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CHOLINESTERASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT LIVER

ASSOCIATION OF ACETYLCHOLINESTERASE WITH THE SURFACE MEMBRANE AND OTHER PROPERTIES OF THE ENZYME

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SUMMARY

Rat liver contains two cholinesterase activities: "true" or acetylcholinesterase (EC 3.1.1.7) and pseudocholinesterase (EC 3.1.1.8). Differences in subcellular distribution are observed between the two activities.

Subcellular fractions rich in surface membrane are observed to be enriched with respect to acetylcholinesterase; this distribution is different from that of pseudocholinesterase and non-specific esterase. The activity in surface membrane preparations cannot be accounted for by contamination of the fraction with membranes derived from other subcellular organelles, or by erythrocyte membranes.

The behaviour of the cholinesterases towards several acetylcholine analogues is described, together with several inhibition and kinetic properties.

INTRODUCTION

Enzymes which could hydrolyse acetylcholine were first found in blood by STEDMAN AND EASSON[1]. NACHMANSOHN AND ROTHERBERG[2] reported that serum cholinesterase hydrolysed butyrylcholine more readily than acetylcholine, whereas erythrocyte cholinesterase hydrolysed acetylcholine readily, exhibited minimal activity towards butyrylcholine and hydrolysed D-acetyl-β-methylcholine (a substrate unaffected by the serum enzyme). Physostigmine (eserine) is a very potent inhibitor of both types of cholinesterase activity.

Several workers have studied methods for differentiating the two types of activity[3–7]. Many of these have used a combination of specific substrates and selective inhibitors. In this study several substrates have been used. The selective inhibitors chosen were: for acetylcholinesterase, 1,5-bis-(4-trimethylammonium phenyl) pentan-3-one diiodide (62C47); for pseudocholinesterase, ethyl propazine methosulphate (Lysivane).

Abbreviations: DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; 62C47, 1,5-bis-(4-trimethylammonium phenyl) pentan-3-one diiodide.

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Some investigators have put forward evidence which indicates cholinesterase to be a pre-requisite for the working of the sodium pump in a variety of tissues[8–13]. BRIGHENTI *et al.*[14] have also shown that in isolated perfused rat liver, acetylcholine may stimulate the production of amylase. In previous studies, acetylcholinesterase activity has been found to be distributed in varying amounts in subcellular fractions of rat liver (see ref. 15). DE DUVE *et al.*[16] suggested that the activity might be associated with the surface membrane.

The present report amplifies, and in general, supports this suggestion[16] and gives further properties of the enzyme species concerned. A preliminary report of some of these studies has appeared[17].

MATERIALS AND METHODS

Isolation of subcellular fractions

Liver tissue was obtained from 6–10-week-old Wistar strain rats of either sex. Surface membrane preparations were made by using the method of COLEMAN *et al.*[18]. Procedures for intermediate fractions are outlined in the appropriate legends. Nuclei were prepared according to the method of BLOBEL AND POTTER[19].

All procedures were carried out at 0–4°. Enzymic assays were carried out on freshly prepared material or on material which had been frozen at —20° (this had minimal effect on the enzymic activities studied).

Preparation of erythrocyte membranes

Blood was obtained from 4 rats by cardiac puncture using heparin as anticoagulant. The blood was centrifuged and the erythrocytes were washed in isotonic Tris–HCl, pH 7.2, with removal of the buffy coat. The erythrocytes were then lysed in distilled water (pH 7.2) and washed until the supernatant was colourless. The membranes were finally suspended in isotonic sucrose (pH 7.2). If stored in hypotonic media the cholinesterase activity is rapidly lost. (T. A. BRAMLEY, personal communication). Partially purified plasma membranes were prepared from the livers of the same rats which were used to provide the blood.

Colorimetric assay of cholinesterase activities

Cholinesterase activities were routinely determined using thiocholine esters by a modification of the method of ELLMAN *et al.*[20]. The incubation system contained: 67 mM phosphate buffer, pH 8.0, 0.3 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 5 mM acetyl- or butyrylthiocholine iodide (as appropriate), 0.1 mM inhibitor (as appropriate), enzyme and water; in a total volume of 3 ml. The system was preincubated for 5 min at 37° and the reaction was started by the addition of substrate. After incubation for 10 min the reaction was stopped with 1 ml ethanol; the tubes were then centrifuged. The yellow colour developed was read against a reagent blank containing buffer, ethanol and DTNB. Controls were carried out to allow for non enzymic substrate breakdown and also for the thiol content of the enzyme. In all experiments the acetylcholinesterase activity is taken as the part of the total cholinesterase activity inhibited by $1 \cdot 10^{-4}$ M 62C47.

Manometric assay of cholinesterases

The method of ALDRICH AND JOHNSON[21] was used.

Assay of K^+-stimulated phosphatase (EC 3.1.3.1)

Tube A contained 100 mM Tris–HCl, pH 8.8, 5 mM $MgCl_2$, 0.5 mM EDTA–Tris (pH 8.8), 14 mM KCl, 6 mM *p*-nitrophenyl phosphate (Tris), enzyme and water in a total volume of 1 ml.

Tube B contained identical components except for the substitution of 0.1 mM ouabain for the 14 mM KCl.

Both tubes were preincubated for 5 min at 37° and the reaction was then started with the substrate. Reaction was stopped after 15 min at 37°, by the addition of 3 ml of 200 mM NaOH and, after centrifugation, the yellow colours developed were read at 420 nm against a reagent blank. The K^+-stimulated activity was calculated from the subtraction of B from A.

Assay of ali-esterase

The incubation medium contained 2 mM 1-naphthyl acetate, 50 mM Tris–maleate, pH 6.8, and the enzyme, in a total volume of 1 ml. The reaction was started by the addition of substrate and terminated after 15 min by the addition of 0.2 ml 5 M $HClO_4$.

5 ml chloroform were added to each tube and shaken to extract the 1-naphthol liberated by enzymic action. The phases were separated by centrifugation and 4 ml of the chloroform layer was removed. To this was added 0.1 ml of Fast Blue R.R. (5 mg/ml in chloroform–methanol, 1:1, v/v), followed by 4 drops of $NaHCO_3$ to neutralize the residual $HClO_4$. 3 ml of methanol were then added and the mixture was shaken and centrifuged to remove the precipitated excess bicarbonate.

The colour was read against a reagent blank at an absorbance of 535 nm. The 1-naphthol standard curve is bimodal, but each mode is linear. In this system the two modes meet at $A_{535\,nm}$ 0.205 and the ratio of the slopes is 0.707. If the correction for values above 0.205 ($A' = 0.707$ (A—0.205) + 0.205) is applied, 1 μmole of 1-naphthol in 7.2 ml gives an $A_{535\,nm}$ of 2.0.

Assay of other enzymes

Other assays used were: 5'-nucleotidase (EC 3.1.3.5), MICHELL AND HAWTHORNE[22]; glucose-6-phosphatase (EC 3.1.3.9), HÜBSCHER AND WEST[23].

Expression of activities

The specific activities of all enzymes are reported in terms of moles of substrate hydrolysed or product formed per h per mg protein at 37°. Relative specific activity refers to the specific activity of the studied material divided by the specific activity of the homogenate.

Chemical estimations

Phospholipids were extracted by the method of GARBUS *et al.*[24], omitting phosphate from the strong salt wash. Organic phosphate was determined by the method of KING[25].

Nucleic acid was extracted by the method of SCHNEIDER[26]. DNA was determined by the method of BURTON[27]. Protein was determined by the method of LOWRY *et al.*[28].

Chemicals used

Substrates and other fine chemicals used in the assays were obtained from Sigma Chemical Co., London. 1,5-bis-(4-trimethylammonium phenyl) pentan-3-one diiodide (62C47) was a gift from Dr. C. T. Openshaw, Burroughs Wellcome Research Centre. Ethylpropazine methosulphate (Lysivane) was a gift of May and Baker Ltd. Eserine sulphate was obtained from the Pharmacy Department, Queen Elizabeth Hospital, Birmingham. All other chemicals were of analytical grade or of the highest grade commercially available.

RESULTS

Subcellular distribution of acetylcholinesterase

Perfused rat livers were homogenised and subjected to differential centrifugation and washing procedures to give a series of primary subcellular fractions (Fig. 1a). The washed nuclear fraction was homogenized vigorously and subjected to further separation procedures (Fig. 1b). All fractions were then assayed for enzymic activity.

Acetylcholinesterase (62C47-inhibited acetylthiocholinesterase) activity appeared in every fraction. A proportion appeared to be soluble after both procedures (fractions S and E), but the major part of the enzyme activity was particulate. From Fig. 1a it can be seen that specific activity is increased in fractions N, L, and Mc. This pattern of distribution is distinctly different from that of glucose-6-phosphatase which is concentrated in fractions L and Mc. Since glucose-6-phosphatase is an enzyme which is an intrinsic part of the endoplasmic reticulum of the hepatocyte, the distribution of acetylcholinesterase suggests that it is probably not a typical enzyme of the endoplasmic reticulum. Under comparable conditions of homogenization and separation, enzymes characterizing certain other subcellular components show patterns of distribution significantly different from acetylcholinesterase: succinate dehydrogenase (mitochondria), fractions M^1, M^2, L; acid phosphatase (lysosomes), fractions M^2, L, Mc; and glucose-6-phosphate dehydrogenase (cytosol), fraction S.

The distribution of acetylcholinesterase in primary fractionation is, however, more reminiscent of enzymes found in the surface membrane. The behaviour of two such enzymes was examined for comparison: 5′-nucleotidase and K^+-stimulated phosphatase. Both these enzymes showed enrichment in fraction N, but differed from acetylcholinesterase in relative enrichment in the later subfractions.

Upon further homogenisation and resolution of the nuclear pellet, the divergence in distribution between acetylcholinesterase and glucose-6-phosphatase became more marked; whereas a greater correlation between acetylcholinesterase and the surface membrane enzymes became more apparent (Fig. 1b).

Density-gradient centrifugation of a partially purified surface-membrane fraction further separated acetylcholinesterase and glucose-6-phosphatase. Under these circumstances the similarity in distribution was still apparent between acetylcholinesterase and 5′-nucleotidase (Fig. 2).

Acetylcholinesterase activity of nuclei

Surface membranes were, in these studies, obtained from fractions rich in nuclei. Contribution of the nuclear membrane to the observed cholinesterase activity is therefore possible. Experiments were designed to investigate this possibility.

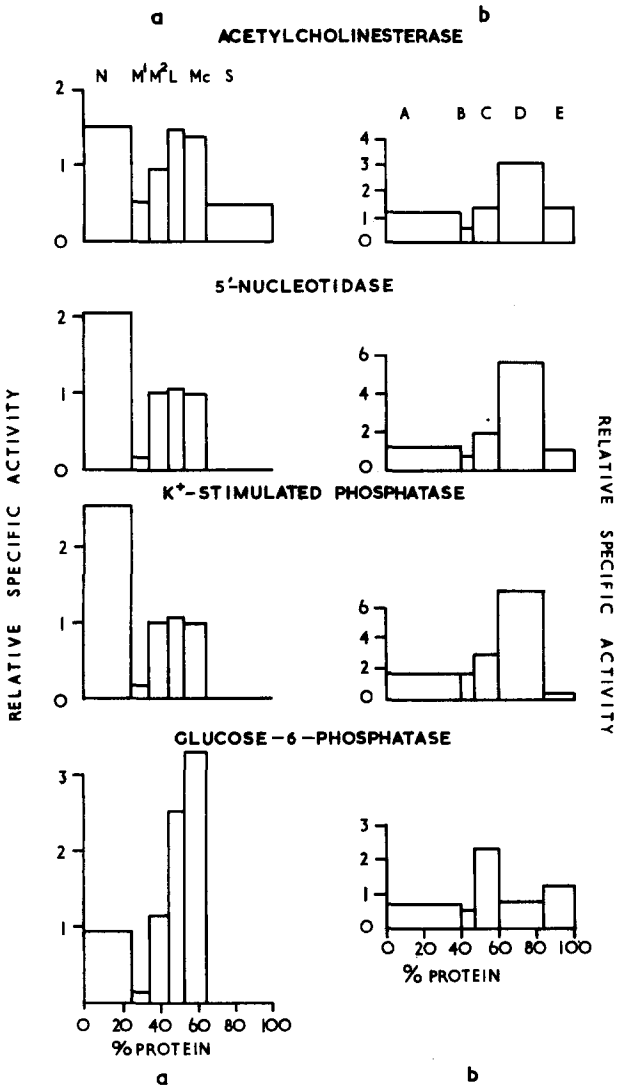

Fig. 1. Distribution of enzymic activities during fractionation of liver tissue. Primary fraction: Nuclear pellet 3 × washed 10000 × g·min (N); mitochondrial fractions 2 × washed, 30000 × g·min, pellet divided into lower zone (M^1) and upper zone (M^2); lysosomal fraction 1 × washed 240000 × g·min (L); microsomal fraction 6000000 × g·min (Mc) and supernatant (S). Secondary fractionation of the nuclear fraction (N) was carried out according to ref. 18 to give Fractions A–E. The names used in ref. 18 to refer to the appropriate fractions in the present experiments are given in brackets. A, 10000 × g·min pellet [P_2]; B, 30000 × g·min pellet [P_3]; C, 600000 × g·min pellet [P_4] lower layers; D, 600000 × g·min pellet [P_4] loose fluffy outer layer (the partially purified surface membrane preparation); E, supernatant [S_4]. Acetylcholinesterase activity is measured as 62C47-inhibited acetylthiocholine hydrolysis. Relative specific activity is expressed relative to homogenate specific activity. Widths of columns represent per cent recovered protein. Mean of three experiments.

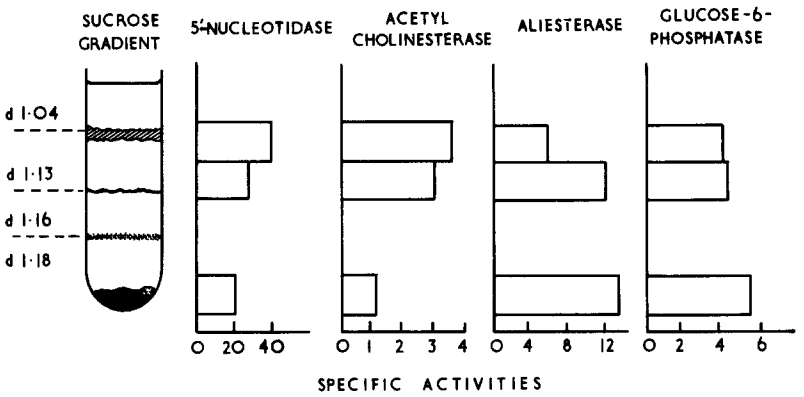

Fig. 2. Distribution of enzymic activities upon density-gradient centrifugation of the partially purified surface-membrane fraction (D). Fractions at each interface as indicated were diluted to bring to approx. 0.3 M sucrose (see ref. 18). Thin-layer floating on *d* 1.13 is referred to in the text as the purified surface membrane preparation. Specific activities are expressed as μmoles/h per mg protein at 37°.

Preparations of intact nuclei were obtained using the method of BLOBEL AND POTTER[19]. In this method the use of high-density sucrose was expected to reduce the contribution of other membranes. This is confirmed by the analytical results (Table I). It is therefore unlikely that the membranes of the nucleus make any significant contribution to the acetylcholinesterase distribution observed in the previous experiments.

TABLE I

COMPOSITION OF A PURIFIED PREPARATION OF RAT LIVER NUCLEI

The recoveries are expressed relative to the original liver homogenate.

Component	% *Recovery*
DNA	90
Acetylcholinesterase	1.8
5'-Nucleotidase	1.6
Glucose-6-phosphatase	3.5

Contribution of erythrocyte cholinesterase to the cholinesterase activity of liver surface membrane preparations

CASTANGA[29] reported that up to 20 % by weight of the liver is contributed by contained blood. The presence of blood within the liver is often ignored in studies involving subcellular fractionation.

Erythrocyte membranes of many animals possess quite high levels of acetylcholinesterase activity. Should erythrocytes become ruptured during the homogenisation and separation procedures used in the preparation of liver surface membranes, then erythrocyte membrane contamination could substantially contribute to the acetylcholinesterase activity of the preparation.

When liver surface membranes and erythrocyte membranes were prepared from

the same rats (see MATERIALS AND METHODS), the specific activities of the preparations obtained were 1.66 (liver) and 5.08 (erythrocytes) μmoles substrate hydrolysed/h per mg protein (mean of two experiments). Thus the erythrocyte surface membrane preparations have 3.5 times the specific activity of the liver surface membrane preparations. This could mean that if the acetylcholinesterase activity of the liver surface membrane preparation were due solely to the presence of erythrocytes, the membrane fraction isolated would be, in terms of protein, approximately one third erythrocyte membrane in origin.

If, however, the liver is carefully perfused with 0.3 M sucrose prior to removal from the animal, the blood content of the tissue is very substantially lowered. The acetylcholinesterase activity of the partially purified surface membrane preparation derived from these perfused livers [1.19 ± 0.37 μmoles substrate hydrolysed/h per mg protein (3 experiments)] is not significantly different from that derived from livers containing much greater amounts of blood at the time of homogenisation, (1.25 ± 0.42 μmoles/h per mg protein (7 experiments)).

When rat blood diluted with 0.3 M sucrose is subjected to the same conditions of homogenisation and fractionation as are used in the preparation of liver surface membranes, the yield of material in the final stage of the preparation is exceedingly small, equivalent to only 0.7 % of the protein content of the final plasma-membrane fraction prepared from non-perfused livers (R. COLEMAN AND R. H. MICHELL, personal communication). Thus the contribution of erythrocyte cholinesterase is likely to be only in the region of 4 % (0.7 × 5.08/1.66) of the total acetylcholinesterase of the surface membrane fraction from non-perfused livers. The contribution of erythrocyte cholinesterase to the total activity of surface membranes derived from perfused liver is likely to be smaller still.

Differentiation of acetylcholinesterase and ali-esterase

In order to determine whether ali-esterase (non-specific esterase) activity might bear some relationship with, or be a contributor to, the observed acetylcholinesterase activity of liver subcellular fractions, assays for esterase activity were carried out in parallel with those of acetylcholinesterase and other enzymes (Figs. 2 and 3). The results show little correlation with acetylcholinesterase activity or with the surface membrane enzymes, but show a strong correlation with glucose-6-phosphatase activity. Thus the activity of esterase, which appears to be located predominantly in the endoplasmic reticulum, has little relationship with the observed acetylcholinesterase distribution.

It was also found that whereas acetylcholinesterase activity in liver is inhibited by $1 \cdot 10^{-4}$ M 62C47, the inhibitor did not inhibit esterase activity at this concentration.

Cholinesterase activity of liver surface membrane with other substrates

In order to confirm the results obtained with the colorimetric assay and to investigate activity towards other substrates, a study was made using the manometric techniques of ALDRIDGE AND JOHNSON[21]. Due to the relative insensitivity of the method related to the amount of material available for study, it was seldom possible to examine the activity towards all substrates in any one preparation. Comparable results with acetylthiocholine as reference substrate were obtained with different

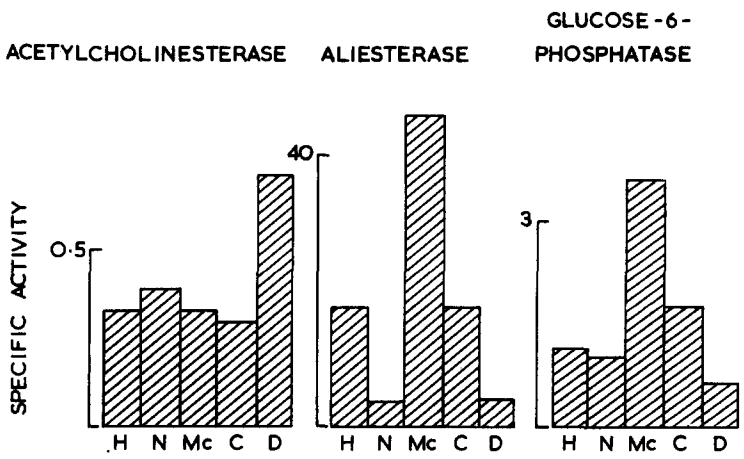

Fig. 3. Distribution of acetylcholinesterase, ali-esterase and glucose-6-phosphatase in subcellular fractions of rat liver. For conditions of fractionation see previous legends. Abbreviations: H, homogenate; N, nuclear fraction; Mc, microsomal fraction; subfractions of primary nuclear pellet, C, 600 000 × g·min pellet lower layers; D, fluffy outer layer (partially purified surface-membrane preparation). Specific activities are expressed as μmoles/h per mg protein at 37°.

preparations. The actual activity towards acetylthiocholine measured by the manometric technique was close to (within 10 %) that measured by the colorimetric assay.

If one assumes that the specificities of the inhibitors are the same in liver as they are in brain (see ref. 5), it is then possible to calculate the relative proportions of each substrate hydrolysed by both enzyme activities (Table II). It can be seen

TABLE II

THE DEGREE OF HYDROLYSIS OF VARIOUS SUBSTRATES BY ACETYL- AND PSEUDOCHOLINESTERASE ACTIVITIES CALCULATED BY THE METHOD OF BAYLISS AND TODRICK[5]

The proportion of acetylcholinesterase, with acetyl β-methylcholine was calculated from the inhibition by 62C47. The residual activity was assumed to be pseudocholinesterase activity. The activity of pseudocholinesterase with benzoylcholine, was calculated from the inhibition produced by Lysivane. Pseudocholinesterase activity with acetylthiocholine was calculated from the average of the inhibition produced by Lysivane and the residual activity in the presence of 62C47. Since the sum of inhibition produced by each substrate equals the uninhibited activity, it can be assumed that the observed hydrolysis of acetylthiocholine was achieved entirely by the joint action of the two cholinesterases. The results represent the means of two experiments using partially purified surface-membrane preparations.

Substrate	% *due to acetylcholinesterase*	% *due to pseudocholinesterase*
Manometric assay		
Acetyl-β-methylcholine	92	8
Acetylthiocholine	45	55
Benzoylcholine	27	73
Colorimetric assay		
Acetylthiocholine	55	45
Butyrylthiocholine	28	72

that the most specific substrates are acetyl-β-methylcholine for acetylcholinesterase and benzoylcholine for pseudocholinesterase.

Determination of acetyl- and pseudocholinesterase activities in surface membrane preparations by the use of the colorimetric assay

Table II also presents results obtained using thiocholine substrates and with 62C47 and Lysivane as specific inhibitors for acetyl- and pseudocholinesterase, respectively. With respect to the relative contribution to the hydrolysis of acetylthiocholine by each enzyme, the results from the colorimetric technique agree fairly well with those of the manometric technique. From the results in Table II, it is clear that the hydrolysis of butyrylthiocholine is largely carried out by pseudocholinesterase.

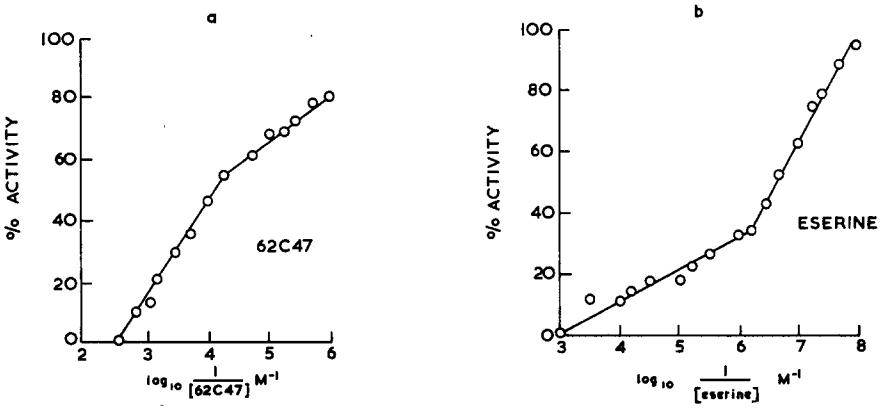

Fig. 4. Inhibition of cholinesterase activity. Activity was measured with acetylthiocholine with the colorimetric assay. Figures represent mean values of two experiments. Enzyme preparation used was the partially purified plasma-membrane fraction (D) (a) with 62C47, (b) with eserine.

When the concentration dependence of inhibition by 62C47 was studied, using acetylthiocholine as substrate (Fig. 4a), a break in the curve was apparent at approx. $1 \cdot 10^{-4}$ M. Inhibition of activity occurred at concentrations in excess of this, possibly due to the action of 62C47 upon the residual (pseudocholinesterase) activity. The addition of $1 \cdot 10^{-4}$ M Lysivane, to the cholinesterase activity remaining in the presence of $1 \cdot 10^{-4}$ M 62C47, produced a complete inhibition of both enzyme activities.

In these inhibition experiments, eserine ($1 \cdot 10^{-4}$ M) was used as a further check on the applicability and effectiveness of both inhibitors. Eserine inhibits the activities of both cholinesterases and the values given by eserine inhibition agreed closely with the sum of the appropriate values obtained with the other, more specific inhibitors.

When the concentration dependence of inhibition was studied in detail (Fig. 4b) a break in the response to eserine was noted at about $1 \cdot 10^{-6}$ M, and represented a decrease of approx. 60% in the rate of acetylthiocholine hydrolysis. Whether this break represents the total inhibition of one of the cholinesterases remains to be established.

Solubilization of acetylcholinesterase activity from surface-membrane preparations

MITCHELL AND HANAHAN[30] reported that treatment of erythrocyte ghosts with hypertonic saline led to a partial solubilization of their cholinesterase activity. Under

similar conditions, 30 % of the acetylcholinesterase activity, 60 % of the protein, 26 % of the phospholipid, but only 6 % of the 5'-nucleotidase were solubilized from rat liver surface membranes (Table III). From these results it appears that the behaviour of liver surface-membrane acetylcholinesterase resembles that of erythrocyte acetylcholinesterase, and is more readily solubilized than 5'-nucleotidase. Treatment of the surface-membrane preparations with isotonic NaCl did not release acetylcholinesterase activity.

TABLE III

EFFECT OF TREATMENT OF SURFACE MEMBRANE PREPARATIONS WITH 1.5 M NaCl

The pellet of partially purified surface membranes was suspended in 1.5 M NaCl and incubated at 0° for 30 min. The membranes were then sedimented (2000000 × *g*·min) and the pellet washed with 30 ml 0.3 M sucrose and re-sedimented. The two supernatants were combined.

	% *Recovered component*			
	Protein	*Phospholipid*	*Acetyl-cholinesterase*	*5'-Nucleotidase*
Sediment	40	74	70	94
Supernatant	60	26	30	6
Recovery	98	100	91	92

Subcellular distribution of cholinesterase activities

Fig. 5 presents preliminary experiments in which the relative distributions of acetylcholinesterase and pseudocholinesterase have been compared.

From previous studies it appeared that butyrylthiocholine was a more effective substrate for pseudocholinesterase than was acetylthiocholine. The inhibition of

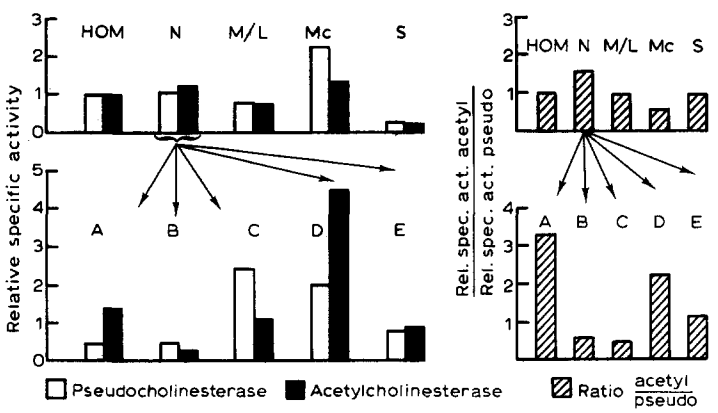

Fig. 5. Cholinesterase activities of subcellular fractions of rat liver. Primary fractionation: nuclear pellet 3 × washed 10000 × *g*·min (N); mitochondrial–lysosomal fraction 1 × washed 240000 × *g*·min (Mc); supernatant fraction (S). Secondary fractionation of primary nuclear fraction according to ref. 18, details of naming *etc.* see Fig. 1. Homogenate activities: pseudocholinesterase (Lysivane-inhibited butyrylcholinesterase), 0.30 μmole/h per mg protein at 37°; acetylcholinesterase (62C47-inhibited acetylcholinesterase), 0.49 μmole/h per mg protein. Recoveries of enzymic activity in primary fractionation: pseudocholinesterase 92 %; acetylcholinesterase 98 %. Recoveries in fractionation of nuclear pellet: pseudocholinesterase 83 %, acetylcholinesterase 97 %. Single experiment.

butyrylthiocholine hydrolysis in the presence of $1 \cdot 10^{-4}$ M Lysivane was therefore taken as a measure of pseudocholinesterase activity. Acetylcholinesterase activity is expressed as the inhibition of acetylthiocholine hydrolysis produced by addition of $1 \cdot 10^{-4}$ M 62C47.

Pseudocholinesterase distribution does not resemble acetylcholinesterase distribution. Pseudocholinesterase activity occurred, on primary fractionation, principally in the microsomal fraction (43 % of recovered activity). The activity in this fraction was enriched 2.2 times. Upon homogenization and further fractionation of nuclear material (which led to purification of acetylcholinesterase) the pattern of distribution again did not correlate with acetylcholinesterase. The divergencies are most easily seen in the ratio of the two activities. Therefore neither in terms of absolute activity nor in terms of ratios of specific activity is there seen a systematic relationship between acetyl- and pseudocholinesterases.

DISCUSSION

Assessment of the progress of purification of a subcellular organelle or membrane is usually based on the selective enrichment of some characteristic morphological, immunological, chemical or enzymic feature of the component in question. At the same time, comparable features of the other subcellular components are progressively decreased in the isolated fraction.

The surface membrane of liver (and several other) cells, contains the enzyme 5'-nucleotidase. This enzyme has frequently been used to assess the degree of purification of surface membranes from cells of liver tissue. An enrichment with respect to acetylcholinesterase was also observed during the isolation of fractions progressively enriched in their content of surface membrane. The specific activity of acetylcholinesterase was greatest in those preparations most highly enriched in surface membrane. These findings therefore support the suggestion of DE DUVE[16] (see INTRODUCTION). The levels of acetylcholinesterase in the most highly purified surface-membrane preparations (1.2–4 μmoles/h per mg protein) were in the same range as that reported by STAHL AND TRAMS[31], for a preparation of surface membrane made by the method of NEVILLE[32]. The specific activity of their starting homogenate was not reported and so the enrichments cannot be compared more precisely.

In the technique used for the preparation of surface membranes, there is a progressive removal of all other cellular components for which analyses have been made[18]. No component other than surface membrane is known to become appreciably enriched under the conditions in which the increase in acetylcholinesterase was noted. In respect of endoplasmic reticulum fragments, which are the major contaminant membranes in the surface-membrane preparation, a significant divergence is only noted between the levels of these (as judged by glucose-6-phosphatase) and acetylcholinesterase enrichment levels.

Since in these studies surface-membrane preparations were obtained from crude preparations of nuclei, the possibility of a contribution of cholinesterase activity by nuclear membranes must be entertained. Assaying for these membranes is difficult since reliable identification procedures on fragmented material are unavailable. An indirect approach therefore had to be used. All indications from this are that nuclear membranes also do not make a significant contribution to the acetylcholinesterase

activity. When Ca^{2+} was present during initial homogenization in order to make nuclear rupture less likely, surface-membrane preparations were obtained which contain no DNA but with high acetylcholinesterase activities (G. E. WHEELER, unpublished observations).

As indicated in Fig. 1, acetylcholinesterase activity is found in all cell fractions. It is possible therefore that all subcellular structures possess some acetylcholinesterase activity, though with specific concentrations much less than that of surface membrane preparations. Since 5'-nucleotidase activity was also found in these fractions, it is also possible that at least a proportion of the acetylcholinesterase activity of these fractions is due to their content of surface-membrane material.

More definite information on this would be gained from the preparation of organelles devoid of surface-membrane contamination, but this is not within the scope of the present study. In the case of nuclei, however, purified preparations (whether expressed in terms of protein or DNA content or in content of membrane phospholipid) contain very little acetylcholinesterase activity. The possibility of contamination by erythrocyte membranes is of particular interest in view of the high acetylcholinesterase activity of these membranes. The minimal differences in acetylcholinesterase content between surface-membrane preparations made from perfused and unperfused livers, and the behaviour of erythrocytes during homogenization in sucrose solution, suggest that erythrocyte membrane contribution to the observed pattern of activity distribution is of only minor importance.

At least two enzymes are involved in the hydrolysis of the substrates used in the present study. Acetyl-β-methylcholine (a highly specific substrate for acetylcholinesterase in brain) is hydrolysed by rat liver surface-membrane preparations. This hydrolysis is scarcely affected by Lysivane. The contribution of pseudocholinesterase activity to the hydrolysis of this substrate is therefore minimal.

From the preliminary experiments reported in Fig. 5, the distribution of pseudocholinesterase activity appears dissimilar to that of acetylcholinesterase, and must reflect differences in intracellular distribution. Pseudocholinesterase activity, whilst complex, appears to follow most closely that of a commonly accepted endoplasmic reticulum constituent, glucose-6-phosphatase. This subcellular distribution is similar to that of ali-esterase. As purification of surface membranes is accomplished, as judged by increasing relative specific activity of 5'-nucleotidase, the acetylcholinesterase: pseudocholinesterase ratio increases. The pseudocholinesterase activity remaining in purified surface-membrane preparations may either represent contamination with other subcellular material, or may be an intrinsic component of the purified membranes.

Preliminary studies indicated that at low substrate concentrations the apparent values for K_m were $0.53 \cdot 10^{-4}$ M for acetylthiocholine and $1.43 \cdot 10^{-4}$ M for butyrylthiocholine. At high substrate concentrations both substrates gave an apparent K_m of $5 \cdot 10^{-4}$ M. The energy of activation for acetylcholinesterase (acetylthiocholine–62C47) was 5.5 kcal/mole and that for pseudocholinesterase was 7.6 kcal/mole (acetylthiocholine–Lysivane) and 10.7 kcal/mole (butyrylthiocholine–Lysivane). (G. E. WHEELER, unpublished observations). These figures show fairly close agreement with erythrocyte acetylcholinesterase (3.7 kcal/mole) and serum pseudocholinesterase (7.7 kcal/mole)[33].

In spite of the general correlation between acetylcholinesterase and 5'-nucleo-

tidase distributions during cell fractionation, the distributions of the two enzymes are not exactly parallel, and there is a marked difference in the final enrichments achieved. One contributing factor must be the amount of soluble acetylcholinesterase in the liver homogenate. This will have the effect of causing the increase of relative specific activity with respect to acetylcholinesterase to be lower that that for 5'-nucleotidase (which has little soluble activity). In this connection, the solubilization of acetylcholinesterase (but not of 5'-nucleotidase) from the particulate material should be noted (Table III). A second factor may be erythrocyte membrane acetylcholinesterase which, whilst contributing to the activity of the homogenate and the nuclear pellet, contributes much less to the activity in the later stages in the preparation. It is also possible that 5'-nucleotidase and acetylcholinesterase may be distributed differently between hepatocyte and reticuloendothelial cells, and membranes from these may be isolated differentially.

The hepatocyte surface membrane has three morphologically distinct regions: the bile canaliculus, the sinusoidal face and that region opposed to other hepatocytes. Due to the presence of desmosomes and junctional complexes, fragmentation of hepatocytes may give rise to segments of surface membrane of a size partly dictated by their morphological location and attachments. Segments of different sizes (and different composition) might be expected to have slightly different properties with respect to differential and density-gradient centrifugation. If, in addition, the enzyme complement of each region were somewhat distinct (a reflection of the different metabolic activity of the various faces of the cell) then a degree of divergence might be expected in the enzymic composition of isolated surface-membrane fractions. Such divergence has been observed for certain enzymes in liver (EVANS[34]) and intestinal mucosa (QUIGLEY AND GOTTERER[35]) and may also be contributing factor in the present situation.

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