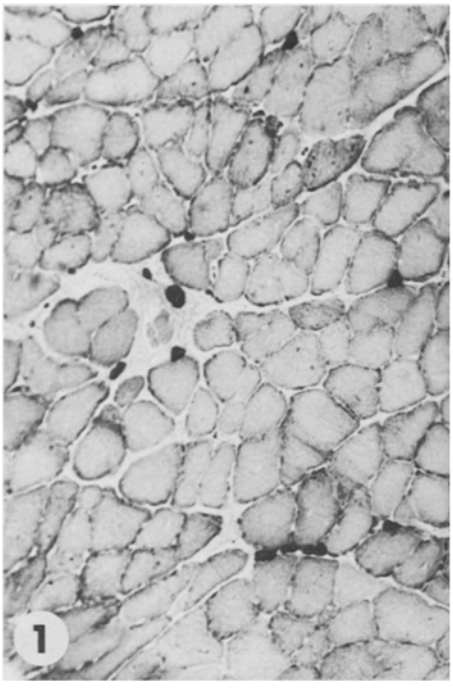
Peroxidase localization

Mast cells were found to be relatively prolific in hamster soleus, gastrocnemius and tongue, as judged by their morphology and metachromatic staining towards Thionine. Those situated between muscle fibres were usually elongate or spindle-shaped, but those associated with blood vessels or loose connective tissue appeared to have the usual shape with an oval or rounded cytoplasm.

They did not exhibit any peroxidase activity when fresh or fixed sections were incubated in the normal way in an aqueous DAB–H_2O_2 media. However, they showed a strong activity in sections incubated on semipermeable membranes mounted on a gelled medium of pH 7.5. The activity appeared to be confined to the mast cell granules (Figs. 1 & 2), but since the granules filled most of the cytoplasm, it was not possible to observe whether other sites in the cell were also reactive.

All the mast cells present in a section appeared to give a positive peroxidase reaction with the membrane technique, as indicated by the non-significant difference shown by Student's *t*-test (one-sample) between the counts of peroxidase-positive cells (190.0 ± 6.2/section) and metachromatic cells (183.4 ± 10.6/section) in 20 alternate consecutive sections of tongue.

Slightly less activity was evident with the pH 5 and 10 gels. Muscle fibres did not exhibit any specific peroxidatic activity with any of the gel media tested. They showed



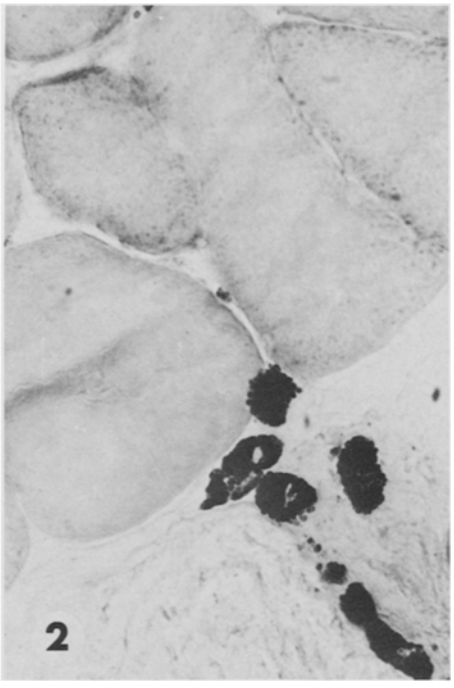


Figure 1. Hamster soleus showing peroxidase-reactive mast cells lying between the muscle fibres. x 100
Figure 2. Peroxidase-reactive mast cells in connective tissue of hamster gastrocnemius. Note the granular reaction. x 400

only the non-specific pale brown colour normally associated with 'background' staining of DAB techniques.

Effect of fixation
Fixation in either glutaraldehyde or formaldehyde abolished the mast cell peroxidase activity, even when the concentrations of the fixative were at the comparatively low levels of 0.75 and 1% respectively. Prolonged washing after fixation did not restore any activity. In contrast, red blood cells showed a strong peroxidatic reactivity in fixed sections, but no reactivity in unfixed sections. In the latter, the intima of blood vessels frequently appeared to have a strong peroxidatic reactivity, which we attribute to adsorbed haemoglobin released from erythrocytes.

Effect of inhibitors
Inclusion of 5–50 mM KCN or 20 mM aminotriazole in the gel media did not visibly affect the peroxidase reactivity of mast cells. However, the reactivity was substantially reduced when tissues were perfused with 20 mM aminotriazole before being subjected to the normal membrane technique (Fig. 3), especially in cells closest to blood vessels where it was often abolished altogether. Some reactivity usually remained in cells

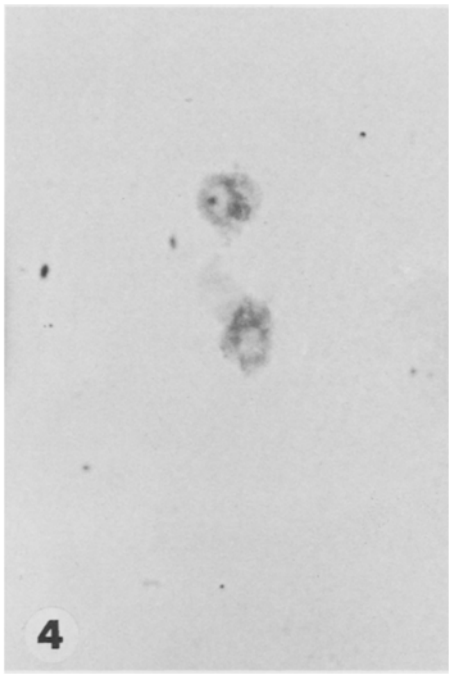


Figure 3. Mast cells from hamster tongue reacted for peroxidase after perfusion with 20 mM aminotriazole. Note the considerable reduction in the amount of reaction product. x 400
Figure 4. As Fig. 3 except that the animal was perfused with 5 mM KCN. Again note the reduction of reaction product. x 400

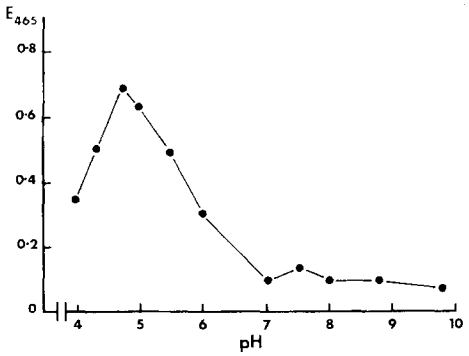


Figure 5. The relative peroxidatic activity of hamster haemoglobin *in vitro* as a function of pH. Reaction conditions as stated in 'materials and methods'. Under these conditions, the maximum activity occurs at about pH 5. In contrast, there is little or no activity at pH 7 and above.

further away from blood vessesls. Perfusion with 5 mM KCN had a similar gradient effect but was much more inhibitory than aminotriazole (Fig. 4).

Haemoglobin

The maximum peroxidatic activity of a suspension of hamster erythrocyte haemoglobin, under the conditions of the assay used, occurred at about pH 5.0 (Fig. 5). Its activity at pH 7 and above was negligible.

Discussion

Peroxidase is not demonstrable, as was originally hoped, within skeletal muscle fibres using the membrane technique described in this paper. This negative finding does not necessarily conflict with our previous report that a peroxidase-like activity can be localized at the ultrastructural level with a more conventional method in the terminal cisternae of the sarcoplasmic reticulum (Christie & Stoward, 1977*a, b*), since such structures are probably beyond the limit of resolution of the light microscope.

However, the technique does reveal that mast cells, in the hamster at least, possess a strong peroxidase activity which is extremely sensitive towards glutaraldehyde and formaldehyde. The enzyme's sensitivity to these fixatives may explain why it has rarely been observed before with 'conventional' techniques. Fixatives markedly inhibit the activity of peroxidases in other cells (e.g. in the lacrimal gland) (Herzog & Fahimi, 1976) but not, it seems, to quite the same extent as mast cell peroxidase.

With the membrane technique, mast cell peroxidase appears to be situated mostly in the granules, but as the granules occupy most of the cytoplasm we were unable, in our light microscope study, to see whether peroxidase activity is present in other sites in the cell as well. In contrast, in an electron microscopic investigation in which the mast cells were fixed with glutaraldehyde and subjected to conventional techniques for localizing peroxidase, Robbins *et al.* (1971) found that the granules were unreactive whereas the nuclear envelope and endoplasmic reticulum showed a moderately strong peroxidase activity. The activity evident in the latter sites is possibly artifactual, arising from the adsorption of peroxidase released from the easily ruptured granules. Comparable artifacts occur in other cells after 'staining' with conventional techniques. Thus according to Fahimi (1973), the peroxidase activity localized in the endoplasmic reticulum and ribosomes of hepatocytes can be atrributed to the diffusion of catalase out of peroxisomes when tissues are washed in buffer after fixation in glutaraldehyde in the first stage of such techniques. Theoretically the peroxidatic reactions demonstrable in mast cell granules with the membrane technique could be due to either: (a) haemoglobin adsorbed onto the granules from lysed red blood cells; (b) myoglobin taken up from 'red' muscle fibres; (c) catalase; (d) dopa oxidase; (e) tyrosinase; or (f) a true peroxidase.

Haemoglobin is probably not responsible for the peroxidatic reactions for at least three reasons. First, in our sections, mast cells in dense connective tissue in regions relatively far removed from a good blood supply give as strong a peroxidatic reaction as that in mast cells close to blood vessels. Second, whereas haemoglobin *in vitro* shows its maximum peroxidation activity at pH 4.8 and virtually no activity at pH 7 and above (Fig. 5), mast cells exhibit their strongest activity at a neutral pH and are

still active at pH 10. And third, the peroxidatic reactions of mast cells are inhibited by low concentrations (5 mM) of perfused KCN, unlike haemoglobin in erythrocytes which requires much higher concentrations, of the order of 100 mM, for its peroxidatic activity to be quenched (Dvorak *et al.*, 1972).

At present we have no firm evidence one way or the other that myoglobin might contribute to the peroxidase activity of mast cells; we are currently investigating this point.

If catalase was responsible for the peroxidatic reactions of mast cells, the third possibility (c) put forward earlier would imply that mast cell granules were equivalent to peroxisomes. This seems unlikely for several reasons. For example, the peroxidatic activity of their catalase content would have been activated by 3% glutaraldehyde (Herzog & Fahimi, 1976), not inhibited as we observed. Further, they would have shown their strongest peroxidatic activity at about pH 10 and no activity below about pH 6, whereas we found that the peroxidatic activity of the granules was in fact more or less the same at pH 5, 7.5 and 10.

The arguments as to whether tyrosinase (d) or dopa oxidase (e) are implicated in the peroxidatic reactions of mast cells, particularly in melanogenesis, have been debated many times in the literature (Okun *et al.*, 1973). They will not be rehearsed again here as our data do not help in resolving the debate.

Thus by elimination, the most probable candidate for the peroxidatic reactions of mast cells in the membrane technique is a peroxidase itself, our last option (e) because: (1) no reactions occur unless H_2O_2 is included in the gel medium; (2) the reactions take place over a wide pH range (cf. Fig. 3 in Herzog & Fahimi, 1976); and (3) the reactions are abolished if tissues are perfused, before incubation, with either 20 mM aminotriazole or 5 mM KCN, both generally considered to be inhibitors of endogenous peroxidases and catalase. The inhibitor evidence, however, is not wholly conclusive since aminotriazole and KCN do not inhibit peroxidases in all situations, for example myeloperoxidase in leucocytes (Rechcigl & Evans, 1963) and peroxidase in Kupffer cells (Fahimi, 1970).

In contrast to its effect in perfusates, neither aminotriazole nor KCN inhibits the peroxidatic reactions of mast cells when it is incorporated into the membrane-covered gelled incubation medium. It is difficult to suggest any convincing explanation for the discrepancy. Nevertheless, other instances are known where specific inhibitors do not exert their expected effects in the membrane technique (see e.g. Meijer & de Vries, 1974).

An interesting feature of the peroxidase membrane technique is its efficiency: with it, all hamster mast cells react. With other methods, only a few do. For example, in the dopa–melanin technique (which is assumed to demonstrate a peroxidase), less than 3% of rat mast cells give a positive reaction, but if the cells are disrupted first, 75–95% react (Okun *et al.*, 1971). This suggests that in the membrane technique the mast cells may undergo a limited lysis. On the other hand, Okun *et al.* (1971) have postulated that the peroxidase in mast cell granules exhibits 'latency' in the same way that acid phosphatase and other acid hydrolases do in lysosomes. However, so far we have been unable to observe any latency, that is, a delay in the reactivity of some cells, in the membrane technique.

At this stage it is not known what the physiological roles of the peroxidase in mast

cells are, or why it should be so abundant. It is hard to believe that it functions solely as a mediator in the conversion of tyrosine to melanin as Okun's group have suggested on several occasions (see Okun *et al.*, 1973), or in the synthesis of catecholamines (Okun *et al.*, 1971), when other cells are much more efficient and available for these tasks. The riddle of the mast cells thus persists.

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