Technical methods

Methylene blue and azure-2 as stains for lipid in osmium-fixed tissues embedded in araldite

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Mixtures of methylene blue and azure-2 are stated to stain neutral lipid droplets an intense dark blue in 0·5-1·0 μm-thick Araldite sections of osmium-fixed kidney (Muehrcke, Mandal, Volini, and Epstein, 1969; Tobian, Ishii, and Duke, 1969). Neither group of investigators, however, detail either the composition of the stain or the method.

A modification of the methylene blue-azure-2 method of Richardson, Jarret, and Finke (1960) is used in this laboratory for general staining 0·5-1·0 μm-thick Araldite sections of osmium-fixed tissue. With this technique, droplets, known to be lipid, are virtually colourless or a pale green.

The present note describes in detail a method of using methylene blue and azure-2 to produce an intense blue-black colouration of lipid droplets.

Tissues, Fixation, and Sectioning

Fatty liver from carbon tetrachloride-treated mice provided suitable material for study, as did pancreatic tissue from tumour-bearing rats, because characteristic small lipid droplets are found in the basal part of acinar cells (Parry, 1972).

Liver was fixed in Palade's (1952) osmium tetroxide fixative; pancreas was fixed in 1% osmium tetroxide in cacodylate buffer, pH 7·3. Both tissues were dehydrated in graded ethanols, cleared in propylene oxide, and embedded in Araldite (Glauert, 1967). Blocks of liver were also fixed in 10% formol saline.

Araldite blocks were sectioned on an ultramicrotome at 0·5 to 1·0 μm, and the sections fixed to slides by gentle heat. Frozen sections were cut at 10 μm from formalin-fixed liver blocks. Some sections were stored in distilled water, others were postfixed in 1% cacodylate-buffered osmium tetroxide for one and a half hr and then stored in distilled water until required.

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Stains

All stains were made up in 1% borax in distilled water.

THE ROUTINE STAIN (LIPID EITHER UNSTAINED OR GREEN)

The stock solutions are 1% methylene blue (MB) (George T. Gurr No. G 183/17612) and 1% azure-2 (A_2) (George T. Gurr No. 21043).

The staining solution (MB-A_2) is made up by mixing equal volumes of the above two stock solutions. (One batch of stock solutions was at least 12 months old. The resulting MB: A-$_2$ stain had a final pH of 7·9. Freshly prepared stock solutions and the MB: A-$_2$ stain prepared from them had a pH of 8·2.)

LIPID STAINS (LIPID AN INTENSE BLUE-BLACK COLOUR)

All stains (made up in 1% borax in distilled water) in this category are alkalinized by the addition of N-NaOH until the final pH is approximately 9·4 (1 drop N-NaOH per ml from a standard Pasteur pipette), (1) 1% MB: 1% A_2 (a mixture of equal volumes of MB and A-2), (2) 1% MB (old stock solution only), and (3) 1% A-$_2$ (old stock solution and fresh stock solution).

Staining

Araldite sections fixed to slides, covered by a drop of stain, are briefly heated in a Bunsen burner pilot light until a trace of vapour rises from the stain. Excess stain is washed off with distilled water. Sections stained by the routine stain may now be heat-dried and mounted. Sections stained by the fat stains, however, are very intensely stained. They may be decolourized by brief treatment with 96% alcohol containing 1 drop per ml, N-NaOH (pH 10·0); stain is rapidly removed and the alcohol should be washed off with distilled water when the section appears a pale blue-grey. Sections may then be mounted in Apathy's mountant or dried and mounted in synthetic non-aqueous mountants such as Depex (George T. Gurr).

Frozen sections are stained by immersion. For lipid staining by the above described stains, sections are immersed in stain for five min; the intensely stained sections are then decolourized by alkaline

Staining Procedure	*Osmium-fixed Araldite Sections (Liver and Pancreas)*	*Formalin-fixed Frozen Sections (Liver)*	*Formalin-fixed/ Osmicated Frozen Sections (Liver)*
MB: A-2 (pH 7·9, 8·2)	–[1]	–	–
MB: A-2 (pH 9·4)	+[2]	–	+
MB (pH 9·4) Old solution	+	–	+
MB (pH 9·4) Fresh solution	–	–	–
A-2 (pH 9·4 { Old solution }	+	–	+
A-2 (pH 7·9, pH 8·2)	–	–	–
H_2O_2 (2 vol 30%: 1 vol H_2O) 1 hr then MB: A_2, pH 9·4	(a)[4] –	0[3]	0
H_2O_2 (1 vol 30%: 2 vol H_2O) 15 min then MB: A_2, pH 9·4	0	0	(b)[5] –
Absolute alcohol (5 min), xylene (5 min), absolute alcohol (5 min), then MB: A_2, pH 9·4	0	0	–
MB: A_2 (pH 7·9) then by wash in 96% alcohol pH 10·0	+	0	0

Table *Results*

[1] – indicates pale green or colourless lipid droplets
[2] + indicates intense blue-black colouration of lipid droplets
[3] 0 = treatment not carried out on this section type.
[4] (a) = most droplets were unstained, but some larger ones retained an intensely blue black stained view. This corresponded with the distribution of pale brown colouration of lipid in unstained, H_2O_2-treated sections.
(b) = Sudan-4 in 70% alcohol stained lipid in these sections.

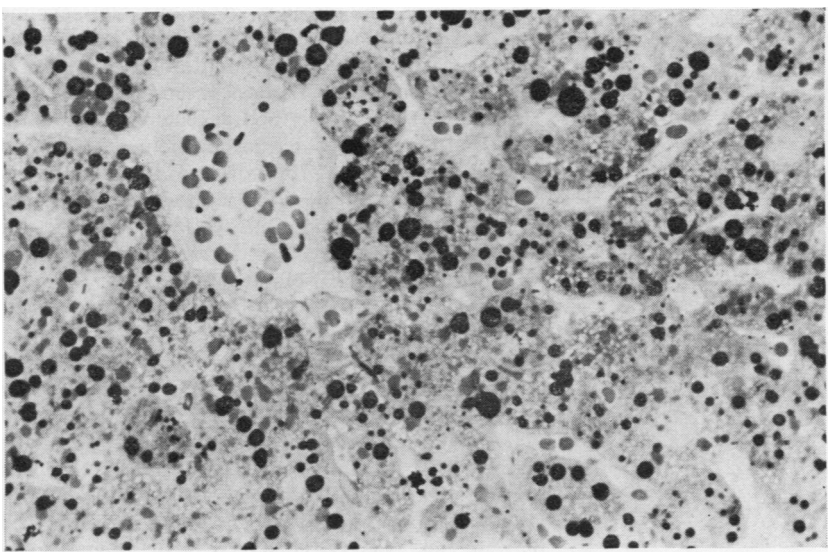

Fig *Liver of CC14-treated mouse. Centrilobular zone, illustrating numerous deeply stained lipid droplets. Erythrocytes in central vein and sinusoids are much paler. (Araldite section, MB-fat stain pH 9·4, × 480.)*

96% alcohol (pH 10·0) until the section is a pale blue-grey. All frozen sections were mounted in Apathy's mountant.

Results and Conclusions

The results are shown in the table, and an example of a stained Araldite section is given in the figure. From these results, the conclusions may be drawn that for lipid staining the following requirements must be met:

1 Osmium must be present, complexed to the lipid, since H_2O_2 oxidation which removes osmium (but not the lipid) abolishes the blue-black lipid staining.

2 The pH at the time of staining must be high (above 8·2), and a pH of 9·4 is found to produce regular blue-black staining of lipid.

3 The essential stain for the reaction is azure-2. The failure of fresh solutions of methylene blue at pH 9·4 to colour lipid confirms this. The success in staining lipid of old stock solutions of methylene blue alkalinized to pH 9·4 may be explained by spontaneous production of azures in such solutions (Baker, 1958).

The remarkable avidity of lipid/osmium complexes for azure-2 is shown by treating routinely

stained sections (MB: A-$_2$, lipid colourless or pale green) with 96% alcohol, pH 10·0. This removes some stain from the section, but the previously pale green or colourless lipid droplets now take on an obvious blue-black colour.

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A modified fluorimetric procedure for the rapid estimation of oxytetracycline in blood

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Chemical methods of determination of terramycin (oxytetracycline or OTC) described in the literature make use of either fluorimetric or colorimetric measurement of a degradation product (Chiccarelli, Woolford, and Avery, 1959; Hayes and DuBuy, 1964). The method described by Hayes and DuBuy is based on the conversion of tetracycline to anhydrotetracycline by heating in an acid medium and the resultant fluorescence of anhydrotetracycline (Hayes and DuBuy, 1964). This method is especially sensitive for the determination of oxytetracycline in biological tissues. Ibsen, Sanders, and Urist (1963) have described a general method for the determination of tetracyclines making use of Mg^{++}-OTC chelate formation. In the present communication we describe a modification of the method of Hayes and DuBuy (1964) adapted for estimating oxytetracycline in serum or plasma obtained from clinical blood samples.

Experimental

All reagents used were AR grade. Terramycin HCl was a gift from Pfizer Ltd. The trichloracetic acid (TCA):HCl reagent used was a modification of the reagent used by Hayes and DuBuy (1964) and was prepared by mixing 3 volumes of 5N TCA with 1 volume of concentrated HCl. In place of TCA:HCl reagent, an $NaWO_4$:H_2SO_4 reagent prepared by mixing 2 volumes of 10% $NaWO_4$ and 1 volume of concentrated H_2SO_4 may also be used without altering the sensitivity of the method.

Procedure

To 1 ml aliquot of human serum (or plasma) obtained at various intervals after administration of an oral amoebic antibiotic[2] preparation containing terra-

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[2]A preparation containing terramycin, chloroquin phosphate, and diiodohydroxyquin in the proportion of 167 mg, 85 mg, and 500 mg respectively.

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