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CHOLINESTERASES

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Dr. M. A. GEREBTZOFF
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INTRODUCTION

1. Scope and limitations of histochemical methods

Progress in microchemistry has brought to biochemists great possibilities of quantitative analysis of biological material. But, with some exceptions, these methods do not permit a precise localization of the substances under investigation. Hence the usefulness of techniques in which the localization in tissues and cells (histochemistry) or even in cellular organites (cytochemistry) is submitted to microscopic control.

There is an intimate interdependence between both procedures. Histochemistry borrows most of its techniques from microchemistry, but only a few of these can be safely adapted to microscopic preparations. Quantitative measurements are in general impossible in histochemistry, or subject to such errors that the logical step is to complement histochemical research with microchemical investigation, as, for instance, Zajicek (1957) did for acetylcholinesterase in blood platelets. More often, histochemistry adds to the results of biochemists the important precision of localization and gives the unsurpassed satisfaction of a beautiful microscopic preparation demonstrating objectively the relations between structure and function.

Thus histochemistry, in spite of its quantitative and even qualitative limitations and of the unreliability of some techniques, holds a central position at the convergence of morphological, physiological and biochemical research.

2. Acetylcholine, choline acetylase and cholinesterases

Cholinesterases may be concentrated at some distance from the acetylcholine-releasing structures; these enzymes are not inevitably concerned with hydrolysis of acetylcholine; and a parallelism between acetylcholine amounts and cholinesterase activity is not always present. But, until now, there has been no histochemical technique for the detection of acetylcholine, with the eventual discrimination between the mediator available for immediate release and the bound precursor suggested by Abdon and Hammerskjöld (1944), Perry (1953), Birks and MacIntosh (1957) and others. Our attempts at detection through gold or platinum complexes have failed.

Acetylcholine-synthesis sites could be studied by the localization of

choline acetylase. While the biochemical surveys of its distribution have given good results in the hands of Feldberg and Vogt (1948), Hebb (1955), Hebb and Silver (1956), though they did not take into account the rate-limiting effect of acetyl coenzyme A concentration (Smallman, 1958), a histochemical method would furnish no indications on the acetylcholine-release sites, since the enzyme appears to be produced in the neuronal cell bodies (Feldberg and Vogt, 1948; Hebb and Waites, 1956). On the contrary, the speed of acetylcholine hydrolysis by 'specific' cholinesterase excludes the existence of an important gap between the structures releasing acetylcholine and those containing the enzyme. Further, the study of cholinesterases constitutes in itself an interesting chapter of enzymology, which, as we learned from a letter Augustinsson wrote us a few months ago, has already inspired more than four thousand articles! Our incursions into biochemistry will be dictated by the limited scope of this book, and readers desiring more extensive information on cholinesterases are referred to treatises and such reviews as those of Augustinsson (1948) and Whittaker (1951), and also, if their interest is centred on the nervous system, to Prosser's Comparative Animal Physiology (1952), to the Neurochemistry of Elliott, Page and Quastel (1955) and to the reviews of Hebb (1957) and Paton (1958).

Among hydrolases, the group of enzymes hydrolysing esters (esterases) is divided into three subgroups: sulphatases, phosphatases and carboxyl-esterases. The last one includes aliesterases (hydrolysing esters of acids with aliphatic chains shorter than 12 C), lipases, cholinesterases, acetyl-esterases, cholesterolesterases, lecithinases, and other enzymes such as atropinesterase. Only cholinesterases and acetylesterases bring about appreciable hydrolysis of acetylcholine, though, as will be shown later, aliesterases may interfere in the histochemical reaction for cholinesterases. Acetylesterase is common in plant tissues but quite exceptional in animals (cobra venom: Bovet-Nitti, 1947); it does not concern us here.

Since the fundamental work of Alles and Hawes (1940), it is known that there are two types of cholinesterase: specific or true cholinesterase, or better, in the nomenclature of Augustinsson and Nachmansohn (1949), acetylcholinesterase (AChE); and non-specific cholinesterase or pseudo-cholinesterase, or simply cholinesterase (ChE). These enzymes differ by the rate of hydrolysis of substrates: it decreases for AChE and increases for ChE with increasing length of the acyl group, from acetyl to *n*-butyryl. AChE is inhibited by an excess of acetyl- or of propionylcholine, while an excess of substrate, even of butyrylcholine, does not inhibit ChE. Biochemists make use of substrates that are hydrolysed by one type of cholinesterase but not by another: for example acetyl-β-methylcholine by AChE, and benzoylcholine by ChE.

About the last substrate, we must add that an excess slightly depresses

the rate of hydrolysis by ChE (Augustinsson, 1948), and that Sawyer (1945) found in the liver of female guinea-pig and rabbit an enzyme hydrolysing it at a higher rate than acetylcholine, but this 'benzoylcholinesterase' might be an aliesterase. On the other hand, the rate is highest for propionylcholine in the case of ChE of rat's heart (Ord and Thompson, 1951).

It seems thus that not only is there no absolute specificity—AChE splits some acetyl esters of bases other than choline—but that the separation between the two types is not as clear as first supposed.

3. Choice of a histochemical technique

In 1948, Gomori proposed a technique based on the precipitation, by cobalt salts, of acids freed by enzymic hydrolysis out of fatty acid esters of choline. This method gave irregular and discordant results.

In the following years appeared two groups of techniques. The first one makes use of specific substrates, and proceeds from the method of Koelle and Friedenwald (1949): frozen sections of tissues are incubated with acetylthiocholine (and, for ChE, with butyrylthiocholine: Koelle, 1950) and copper glycocollate. The freed thiocholine forms with the copper ions an insoluble mercaptide which is transformed into copper sulphide. In an improved form (Koelle, 1951), it has been adopted by Gomori (1952) and is now extensively used either in the last original modification, or in a modified form tending towards better specificity (Holmstedt, 1957) or better localization of the enzymes (Couteaux, 1951: Coërs, 1953; Gerebtzoff, 1953 and 1956). The other group employs non specific substrates and then differentiates between the various carboxylesterases with the help of specific activators and inhibitors: Barnett and Seligman (1951), Holt and Withers (1952) incubate the sections with indoxyl and 5·3-bromoindoxyl acetate and oxidize the freed indoxyl fraction to indigo and indigo blue; Ravin, Zacks and Seligman (1953) couple with an azoic dye the β-naphthol freed from β-naphthyl acetate. Apart from these trends, the technique of Crevier and Belanger (1955) has its source in the researches of Wilson (1951) on the esteratic site of AChE. It consists of an incubation with thiolacetic acid and lead nitrate, seems to give good results, but has been tried only on a very limited selection of tissues.

Each method has its drawbacks. Neither substrates nor inhibitors are really specific, and only a combination of both gives more or less definite results. Sensitivity must be sacrificed to precision in localization. Long practice is necessary to evolve the technique most appropriate for the material studied and the ends to be attained. We shall now set forth the standard technique used in our laboratory. Modifications for embedding in paraffin and for smears will be found in our 1956 paper.

The best results are obtained with tissues removed half an hour or less after death, but they may be fair even when necropsy takes place the next day, provided the body is kept in a cold room.

1. Pieces no thicker than 5 mm are fixed in 10 per cent neutralized formalin for 4–6 hr. Frozen sections are cut at 20–25 and sometimes at 50μ and the sections are kept 30 min in the same medium.

2. Wash thoroughly (30 min) in doubly distilled water.

At this stage, controls with inhibition of ChE by di*iso*propylfluorophosphonate (DFP) are separated: some sections are washed for only 5 min, treated 30 min in DFP 10^{-7} M (at 37°C for homeotherm tissues, at laboratory temperature for poïkilotherm tissues), and washed again for 5 min. The DFP solution is freshly prepared by successive dilutions in doubly distilled water of a stock solution 10^{-1} M in anhydrous propylene glycol, kept in the icebox. The control sections are then submitted to the same procedures as the other sections.

3. The substrate includes:

Buffer solution	2·5 cm³
Doubly distilled water	1·9 cm³
Glycocolle 3·75 per cent	0·1 cm³
Copper acetate 0·1 M	0·1 cm³
Acetylthiocholine (for AChE detection), or	0·4 cm³
Butyrylthiocholine (for ChE detection)	

The buffer solutions are prepared with 0·1 N acetic acid and 0·1 M sodium acetate. We incubate tissues with high cholinesterase activity at pH 5, other tissues at pH 6·2 and even 6·8. The buffer and glycocolle solutions may be prepared in advance and kept in the icebox.

Acetyl- and butyryl-thiocholine are prepared in the following way:

Acetylthiocholine iodide	15 mg
or	
Butyrylthiocholine iodide	18·5 mg
Doubly distilled water	0·78 cm³
Copper acetate 0·1 M	0·26 cm³

Centrifuge 10–15 min at 3500–4000 rev/min.

These quantities are adequate for 2 × 5 cm³ of substrate.

If interference by aliesterases is suspected, controls may be made by replacing in the substrate the 1·9 cm³ of water by the same volume of a 1·7 mg per cent solution of eserine sulphate. It is well known that at this final concentration (10^{-5} M) eserine inhibits cholinesterases, but remains without effect on other esterases (Mendel and Gunter, 1946; Verne, 1954; Chessick, 1954).

The optimal length of incubation (at 37°C for warm-blooded, at room temperature, with an optimum at 22–23°C, for cold-blooded animal

tissues) varies from one material to another. In general, we do not go beyond 2 hr, but sometimes we leave the sections in the substrate as long as 18 hr (embryonic tissues, for instance).

4. The sections taken out of the substrate after different times of incubation are washed for half a minute in doubly distilled water, plunged for a short time into 5–10 per cent pure ammonium sulphide, and washed thoroughly in doubly distilled water. Dehydrate, clear and mount in Canada balsam.

Overstainings after the last washing give satisfactory contrasts. Silver impregnations of nerve fibres may also be attempted, especially by a modified Bodian technique (Csillik and Sávay, 1958).

By lowering the pH of the substrate—with the resulting loss in sensitivity and gain in precision of localization—and by taking out sections at different times of the incubation, it is possible to obtain quite satisfactory preparations. This last procedure permits the study of the evolution of the histochemical reaction and thus to reach a clearer idea of enzymatic activity, the appreciation of which however remains subjective.

A recent paper by Miss L. Arvy (1958) gives a critical review of histochemical (and biochemical) techniques for the detection of esterases, as well as a survey of the repartition of these enzymes throughout the animal kingdom. It includes many details outside the scope of this book.

Part I

HUMORAL CHOLINESTERASES

After the demonstration, by Alles and Hawes (1940), of two types of cholinesterases in human blood, the link between ChE and serum, and between AChE and erythrocytes seemed so strong that Zeller and Bissegger (1943) gave to ChE the name of cholinesterase type *s* (serum) and to AChE that of cholinesterase type *e* (erythrocytes). But it was soon shown that the serum contains a mixture of ChE and AChE (Mendel, Mundell and Rudney, 1943; Hawkins and Mendel, 1947; Augustinsson, 1948), with a predominance of ChE in most species, including man, and of AChE in other: for instance, the rabbit. Some sera have almost no cholinesterases: those of ruminants, birds and fishes. As for erythrocytes, we shall see that they are not the only blood-corpuscles that may contain AChE; and this enzyme is scarce in, or absent from the erythrocytes of birds and fishes.

While serum (or, better, plasma) cholinesterases escape the eye of the histochemist, and the AChE bound to the cell membrane of erythrocytes (Croft and Richter, 1943) is in such a diffuse form that it cannot be clearly demonstrated by his techniques, we believe that histochemistry can at least bring a contribution to the knowledge of the origins of these 'humoral' or 'circulating' cholinesterases.

CHAPTER I

Plasmatic and Hepatic Cholinesterases

1. The probability of a hepatic origin of plasmatic cholinesterases

Hepatic cholinesterases belong to the cells of the liver and ought to be included among tissue cholinesterases. But there is a strong presumption that plasmatic cholinesterases have their origin in that organ, and we must describe them here. This presumption is based on the parallelism, for each animal species, between the nature and activity of cholinesterases in liver and in serum, and on the modifications of serum ChE activity in hepatic diseases (for references: Augustinsson, 1948 and 1950; Gajdos, 1950; Gerebtzoff, 1954). There seems to be one exception: in the liver of the guinea-pig, the dominant esterase is the 'benzoylcholinesterase' of Sawyer (1945), which could even be the only cholinesterase of this organ (Goutier-Pirotte and Goutier, 1956), while the plasma is rich in ChE. The last cited authors believe that, in this special case, plasmatic ChE has its origin in the intestinal wall. There is some ChE and AChE in the liver of the fœtus, but their amounts fall down during the last days before and the first days after birth.

The liver being formed of lobules having a vascular polarization, with afferent vessels at the periphery and an efferent centrolobular vein, the cholinesterase activity must be highest in the centre of lobules if there is really an enzymatic flow from the liver to the blood. Research on the liver of the cat (Koelle, 1951) seemed to confirm this. Our investigations (Gerebtzoff, 1954) showed also a centrolobular enzymatic predominance, but, curiously enough, they concerned the most unfavourable material: the guinea-pig's liver, where incubation with butyrylthiocholine revealed the presence of what seemed to be ChE but must have been benzoyl-cholinesterase, with the highest activity near the centrolobular vein (Fig. 1); and the liver of the rabbit with the same central predominance, of AChE this time (Fig. 2).

Bertrand (1954) studied in our laboratory the localization of ChE proper in the liver of mice and found that the most active zone, while remaining the same in each lobule, varied considerably from one animal to another. ChE was concentrated midway between the periphery and the centre of the lobule (Fig. 3), was abundant in the whole lobule (Fig. 4), or was most active at its periphery (Fig. 5). He found centrolobular and midway localizations in rabbits, and the last one in a young cat. On the whole, his

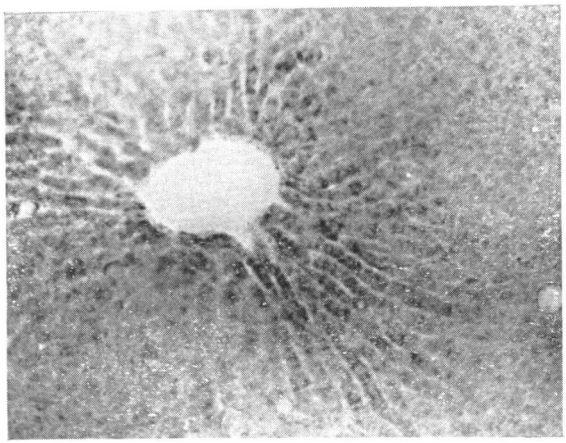

FIG. 1. Parenchymatous localization of ChE in the liver of the guinea-pig. Incubation: 90 min. × 150
Unless otherwise specified, slides were incubated with acetylthiocholine (AcThCh) for the detection of AChE, and with butyrylthiocholine (BuThCh) for that of ChE; and usually at pH 6·2 *and* 37°C.

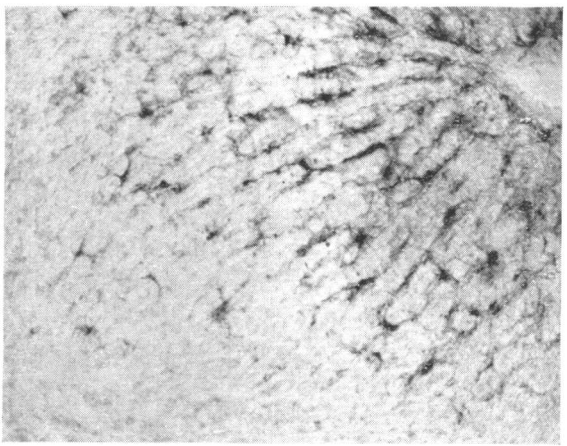

FIG. 2. Histiocytary localization of AChE in the liver of the rabbit. Incubation: 90 min. × 150

work appeared to have obscured the question of the hepatic origin of plasmatic cholinesterases.

2. The dual localization of hepatic cholinesterases

In comparing the results of Koelle, Gerebtzoff and Bertrand, it becomes evident that not only intralobular but even cellular localization varies

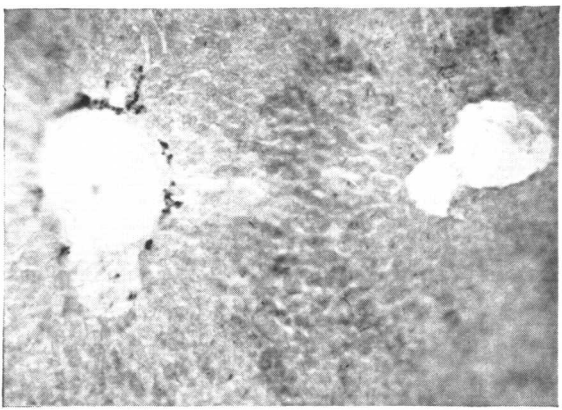

FIG. 3. Midway lobular localization of ChE in the liver of a mouse after 2 days of fasting. Incubation: 90 min. × 150

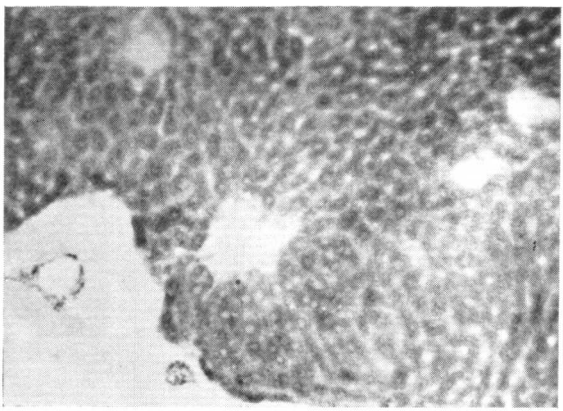

FIG. 4. The whole lobule is filled with ChE in the liver of a mouse 6 hr after a meal. Incubation: 90 min. × 150

from species to species. The enzyme is inside parenchymatous cells in the liver of the guinea-pig (Fig. 1), in histiocytes (endothelial and Kuppfer cells) limiting the hepatic sinusoids of cats and rabbits (Fig. 2), in parenchymatous cells and in histiocytes surrounding the portal veins in the

liver of the mouse (Fig. 3–6). In the guinea-pig, rare Kuppfer cells give exceptionally a positive reaction for ChE.

ChE (and benzoylcholinesterase) located in parenchymatous cells is dispersed in the cytoplasm as minute granules. This diffuse aspect is readily explained by the fact that most of the enzyme is concentrated on

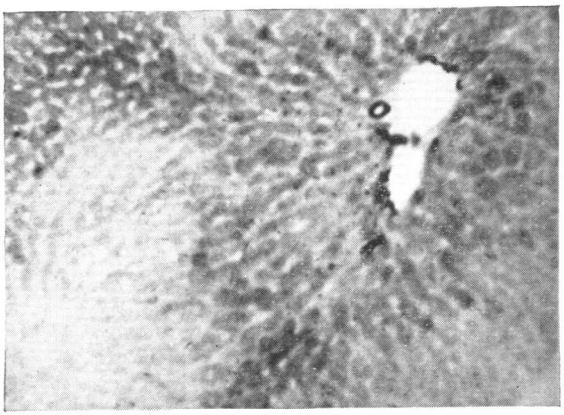

FIG. 5. Peripheric lobular localization of ChE in the liver of a mouse 1 hr after a meal. Incubation: 90 min. × 150

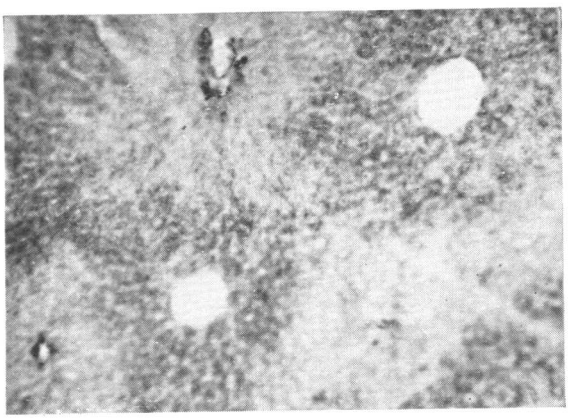

FIG. 6. Centrolobular localization of ChE in the liver of a mouse in deep inanition. Incubation: 120 min. × 150

microsomes, as shown by Goutier and Goutier-Pirotte (1955) for guinea-pig liver cholinesterase by means of a fractionation technique. In histiocytes, the enzyme, if present, is so densely packed in the scant cytoplasm, that it is impossible to get a clear picture of its distribution. As the same

fractionation study gave identical results for AChE of rabbit's liver, it is probable that the same cytochemical conditions apply to both types of localization. But we shall see that this does not imply that they follow a similar pattern in experimental conditions.

3. Hepatic and plasmatic cholinesterases and assimilation of food

The rabbit, in the liver of which Bertrand had found a midway lobular localization of AChE had been operated (cervical radicotomy) 2 days before and had not eaten since; the young cat presenting the same localization had been weaned too soon and had not eaten for 3 days. These observations and the fact that the rat's hepatic and plasmatic ChE sinks

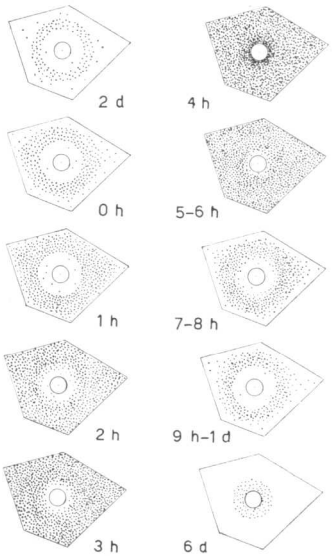

FIG. 7. Evolution of ChE activity and lobular localization in the liver of mice at different periods of fasting.
d: days, h: hours after the last meal.

down after one day of fasting (Harrisson and Brown, 1951) and that this activity rises in the liver of under-nourished children parallel with the level of food intake (Waterlow, 1950) suggested to Bertrand that the variability in lobular localization might be related to feeding.

These experiments were made on a group of over 60 white mice, fed with a diet consisting essentially of bread, and receiving as much water as they wanted. The first step was to study the eventual effects of fasting. After 1 day of (rather relative) fasting, and more clearly so after 2 days, the diffuse localization of ChE in the lobule, encountered generally in normally fed mice, has given way to a midway localization (Fig. 3). In the subsequent experiments, the animals are first brought to this 'resting