A Simultaneous Coupling Azo Dye Technique Suitable for Whole Mounts

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With one plate (fig. 1)

SUMMARY

A method based on the familiar coupling azo dye technique has been devised for the study of enzyme distribution in thick specimens. Its application to the study of esterase distribution in whole tadpoles of *Xenopus laevis* is described in detail. It can also be used to show the distribution of alkaline phosphatase, both in *Xenopus* tadpoles and in limbs dissected from chick embryos. It has proved useful in embryological studies both for teaching and for research.

ORDINARY section techniques have certain disadvantages when a three-dimensional picture is the primary requirement. These disadvantages are particularly serious in many studies of enzyme distribution, where ordinary paraffin embedding procedures cannot be used. An attempt was therefore made to design a suitable histochemical technique which would eliminate the need to cut sections.

This technique, which is described below, was originally devised to study the distribution of cholinesterase in developing tadpoles of the toad, *Xenopus laevis*. It has since been applied to other enzyme studies, and the general principle of it could be extended much farther.

The method is based upon the now widely used simultaneous coupling azo dye technique for esterases and phosphatases first introduced by Menten, Junge, and Green in 1944 and since improved and extended by Nachlas and Seligman (1949), Gomori (1952), Pearse (1954), and others. Thus, in the established technique for esterases, sections are incubated with α-naphthyl acetate in the presence of a suitable diazonium salt. As the esterase hydrolyses this substrate, free α-naphthol is released which very rapidly couples with the diazonium salt to give a brightly coloured precipitate of an azo dye at or close to the site of enzyme activity. The important modification made in applying this technique to whole tadpoles was to pre-incubate in a buffered solution of the diazonium salt before introducing the substrate, in order to ensure that an adequate concentration of the coupling agent was present throughout the tissue before any release of α-naphthol took place. Various minor modifications were also made in order to reduce the background staining of tissues by the diazonium salt, since even slight general coloration in a thick specimen seriously impairs the quality of the microscopic image. Thus the use of acetone to dissolve the substrate was eliminated, and phosphate

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was replaced by tris(hydroxymethyl)aminomethane as the buffer. Sites of alkaline phosphatase activity can be demonstrated by a precisely similar method but with α-naphthyl phosphate in place of the acetate.

Details of Technique

The technique, as used to study cholinesterase distribution in the *Xenopus* tadpole, is as follows:

1. Fixation for 2–24 h in neutral 10% formalin at 4° C has proved most satisfactory.

2. After washing for a few minutes in tap-water, the tadpoles are skinned under a dissecting microscope. As much as possible of the intestine must also be removed, since this contains a very active esterase which is not rendered insoluble by the fixation employed.

3. After a total period of ½ h in several changes of tap-water the tadpoles can be transferred to the diazonium salt solution for a period of 30–40 min. They are then transferred to the complete incubation medium and studied under a dissecting microscope. When the azo dye has reached the required intensity (usually after 2–20 min) they are returned to the diazonuim salt solution for 20–30 min. It should be noted that further intensification of the colour continues for several minutes after removal from the complete incubation medium.

4. After a thorough wash in water it is an advantage to fix the preparations in formaldehyde-calcium for at least a few days at room temperature before attempting to make permanent mounts.

5. Direct mounting of the preparations in glycerol produces gross distortion, because water diffuses out much more rapidly than glycerol diffuses in. This effect can be avoided by passing the tadpoles through a graded series of water-alcohol-glycerol mixtures. Alcohol reduces the viscosity of the water-glycerol mixtures and markedly reduces the rapid extraction of water from the tissues by the glycerol. The optimum number of mixtures in the series and the time taken in each will depend upon the size of the specimen. A typical series used is:

water : alcohol : glycerol by volume: 5 : 5 : 1 → 5 : 5 : 3 → 3 : 7 : 5

→ 3 : 7 : 10 → ½ : 9½ : 10 → ½ : 9½ : 20.

This actual series was chosen merely because it was easily made up by mixing pure glycerol with the aqueous alcohol solutions kept in stock (50%, 70%, and 95%). The best results are obtained by placing tadpoles immersed in the final solution (preferably on cavity slides) in a desiccator over anhydrous calcium chloride, which rapidly takes up both water and ethyl alcohol.

6. Dissection of total preparations is not difficult, especially if the final dissecting is done in a glycerol-water mixture. Small pieces of tissue, obtained by blunt dissection, can be placed directly in the fourth solution of the series

given above on a microscope slide for final dissecting or teasing. Excess fluid can then be removed and the tissue mounted in glycerin jelly. Alternatively, the whole tadpole (or parts of it) can be embedded in gelatin and cut on the freezing microtome.

7. Supplementary techniques of various kinds can be applied before mounting. The azo dye is soluble in most lipid solvents, but not in water or ethyl alcohol at room temperature. Both the Bodian and Holmes silver techniques for nerve-fibres have been successfully applied to small pieces of tissue dissected from esterase preparations of tadpoles.

8. The solutions required are:

(*a*) The stock buffer solution, consisting of 0·2 M tris(hydroxymethyl)-aminomethane (M = 121; obtainable sufficiently pure from L. Light & Co., Colnbrook, Bucks.) made up in distilled water. This solution keeps at room temperature for several weeks or at 4° C for several months.

(*b*) A working buffer solution, best made up weekly:

10 ml stock buffer solution
1 ml N/1 hydrochloric acid
9 ml distilled water
} pH = 8·1.

(*c*) The diazonium salt solution:

3 ml buffer solution of pH = 8·1.
20–30 mg 'Red TR'.
Made up to 10 ml, filtered, and used within a few hours.

The full designation of 'Red TR' is Brentamine fast red TR salt. It is the stable diazonium salt of 5-chloro-*o*-toluidine and can be obtained from the Academic Relations Department, Imperial Chemical Industries, Hexagon House, Blackley, Manchester, 9. Of the many diazonium salts tested, this one gave by far the best total preparations.

(*d*) The complete incubation medium, which should be made up immediately before use by the following procedure.

Place a few crystals (5–10 mg) of purified α-naphthyl acetate (British Drug Houses) in a test tube and pulverize them. (This is easily done by using a second, longer, and thinner test tube.) Add equal volumes (5–10 ml) of distilled water and unfiltered diazonium salt solution (*c*); cork the test tube, shake vigorously for ½ min, and filter. Some commercial samples of α-naphthyl acetate may need to be recrystallized from alcohol, but most samples can be adequately purified by two or three washes with very small volumes of alcohol.

For the study of alkaline phosphatase activity the same technique can be used but with sodium α-naphthyl phosphate (G. T. Gurr) as the substrate and sodium diethyl barbiturate as the buffer. Thus in place of solutions (*c*) and (*d*) above the following two solutions are used:

(*e*) 4 ml N/10 sodium diethyl barbiturate.
20–30 mg Red TR.
Made up to 10 ml and filtered.

(*f*) 4 ml N/10 sodium diethyl barbiturate.
10–15 mg Red TR.
10 mg sodium α-naphthyl phosphate.
Made up to 10 ml and filtered.

This procedure has also been used to demonstrate the alkaline phosphatase activity associated with calcification in bones dissected from the limbs of chick embryos, the dissection being made before fixation in cold formalin.

Discussion

The successful application of the technique depends upon a number of conditions being fulfilled:

(*a*) Enzymes giving a positive reaction must not be destroyed by fixation and yet must be rendered insoluble in aqueous solutions. Structures found to contain any enzyme soluble after fixation must be removed before incubation, e.g. the intestine of the *Xenopus* tadpoles.

(*b*) The tissue must have an open structure to allow ready access of the reagents (otherwise activity in the superficial layers only will be revealed) and there must be no membranes present which are impermeable to either the substrate or the diazonium salt. These requirements can often be met by preliminary dissection before incubation, e.g. skinning of the tadpoles and dissection of the bones from chick limbs.

(*c*) The tissue must not contain a high proportion of fat, because the azo dye has an appreciable lipid solubility and fat tends to slow down diffusion of the reagents.

(*d*) The enzymes giving a positive reaction must be fairly sharply localized. If there is a significant amount of enzyme diffusely distributed it becomes difficult to distinguish any details in a thick specimen.

Just how far these conditions are fulfilled is usually obvious from a study of the specimen both during incubation and after final mounting in glycerol. As a further check, a treated specimen can be embedded in gelatin and frozen sections cut: it is then easy to assess how far adequate penetration of the reagents has occurred.

Fig. 1 (plate). A, B, and C are whole mounts, showing alkaline phosphatase distribution, prepared by students in an anatomy class. A, tibia and fibula; B, radius, ulna, and carpus, both from 8-day chick embryos; C, a culture of a phalanx.

D, part of a skinned, third-form *Xenopus* tadpole showing the esterase concentrated at the myocommata, where the myoneural junctions are. (The black area at the lower left is due to the melanin of the abdominal wall.)

E, the ciliary ganglion dissected from an esterase preparation of a *Xenopus* tadpole. The efferent nerve-fibres, which by-pass the ganglion and are presumably cholinergic, are much more deeply stained than the sensory neurones.

F, the end of a single myotomal muscle-fibre which had been teased out from an esterase preparation and silvered by the Bodian technique. The end of the muscle-fibre is deeply stained owing to esterase activity associated with the endings of the motor nerve; this is shown up by the silver stain.

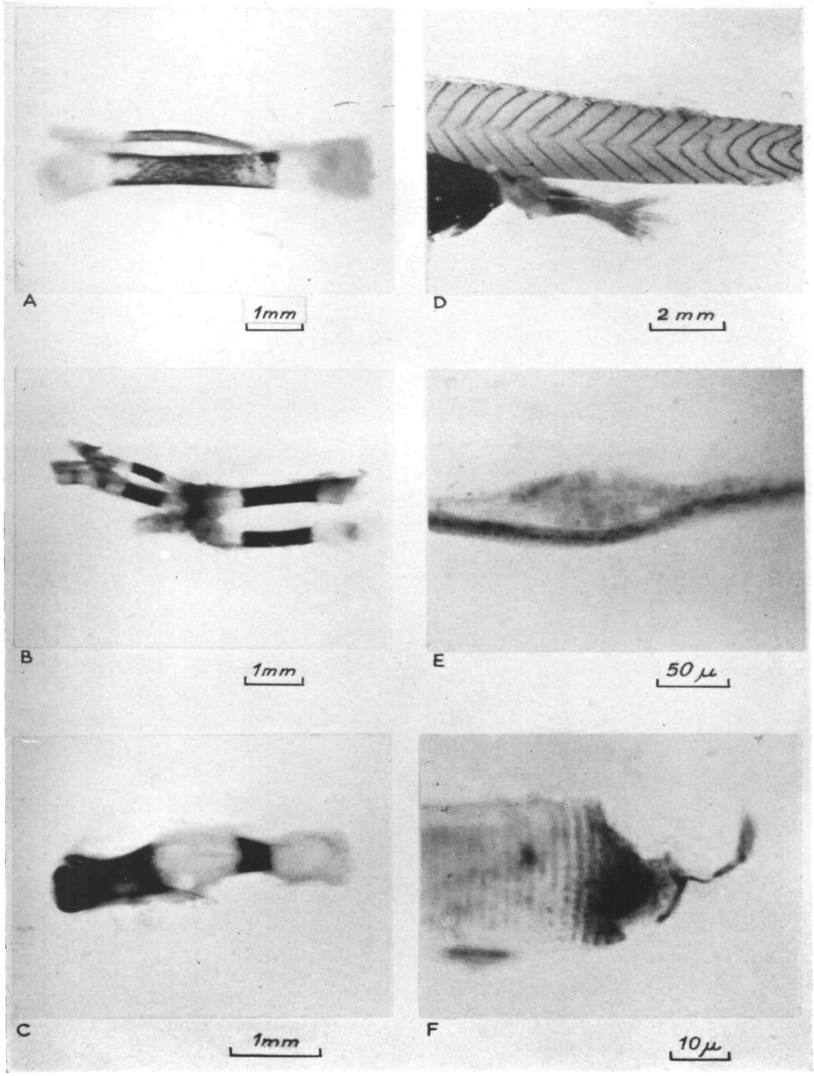

FIG. 1

R. P. LEWIS

It should be emphasized that this technique was not designed for cytological studies of enzyme distribution, although the localization of the azo dye is often extremely good, particularly in superficial structures. Its chief value is as a general method, especially for following gross changes in the three-dimensional distribution of an enzyme. As such it has proved very useful in preliminary studies of enzyme changes during embryonic development (Lewis and Hughes, 1957). It has also proved useful for teaching several embryological problems to advanced students, e.g. the spread of alkaline phosphatase activity during calcification and progressive development of motor endings in skeletal musculature, in chick limbs and *Xenopus* tadpoles respectively. Examples from both research and teaching material are shown in fig. 1.

The complete incubation medium developed for the esterase technique (solution *d* described above) has also proved very suitable for studies in ordinary sections. It appears to be more stable than the one recommended by Pearse (1953) and to give less background staining due to decomposition of the diazonium salt.

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REFERENCES

GOMORI, G., 1952. *Microscopic histochemistry*. Chicago (University Press).
LEWIS, P. R., and HUGHES, A. F. W., 1957. In *Metabolism of the nervous system*. London (Pergamon Press).
MENTEN, M. L., JUNGE, J., and GREEN, M. H., 1944. J. biol. Chem., **153**, 471.
NACHLAS, M. M., and SELIGMAN, A. M., 1949. J. Nat. Cancer Inst., **9**, 415.
PEARSE, A. G. E., 1953. *Histochemistry, theoretical and applied*. London (Churchill).
—— 1954. Internat. Rev. Cytol., **3**, 329.