CHAPTER 7

HISTOCHEMICAL DEMONSTRATION OF MUSCLE LIPASE

Lipase, which catalyzes the hydrolysis of fat into its component fatty acids and glycerol, was demonstrated histochemically by Gomori (1953) using Tween 80 as substrate. In this method, the liberated fatty acids are made to react with calcium chloride for the formation of a calcium soap, which is converted to a lead soap and finally demonstrated as brownish black precipitates of lead sulphide. In this chapter are reported the results of an attempt to demonstrate lipase activity by the conversion of the calcium soap into a coloured calcium compound.

Sodium alizarin sulphonate has been widely used as a reagent for calcium and is the basis of a variety of techniques for staining bone in whole specimens (Hood and Neill, 1948; Sedra, 1950; Edwards, 1953). Crosti (1960) has studied the early presence of calcium as bone salts in the forelimbs of chick embryos employing the alizarin red S technique. Williams (1946) made use of alizarin red S for differentiating various scale types <u>in situ</u> in pieces of skin. Similar studies were carried out by Dietrich (1953) on the histological development of the scales of the bass. Cameron (1930) was the first to investigate histochemical techniques for calcium on gelatin models using anthraquinone dyes, and concluded that alizarin red S is a specific reagent for calcium. Dahl (1952)

developed an alizarin staining technique which is specific for calcium under the usual conditions of normal and pathological calcification. McGee-Russell (1958) in an evaluation of the various histochemical methods for calcium considered the one employing sodium alizarin sulphonate the best. His investigations showed that the method using this dye was superior to all the others which are based on the conversion of calcium salts to salts of other metals. This dye has also been used in histochemical studies by adding it to the substrate medium, as was done by Bourne (1943) in his studies on the distribution of alkaline phosphatase in various tissues.

In the present investigation, lipase has been demonstrated histochemically in muscle using Tween 85 as substrate in the incubation medium and alizarin red S to colour the calcium soap formed.

MATERIALS AND METHOD

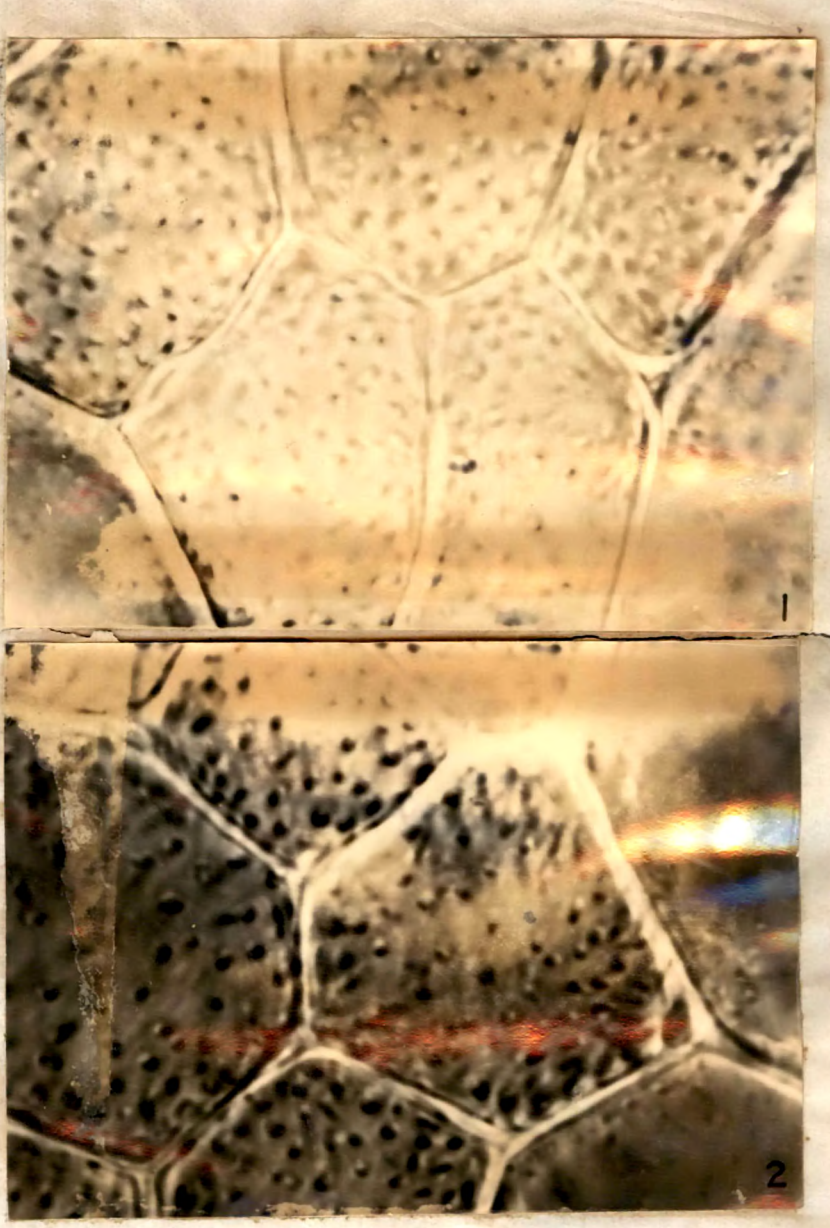
The lateral line muscle (red) of the fish and the breast muscle of the pigeon were used. The procedure employed in the technique is as follows. Fresh frozen sections 10 to 15 μ thick were cut directly into cold 6% neutral formalin and fixed for 4 hours at 5^{o}C. Sections were mounted on clean slides and, after being allowed to dry, were given a thin coating of 1% gelatin and fixed for 30 minutes in cold neutral formalin. The slides were then washed in running water for half an hour, rinsed with distilled water, placed in borate buffer pH 8.0 with Versene 0.002 M for 1 to 15 minutes at

5°C, and again washed in distilled water. The control sections after boiling for 10 to 15 minutes were incubated along with the sample for about 16 hours at 37°C (George and Iype, 1960) using Tween 85 (Polyoxyethylene sorbitan trioleate) instead of Tween 80 (Polyoxyethylene sorbitan monooleate), both products of the Atlas Powder Co., U. S. A., as substrate. After washing in distilled water, the slides were immersed in 1% alizarin red S at pH 6.3 to 6.8 for 30 seconds to 1 minute, rinsed rapidly in distilled water and finally mounted in glycerine jelly.

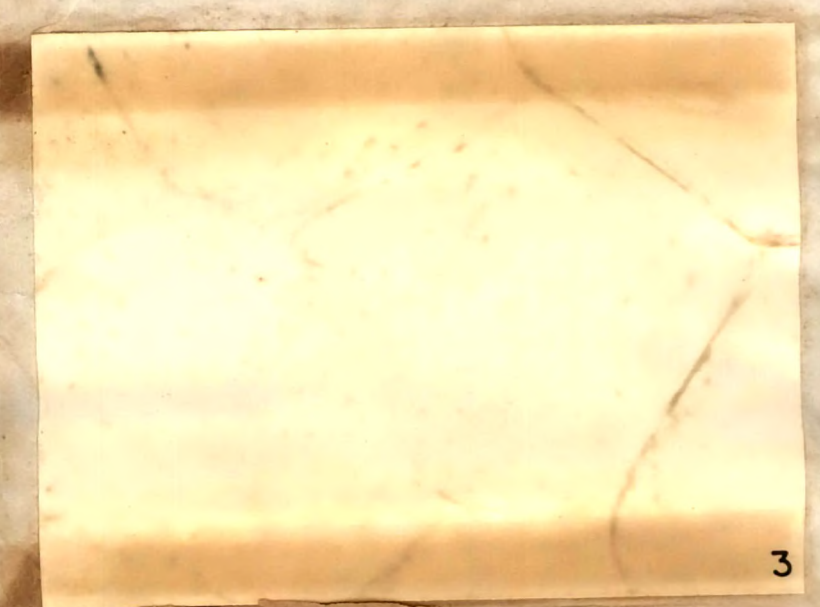
RESULTS

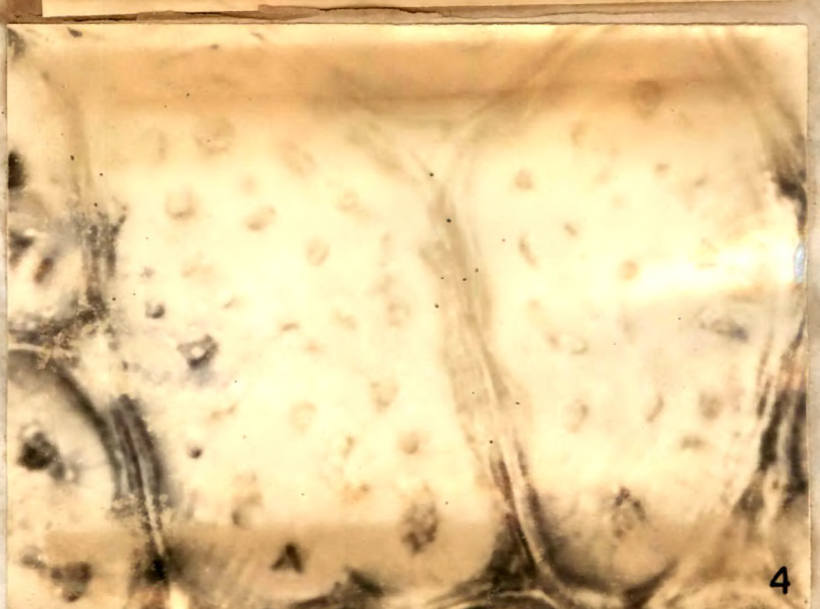
Fatty acids liberated by the hydrolysis of Tween 85 by lipase present in tissue react with calcium chloride in the incubation medium to form a calcium soap. This insoluble calcium soap formed at the sites of lipase activity is then made more easily detectable by converting it to a coloured compound using alizarin red S. In stained sections rapidly rinsed in distilled water, the surplus stain is removed and the background becomes faint pink. The sites where calcium soap is formed appear orange red in sharp contrast to the faint pink background. Localization of lipase activity can be clearly seen in mitochondria (Figs. 1 & 4).

As controls several means were employed to inactivate the enzyme. Some sections were boiled in water prior to incubation, some incubated in a medium to which no substrate was added, and some in a medium lacking calcium chloride.



Figs. 1 & 4 Transverse section of breast muscle of pigeon and the red lateral line muscle of fish respectively showing localization of lipase activity in the mitochondria. 1000 X.





Figs. 2 & 5 Control sections of breast muscle of pigeon and red lateral line muscle of fish respectively not treated with Versene, showing the staining reaction due to the endogenous Ca^{++}. 1000 X.

Figs. 3 & 6 Control sections of the breast muscle of pigeon and the red lateral line muscle of fish respectively, treated with Versene showing absence of lipase activity after removal of endogenous calcium. 1000 X.

In control sections untreated with Versene, it was found that there was considerable background colouration due to the uptake of alizarin by the calcium present in the muscle. Similarly, the mitochondria appeared to be darkly coloured owing to the presence of calcium in them (Figs. 2 & 5). This staining reaction could not be mistaken for lipase activity since the reaction at enzyme containing sites was completely destroyed by boiling the sections before incubation, whereas this was not. Such colouration was absent in controls treated with Versene in borate buffer (Figs. 3 & 6); in this situation calcium was effectively complexed by Versene so that it could no longer display its cationic properties. The complex formed by Versene with calcium was readily dissolved when the sections were washed in water, in keeping with the water soluble nature of Versene (Bersworth, 1954).

DISCUSSION

In the present investigation the localization of lipase in pigeon breast muscle and in the red muscle of fish was clearly established. The narrow red fibres have a characteristic arrangement of mitochondria which facilitates the identification of enzyme activity originating in these organelles. Lipase activity was demonstrated with alizarin red S at sites which correspond to the distribution of mitochondria. In the broad white fibres of the pigeon breast muscle, the distribution of lipase activity again suggests localization in the mitochondria. Hitherto, lipase activity in muscle fibres has

been demonstrated in the form of precipitates of lead sulphide crystals scattered about in the muscle fibres. A majority of these precipitates are artefacts and do not represent the precise cytochemical localization of lipase activity.

According to McGee-Russell (1958) the most satisfactory pH for staining with alizarin red S is 4.1 to 4.3, but in the present study a pH of 6.3 to 6.8 as suggested by Dahl (1952) gave more satisfactory results. Similarly, a quick rinse in acetone immediately after staining as recommended by McGee-Russell was not suitable, because acetone caused considerable shrinkage of the muscle fibres. Dahl (1952) did not recommend the use of any adhesive such as gelatin or egg albumen since it was found to take up stain. Nevertheless, for the histochemical localization of lipase activity, a coating of dilute gelatin over the sections is essential, since gelatin will hold in place the calcium soap formed and prevent its diffusion (George and Iype, 1960).

It was, however, necessary to confirm that the calcium alizarate obtained was due to the reaction of the calcium soap formed and not to the endogenous calcium of the muscle itself. To remove this endogenous calcium from the muscle, sections were treated with acetic acid in concentrations of 1 to 10% for varying intervals prior to incubation. By this procedure it was found that there was some inhibition of lipase activity. On the other hand, if the concentration of acetic acid and the time of extraction was reduced, calcium

was not completely removed from the muscle. This necessitated the use of a metal complexing agent to bind the calcium. Therefore, Versene 0.002 M in borate buffer pH 8.0 for 1 to 15 minutes at 5°C (Wills, 1960) was used. At 37°C, the insect fat body lipase has been found to be completely inhibited by the addition of 0.005 M Versene, 35% inhibited by a 0.002 M solution and 5% inhibited with a 0.001 M solution of this chelator (Hegdekar, 1964). Wills (1960) reported a 70% inhibition of lipase activity by 0.002 M Versene in 15 minutes at 37°C, whereas at 5°C there was absolutely no loss of activity. He therefore pointed out that temperature and the duration of treatment in Versene were important factors in the inhibition of lipase activity and recommended the use of borate buffer at pH 8.0 with 0.002 M Versene at 5°C. Such a treatment permitted the complete removal of calcium without having any adverse effect on lipase activity.

This chapter has dealt with the suitability of alizarin red S for demonstrating mitochondrial lipase in skeletal muscle fibres. The staining reaction has been found to be permanent with no loss in intensity of colour and very slight diffusion of the stain after long periods of time. The advantage of this method over the lead nitrate technique of Gomori (Pearse, 1960) is that it avoids the production of the artefacts obtained with lead nitrate and the problem of their removal by washing. The lipase demonstrated using Tween 85 as substrate has been shown to represent true lipase (George and Ambadkar, 1963) and not lipase in combination with some

nonspecific esterases, as when Tween 80 is used as in Gomori's original method. In this respect also the present method is to be preferred over the previous ones.