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PROPERTIES AND CLASSIFICATION OF THE SOLUBLE ESTERASES OF HUMAN LIVER

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Abstract—Water-soluble enzymes of human liver were separated by zone electrophoresis in starch gel and compared with those of serum. Three separate zones of esterase activity in the liver extracts were found. An attempt was made to identify these enzymes by using several series of substrates and inhibitors in conjunction with various histochemical staining techniques. One enzyme, migrating at pH 7·2 toward the cathode, reacts like an acetylesterase. Another enzyme, migrating toward the anode at a rate approximating that of serum albumin, reacts like an aliesterase. A third region of activity contains a mixture of esterases and acid phosphatase. The organophosphorus anticholinesterases that were tested showed different patterns of inhibition of the liver esterases. Observations on alkaline phosphatase, sulphatase, glucosidase, galactosidase, and leucine aminopeptidase were recorded for comparison with the data on esterases.

THERE ARE three types of esterases in the serum of animals; i.e., aromatic esterases (A-esterases), aliphatic esterases (B-esterases), and cholinesterases (C-esterases). Aromatic esterases hydrolyse phenyl and *p*-nitrophenyl esters, and are neither inhibited by eserine nor by organophosphorus compounds. Aliphatic esterases preferentially hydrolyse aliphatic esters (tributyrin, etc.) and are inhibited by organophosphorus compounds but not by eserine. Cholinesterases (i.e., acetylcholinesterase and pseudocholinesterase) hydrolyse choline esters at a higher rate than both aliphatic and aromatic esters and are inhibited by eserine and organophosphates.[1–3] Enzymes of these three classes are not found in the sera of every species, however, and the substrate specificities of corresponding enzymes may vary from species to species.[4, 5] Human serum contains pseudocholinesterase and aromatic esterase.[6] Since it has been suggested that these enzymes are formed in the liver,[7] we expected to find them in extracts of human liver.

Starch gel electrophoresis was first used successfully by Smithies for the separation of serum proteins.[8, 9] Subsequent investigators adapted this procedure to the study of enzymes (chiefly esterases) of mouse and rat tissue,[10, 11] human serum,[12] maize,[13] unicellular organisms,[14] and the silk moth.[15] The present paper deals with the application of this method to the water-soluble enzymes of the human liver. A preliminary report on this work has been published elsewhere.[16]

METHODS AND MATERIALS

Samples of human liver were obtained at autopsy from unselected cases in the pathological laboratory of a general hospital and were frozen immediately on removal. The preparation of the homogenates has been described.[16] The basic procedure was to homogenize the tissues in distilled water, then to freeze and thaw the homogenates, centrifuge them at 11,000 × *g* and concentrate the supernatant.

Alternative procedures were tested, substituting isotonic sucrose solution (0·25 M), physiological saline, or 0·06 M phosphate buffer for water in homogenizing the liver samples. The extracts had a granular appearance and contained particles that were exceedingly difficult to separate by centrifugation; upon electrophoresis they showed gross streaking and tailing in all zones. These solutions yielded a poorer extraction of the esterases, as evidenced by the fact that the enzymic activity of the various zones was relatively low, requiring a long incubation period. The aqueous extract was by far the better preparation, producing a clear, homogeneous sample which, on electrophoresis, was resolved into sharp, distinct bands in the gel.

Aware that our treatment of the liver enzymes in the extraction might alter their properties in some way, we subjected human serum to the same procedures as the liver tissue and subsequently compared the untreated and treated serum electrophoretically. The staining and migrating properties of the serum esterases remained unchanged.

Electrophoresis

Zone electrophoresis was carried out with hydrolyzed potato starch (Connaught Medical Research Lab., Toronto, Canada) according to the method of Smithies.[8, 9] Modifications of this procedure have been described.[16]

Protein staining

A saturated solution of amido black B in methanol : water : glacial acetic acid (50 : 50 : 10) was used to locate the proteins. After treating the gels with this solution for 3 min, the dye solution was poured off and excess dye was removed by continuous washing with the same solvent mixture. Lipoproteins were detected by sudan black B in 55% ethanol, the starch strips incubated for 1 hr. The methanol–water–acetic acid solution was used after all procedures to harden and preserve the gel.

Histochemical procedures

Esterase activity was demonstrated by a number of different procedures. One was a modification of the methods of Hunter and Markert[10] and Nachlas and Seligman.[17] The substrates tested were α-naphthyl acetate, β-naphthyl acetate, 6-bromo-2-naphthyl acetate (Dajac), naphthol AS acetate (Dajac), naphthol AS-D acetate (Sigma), β-naphthyl benzoate (Eastman Kodak), β-naphthyl caprylate, β-naphthyl laurate, β-naphthyl myristate, and β-naphthyl stearate (Dajac). Starch strips were incubated for varying intervals in a mixture of two solutions. Five millilitres of a 1·0% solution of the substrate in acetone, and 20–30 mg of either naphthanil diazo blue B (tetrazotized *o*-dianisidine) (Sigma) or Fast Blue RR (diazotized 4-benzoyl-amino-2, 5-dimethoxyaniline) (Dajac) were dissolved separately in 100 ml of 0·2 M phosphate buffer, pH 6·8. Five millilitres of 1·0% calcium chloride was added to the dye solution, and the two solutions were poured simultaneously over the gel. The reaction for the above procedure depends upon the enzymic hydrolysis of the ester, releasing naphthol which simultaneously couples with the dye, precipitating at or near the site of the enzyme.[18] A choline ester, β-carbonaphthoxycholine iodide (Sigma)[19], also was used with this procedure, the reaction being analogous to that of β-naphthyl acetate.

In addition, indoxyl acetate (Allied Chemicals)[20] and 5-bromoindoxyl acetate (Sigma)[21] were used to localize esterases. A 0·5% solution of the substrate in absolute

ethanol was mixed with 5 ml 1·0% calcium chloride, 35 ml physiological saline, and 45 ml 0·025 M borate buffer, pH 8·3. The gel strip was incubated in the medium for 20 min. The enzymic hydrolysis of the ester produced two indoxyl radicals which link together to form indigo blue that precipitates at the enzyme site.[20] Also employed for esterases was Gomori's modification of Koelle and Friedenwald's procedure utilizing acetylthiocholine as the substrate.[22]

The activities of other hydrolytic enzymes were demonstrated by the procedures of Laufer[15] developed from those of Hunter and Markert. Alkaline phosphatase was demonstrated, with 30 mg of sodium or calcium β-naphthyl acid phosphate (Dajac) and 30 mg of naphthanil diazo blue B in 0·2 M Tris-HCl buffer at pH 9·4; for acid phosphatase, the substrate was sodium or calcium α-naphthyl acid phosphate (Dajac) in 0·2 M sodium acetate-acetic acid buffer at pH 5·0. Leucine aminopeptidase activity was demonstrated by the use of L-leucyl-β-naphthylamide (Dajac) as a substrate. The reacting medium was identical with that above employing the naphthyl esters. The pH of the final mixture was 7·6. In the same manner, 6-bromo-2-naphthyl-β-D-glucopyranoside and 6-bromo-2-naphthyl-β-D-galactopyranoside were used to detect glucosidase and galactosidase, respectively.

The presence of sulphatase was demonstrated by a modification of the methods of Seligman *et al.*[23] and Seligman and Nachlas.[24] The substrate, 6-bromo-2-naphthyl sulphate, in a concentration of 1·0% in 0·2 M sodium acetate-acetic acid buffer, pH 6.1, was incubated with the gel strip for 4 hr with occasional shaking. At the end of this time 1·0% naphthanil diazo blue B in 0·2 M phosphate buffer, at pH 7·6, was added and allowed to react with the hydrolyzed substrate in a post-hydrolysis coupling reaction as discussed by Pearse.[18]

To aid in the detection of lipase, sodium cholate (Mann Research Labs.), in a concentration of 1·0%, was added to the substrate-dye solution. The addition of this compound to the system serves to activate lipases and to inhibit esterases.[25, 26] The substrates used to detect lipase activity were the β-naphthyl esters of caprylic, myristic, lauric, and stearic acids.

Identification of serum esterases

In an experimental series performed before samples of human liver became available, starch gel electrophoresis was used for the separation of serum esterases in order to elute the enzymes from the gels and test them *in vitro*.

After electrophoresis the gels were stained for a short time with β-naphthyl acetate in order to locate the enzyme zones. The moment the zones were faintly visible, the staining solution was discarded, the gels rinsed with physiological saline, and the stained areas cut out of the gels.

The excized zones were frozen to − 20 °C, then homogenized in a Servall Omni-Mixer with 50 ml of 0·06 M phosphate buffer. Each homogenate was frozen again to −20°C. After thawing, the homogenates were centrifuged at 18,000 × *g* for 30 min in a refrigerated centrifuge at 0 °C. The supernatant (approximately 40 ml) was removed and the sediment discarded. The eluates were concentrated in a viscose dialysis bag against polyethylene glycol (Carbowax). Final volumes generally were 15 ml.

Measurements of enzyme activity were carried out in a Beckman DK2 recording spectrophotometer with a time-drive accessory, at a temperature of 25 °C, and absorption cells with a light path of 10 mm. Pseudocholinesterase activity of the eluate was

detected by a modification of a previously described procedure,[27, 28] using benzoylcholine in a final concentration of 5×10^{-5} M. Dibucaine number (DN) were determined as described earlier,[29] and the ability of tetraethylpyrophosphate (TEPP) to inhibit the enzyme was tested.

Aromatic esterase activity was detected in the concentrated eluates by following the change in absorbance during the hydrolysis of phenyl acetate at a final concentration of $2{\cdot}3 \times 10^{-3}$ M.[30] Tests were done in the absence and presence of 10^{-3} M calcium chloride. Sometimes disodium versenate was added to the reaction mixtures in a final concentration of 10^{-3} M.[31]

Inhibition studies

Further to characterize the esterases, the gels were exposed to the following cholinesterase inhibitors in physiological saline (concentrations are listed in Table 2): Eserine, (Nutritional Biochemicals Corp.) RO2-0683 (dimethylcarbamate of 2-hydroxy-5-phenyl-benzyl trimethylammonium bromide; Roche).[32] BW 284c51 (1, 5-*bis*(4-allyl dimethylammonium phenyl)pentan-3-one diiodide).[33] BW 62c47 (1, 5-*bis*(4-trimethylammonium phenyl)pentan-3-one-diiodide).[33] Decamethonium bromide.[12] *p*-Chloromercuribenzoate; (Sigma).[34] Sodium fluoride.[35] TEPP; (K and K Labs).[36] DFP (diisopropylfluorophosphate).[37] Malathion (O,O-dimethyl-S-(N-methylcarbamylmethyl)thiothionophosphate; Green Cross).[36] Phosdrin (dimethyl-1-methyl-2-carbomethoxyvinyl phosphate).[36]

The gel strips were placed in solutions of the respective inhibitor concentrations for 20 min prior to putting them into the substrate-dye solution. β-Naphthyl acetate was the substrate used, since it was hydrolysed by all liver and serum zones. Where the inhibition was found to be reversible (the first five compounds above), inhibitor of the same concentration was added to the staining solution.

In the manner described above, the presence of sulphatase activity was confirmed by the use of an inhibitor, sodium sulphite (BDH Analar), in a concentration of 0·0025 M.[38]

RESULTS

Fig. 1 is a drawing based on previous observations,[16] the purpose of which is to orient and to define the zones and enzyme activities that are used in this paper. We will designate the enzymes contained in the zones by the zone number: the two esterases of human serum are aromatic esterase (zone S-1) and pseudocholinesterase (zone S-2), as determined by the elution studies.

The activity of zone S-2 toward benzoylcholine, inhibition by TEPP, and the determination of identical DN (within experimental error) to the crude serum shows that this eluate contains pseudocholinesterase (Table 1). This preparation did not hydrolyze phenyl acetate at a measureable rate.

The eluate of serum zone S-1 hydrolysed phenyl acetate at an increased rate on the addition of calcium ions (Table 1). In the presence of calcium the addition of disodium versenate produced a 100% inhibition of the activity. This preparation did not hydrolyse benzoylcholine. The properties of the eluate of S-1 are analogous to those found for aromatic esterase.

The distribution of the liver esterases as compared with the serum esterases is seen in Fig. 1. The liver esterases are found in three distinct zones (L-1, L-2, and L-3)

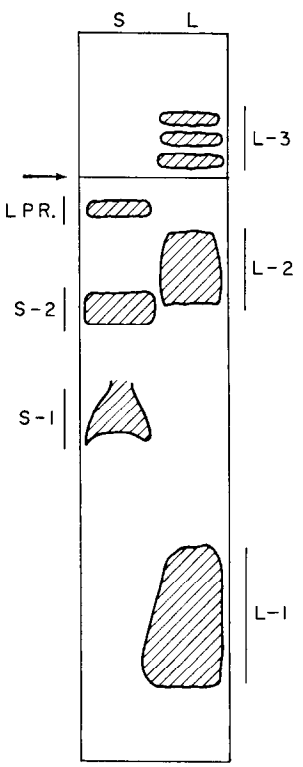

FIG. 1. The esterase patterns of human liver homogenates and serum in starch gel after electrophoresis as detected by β-naphthyl acetate. Abbreviations L and S indicate liver and serum samples, respectively; zones L-1, L-2, and L-3 indicate liver esterases; zone S-1 is A-type esterase; zone S-2 is cholinesterase of human serum. LPR indicates a lipoprotein fraction present in serum (identified by sudan black B). The arrow points to the origin. The anode is toward the bottom of the picture.

TABLE 1. THE HYDROLYSIS OF BENZOYLCHOLINE AND OF PHENYL ACETATE *in vitro* BY HUMAN SERUM AND BY ELUATES FROM STARCH GEL AFTER ELECTROPHORESIS*

	Benzoylcholine†		Phenyl acetate‡		
		Presence of 10^{-5} M dibucaine	10^{-3} M Ca added	No Ca added	Presence of 10^{-3} M EDTA
Eluate of S-1 diluted 1 : 2	0·000	0·000	0·049	0·023	0·000
Elute of S-2 diluted 1 : 2	0·183	0·031	0·000	0·000	0·000
Normal serum diluted 1 : 1000	0·172	0·029	0·138	0·056	0·000

* The enzymic activities are stated as changes of ultraviolet absorbance (A).
† Δ A_{240} mμ/15 min.
‡ Δ A_{269} mμ/3 min.

and show electrophoretic properties completely different from those of serum esterases. Zone L-3 is composed of three bands which have migrated toward the cathode rather than the anode. Zone L-3 does not appear if the borate buffer pH 8·3 is used instead of the phosphate buffer pH 7·2. Zones L-1 and L-2, while appearing here as solid bars, are composed also of bands that are only sometimes visible, and then immediately after the beginning of the staining reaction. Subsequent washing with the solvent mixture lightens the stain and these bands disappear.

On occasion an esterase, migrating and staining like zone S-2, was found in liver preparations. The staining reactions were faint enough to suggest that this was due to serum trapped in the liver tissue. This esterase did not show when the sample was taken from the drained upper portion of a bottled specimen of liver.

The specificity studies of the esterases are summarized in Figs. 2 and 3 and Table 2.

TABLE 2. THE SUBSTRATE SPECIFICITY OF THE ESTERASE ZONES OF LIVER AND SERUM IN STARCH GELS*

Substrates	Liver zones			Serum zones	
	L-1	L-2	L-3	S-1	S-2
α-Naphthyl acetate β-Naphthyl acetate 6-Bromo-2-naphthyl acetate	+	+	+	−	+
β-Naphthyl acetate (omission of calcium)	+	+	+	−	+
Indoxyl acetate 5-Bromoindoxyl acetate	+	+	+	−	+
β-Naphthyl caprylate β-Naphthyl myristate β-Naphthyl laurate β-Naphthyl stearate	+	+	−	−	+
Naphthol AS acetate	−	+	+	−	+
Naphthol AS-D acetate	+	+	+	−	+
β-Carbonaphthoxycholine iodide	−	+	−	−	+
Acetylthiocholine	−	−	−	−	+
β-Naphthyl benzoate	−	+	−	−	−

* + indicates the presence, − the absence, of a staining reaction as a result of esterase activity. All substrates were incubated with the gels for varying time periods. Prolonged incubation (especially with the long-chain, fatty-acid esters) resulted in staining in liver zone L-1.

The substrates 5-bromoindoxyl acetate and acetylthiocholine do not appear in the figure but they may be found in the table. The results of the inhibition studies are listed in Table 3.

Fig. 4 is a composite picture of the results of the substrates employed to demonstrate the presence of enzymes other than esterases. In Figs. 2, 3, and 4 the enzyme zones do not always seem to be equidistant from the origin. This is owing to the fact that a series of gels was tested with the different substrates. Due to current fluctuations, the distance of migration was not always the same in each gel. In addition, treatment of the gel with the solvent system causes shrinkage as well as hardening of the gel and this was not uniform for each gel.

The zones of liver and serum enzymes appear very broad, owing to diffusion artifacts caused by long periods of incubation required with some of the substrates. This may be overcome partly by a selection of the right pH, temperature, and a substrate

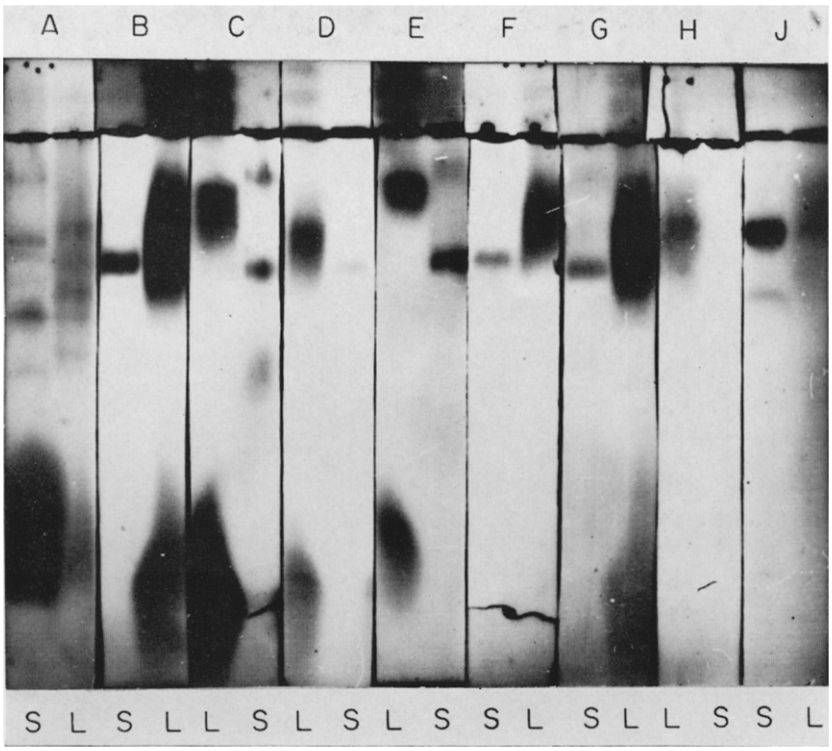

FIG. 2. Substrate specificities of the esterases of human liver homogenate and serum in starch gel after electrophoresis. Section A shows the location of the proteins. The substrates used in the other sections are as follows: B, β-naphthyl acetate; C, β-naphthyl acetate; D, 6-bromo-2-naphthyl acetate; E, indoxyl acetate; F, naphthol AS acetate; G, naphthol AS-D acetate; H, β-naphthyl benzoate; J, β-carbonaphthoxycholine iodide. S and L indicate serum and liver respectively. Note: In the serum portion of J, the second band beneath the pseudocholinesterase is not normally seen but may be due to hydrolysis by aromatic esterase during the prolonged incubation time required by this substrate.

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FIG. 3. Substrate specificity of esterases of human liver homogenate and serum in starch gel after electrophoresis, toward long-chain, fatty-acid esters as compared with β-naphthyl acetate. The substrates used in the sections are: A, β-naphthyl acetate; B, β-naphthyl caprylate; C, β-naphthyl myristate; D, β-naphthyl laurate; E, β-naphthyl stearate. S and L indicate serum and liver, respectively.

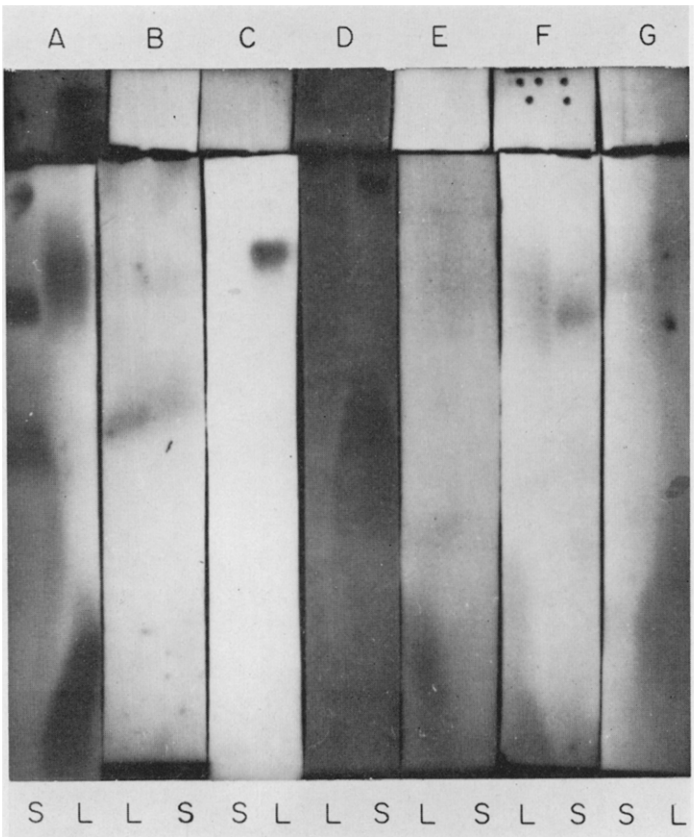

FIG. 4. Identification of various enzymes separated from the esterases of human liver homogenate and serum in starch gel by electrophoresis. The enzymes shown in each section are: A, esterases; B, alkaline phosphatase; C, acid phosphatase; D, sulphatase; E, leucine amino peptidase; F, galacto, sidase; G, glucosidase. The substrates are listed in the text. S and L indicate serum and liver- respectively.

with a large, insoluble breakdown product that will not diffuse from the position of the enzyme. One other cause of the wide zones is the presence of the bars within the zones, which may represent more than one enzyme.

Alkaline phosphatase was found in both serum and liver, localized in distinct zones that have the same electrophoretic mobility, suggesting that the enzymes from these two sources are identical. Acid phosphatase, on the other hand, was found only in the liver preparations, in liver zone L-2, as a narrow band.

TABLE 3. THE INHIBITION OF THE ESTERASE ZONES OF LIVER AND SERUM IN STARCH GELS

Inhibitor	Concentration	Liver zones			Serum zones	
		L-1	L-2	L-3	S-1	S-2
Eserine	1×10^{-4} M	−	+	−	+	−
	1×10^{-6} M	+	+	+	+	−
Decamethonium bromide	$1{\cdot}5 \times 10^{-5}$ M	+	+	−	+	−
p-Chlorohydroxymercuribenzoate	1×10^{-3} M	+	+	+	−	+
TEPP	$3{\cdot}65 \times 10^{-5}$ M	−	+†	−	+	−
DFP	1×10^{-4} M	−	−	−	+	−
	1×10^{-6} M	+	−	+	+	−
Malathion	1×10^{-3} M	−	−	−	+	−
Phosdrin‡	1×10^{-4} M	+	−	−	+	−
	1×10^{-7} M	+	+	−	+	+
	1×10^{-8} M	+	+	+	+	+
Sodium fluoride	2×10^{-1} M	+	+†	+	+	+†
	1×10^{-2} M	+	+†	+	+	+†

+ indicates the presence, − the absence of a staining reaction as a result of esterase activity. The substrate used to detect activity in all cases was β-naphthyl acetate with calcium added. RO2-0683 (4×10^{-6} M), BW 284c51 ($1{\cdot}75 \times 10^{-5}$ M), and BW 62c47 ($1{\cdot}75 \times 10^{-5}$ M) reacted similarly to eserine.

† Partial inhibition of the zone.

‡ Phosdrin was pretreated with ferricyanide (10^{-4} M).

As can be seen from Fig. 4, sulphatase activity was found only in serum, in the region of zone S-1. The identity of this enzyme was confirmed by the use of the inhibitor sodium sulphite, which completely inhibited the sulphatase but had no effect on aromatic esterase.

The results with 6-bromo-2-naphthyl-β-D-gluco- and galactopyranoside, supposedly substrates for glucosidase and galactosidase, can be seen in Fig. 4. They are both hydrolyzed by liver zones L-1 and L-2 and by serum zone S-2.

The long-chain, fatty-acid esters required an incubation period of 3 hr before a stain could be detected. The addition of sodium cholate to the substrate-dye solution accelerated the enzyme action and thus reduced the incubation period to 1 hr.

DISCUSSION

Serum enzymes

The serum enzymes, pseudocholinesterase and A-esterase, have been eluted from the gel and identified in solution. Many characteristic properties of these enzymes with respect to inhibitors and substrates have been demonstrated on the gels. There is no reason to expect different behaviour from enzymes in solution and enzymes contained in the gel.

A schematic drawing of data from the literature[39–41] on serum esterases separated by starch gel electrophoresis is shown in Fig. 5 in comparison with our results. The reported electrophoretic mobility of pseudocholinesterase (S-1), slight differences of buffers allowed, appears to be very similar. The only exception is that some authors (Fig. 5, B and D)[39, 41] have identified two pseudocholinesterase bands, while we have

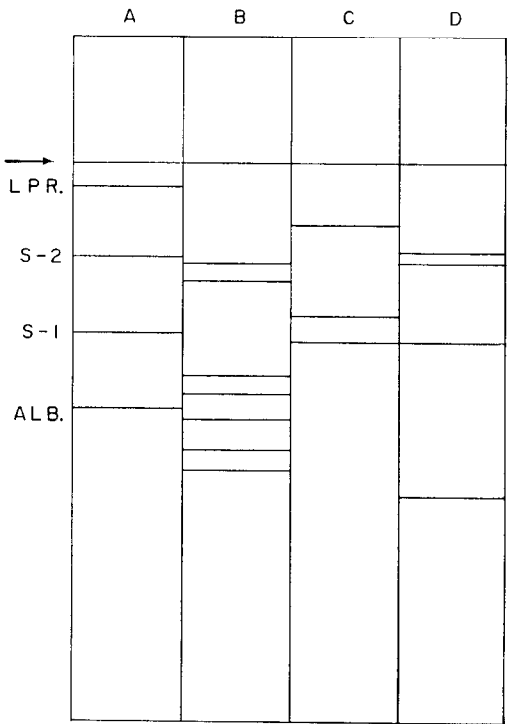

FIG. 5. A schematic representation of human plasma esterases separated by starch gel electrophoresis; comparison of the results from different laboratories. A refers to data described in this paper and may be considered to represent a gel like that in Fig. 2C. The position of the mid portion of each zone is indicated by a line. B, data by Paul and Fottrell;[39] C, data by Lawrence *et al.*;[40] D, data by Dubbs *et al.*[41]

seen no more than one band of enzyme identifiable as pseudocholinesterase. Svensmark[42] has shown that treatment of serum with the enzyme sialidase will alter the electrophoretic mobility of pseudocholinesterase without changing the activity. One wonders whether the loss of a sialic acid moiety from some pseudocholinesterase molecules might account for the appearance of two bands in the gels. There is agreement in the S-2 zone with the exception of Fig. 5B. The observation of so many bands in this strip makes it difficult to classify the esterases by our system.

Slight esterase activity has been noted in the albumin zone. This previously had been considered to be protein binding of the naphthol-dye complex, but Erdös and Boggs[43] have shown that albumin is able to hydrolyze naphthyl acetate.

The hydrolysis of the naphthyl pyranosides (Fig. 4, F and G) at the precise location of pseudocholinesterase requires further study. These pyranosides are supposed to be

substrates of galactosidase and glucosidase.[15] Whether a glycoside linkage is sufficiently similar to an ester linkage to be acted upon by pseudocholinesterase is not known.

The hydrolysis of the fatty-acid esters is a property of aliesterases,[44] but these do not seem to be present in human serum.[45] Saifer and Perle[26] state that there are four types of esterase capable of acting on long-chain, fatty-acid esters when these esters are present in serum: clearing-factor lipases, pseudocholinesterases, liver esterases, and pancreatic lipases. Pseudocholinesterase, under their experimental conditions, had no effect on phenyl laurate. The first enzyme, clearing-factor (or lipoprotein) lipase, is not normally found in blood.[46] Our results show activity toward the long-chain, fatty-acid esters in the pseudocholinesterase zone. The sodium cholate activation of this enzyme is also peculiar in that this phenomenon is generally supposed to occur only in the case of lipases.[25] Cholate inhibits esterases.[25] Further investigation is in progress.

Liver esterases

The results have indicated that liver esterases are markedly different from serum esterases, electrophoretically and in substrate specificity. Before specific observations can be contemplated, some literature reports on liver esterases must be recalled. According to Hofstee,[44] more than one esterase is found in liver. One of these esterases belongs to the acetylesterases which attack aliphatic and aromatic esters of acetic acid and are not inhibited by low concentrations of eserine. Another liver esterase preferentially splits long acyl-chain esters of the type of *m*-hydroxybenzoic esters of normal fatty acids, 2 to 14 carbons in length. Lipase will not hydrolyze these substrates, but this liver esterase, serum cholinesterase, trypsin, and chymotrypsin will.[44] This liver esterase is inhibited by DFP, other organophosphorous compounds, and eserine. In addition, an aliesterase (B-type esterase) is also found in the liver, hydrolyzing aliphatic and aromatic esters (triacetin, ethyl butyrate, *p*-phenyl and *p*-nitrophenyl acetate, and butyrate) and inhibited by TEPP, DFP, other organophosphates, and concentrations of eserine above 10^{-5} M.[36, 47] In two well known classifications, aliesterases and lipases have been grouped together as a subgroup of esterases.[48, 49]

Since our extraction procedure employed only water, one would not expect to find all of the liver esterases in the gels, but only those that are loosely bound and water-soluble. Microsomal esterases are not likely to be in evidence.

The patterns of inhibition and substrate specificity are compatible with the assumption that liver zone L-1 is an aliesterase. This zone hydrolysed the long-chain, fatty-acid esters and all of the acetate esters except naphthol AS acetate but did not hydrolyze naphthyl benzoate or the choline esters. This zone was blocked by the organophosphorus compounds that inhibited pseudocholinesterase, with the exception of Phosdrin and low concentrations of DFP. Decamethonium and low concentrations of eserine had no effect on zone L-1 but did inhibit zone S-2. Sodium fluoride, according to Hofstee,[35] will inhibit liver esterase, but there was no inhibition of L-1 under our conditions. By these criteria, cholinesterase, acetylesterase, and Hofstee's liver esterase are ruled out, and one might consider the enzyme to be an aliesterase which, however, is not known to be activated by sodium cholate. Hence this activating effect cannot be explained, unless a lipase also is present.

There are other indications that L-1 may contain more than one enzyme. Besides the esters mentioned above, zone L-1 also hydrolyzed 6-bromo-2-naphthyl gluco- and

galactopyranoside and L-leucyl-β-naphthylamide. The hydrolysis of the first two compounds requires further study, while that of the last substrate would lead one to suspect the presence of leucine aminopeptidase, although with reservations, since liver esterase reportedly splits C—N bonds.[50] However, the presence of liver esterase is unlikely, in view of the lack of fluoride inhibition.

In contrast to zone L-1, liver zone L-3 hydrolysed all of the substrates tested, with the exception of acetylthiocholine. This zone showed a resistance to all anticholinesterases except DFP, Malathion and Phosdrin at 1×10^{-4} M. TEPP and sodium fluoride seem to inhibit a part of this zone. This might indicate the presence of more than one enzyme, especially since only a portion of L-2 hydrolysed sodium α-naphthyl acid phosphate at pH 5·0, indicating acid phosphatase activity (Fig. 4C), Sodium cholate has an activating effect on the enzyme(s) of this zone toward the long-chain, fatty-acid esters, indicating possible lipase activity. Cholinesterase activity also is present, as is seen by the splitting of β-carbonaphthoxycholine iodide but not as seen by acetylthiocholine.

Liver zone L-3 hydrolyzed only the acetate esters and is susceptible to all of the anticholinesterases except low concentrations of DFP and Phosdrin. High concentrations of eserine (10^{-4} M) inhibit, but 10^{-6} M solutions do not. This apparent specificity of substrates and inhibitors would classify this zone as an acetylesterase.

There are obvious difficulties in our attempts to identify the electrophoretically separated zones with known esterases. One major difficulty results from the fact that many of the data in the literature describing enzyme specificities were obtained with crude extracts of tissue. The observations thus stress the need to characterize esterases in the future by both physical properties and enzymic activities.

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REFERENCES

1. K.-B. AUGUSTINSSON, *Nature (Lond.)* **181**, 1786 (1958).
2. K.-B. AUGUSTINSSON, *Acta chem. scand.* **13**, 571 (1959).
3. W. N. ALDRIDGE, *Biochem. J.* **53**, 62 (1953).
4. D. H. ADAMS, *Biochim. biophys. Acta* **3**, 1 (1949).
5. R. O. DAVIES, A. V. MARTON and W. KALOW, *Canad. J. Biochem.* **38**, 545 (1960).
6. A. V. MARTON and W. KALOW, *Canad. J. Biochem.* **37**, 1367 (1959).
7. R. V. LAMOTTA, H. M. WILLIAMS and H. J. WETSTONE, *Gastroenterology* **33**, 50 (1957).
8. O. SMITHIES, *Biochem. J.* **61**, 629 (1955).
9. O. SMITHIES, *Biochem. J.* **71**, 585 (1959).
10. R. L. HUNTER and C. L. MARKERT, *Science* **125**, 1294 (1957).
11. C. L. MARKERT and R. L. HUNTER, *J. Histochem. Cytochem.* **7**, 42 (1959).
12. W. KALOW, *Ciba Foundation Symposium on Biochemistry of Human Genetics* pp. 39–56, Churchill, London (1959).
13. D. SCHWARTZ, *Proc. Nat. Acad. Sci., Wash.*, **46**, 1201 (1960).
14. S. L. ALLEN, *Genetics* **45**, 1051 (1960).
15. H. LAUFER, *Ann. N. Y. Acad. Sci.* **89**, 490 (1960).
16. D. J. ECOBICHON and W. KALOW, *Canad. J. Biochem.* **39**, 1329 (1961).
17. M. M. NACHLAS and A. M. SELIGMAN, *J. nat. Cancer Inst.* **9**, 415 (1949).
18. A. G. E. PEARSE, *Int. Rev. Cytol.* **3**, 329 (1954).
19. H. A. RAVIN, K. G. TSOU and A. M. SELIGMAN, *J. biol. Chem.* **191**, 843 (1951).
20. S. J. HOLT, *Nature Lond.*, **169**, 271 (1952).

21. B. PEARSON and F. GROSE, *Arch. Path., Chicago*, **67**, 324 (1959).
22. G. GOMORI, *Microscopic Histochemistry* p. 210, Univ. of Chicago Press (1958).
23. A. M. SELIGMAN, M. M. NACHLAS, L. H. MANHEIMER, O. M. FRIEDMAN and G. WOLF, *Ann. Surg.* **130**, 333 (1949).
24. A. M. SELIGMAN and M. M. NACHLAS, *Cancer Res.* **10**, 240 (1950).
25. H. J. RADERECHT and H. MOSKAU, *Clin. chim. Acta* **4**, 221 (1959).
26. A. SAIFER and G. PERLE, *Clin. Chem.* **7**, 178 (1961).
27. W. KALOW and H. A. LINDSAY, *Canad. J. Biochem.* **33**, 568 (1955).
28. W. KALOW, K. GENEST and N. STARON, *Canad. J. Biochem.* **34**, 637 (1956).
29. W. KALOW and K. GENEST, *Canad. J. Biochem.* **35**, 339 (1957).
30. H. A. LINDSAY, Thesis, Univ. of Toronto, Ontario (1955).
31. A. V. MARTON and W. KALOW, *Biochem. Pharmacol.* **3**, 149 (1960).
32. R. D. HAWKINS and J. M. GUNTER, *Biochem. J.* **40**, 192 (1946).
33. B. J. BAYLISS and A. TODRICK, *Biochem. J.* **62**, 62 (1956).
34. T. P. SINGER, *J. biol. Chem.* **174**, 11 (1948).
35. B. H. J. HOFSTEE, *J. biol. Chem.* **207**, 211 (1954).
36. B. HOLMSTEDT, *Pharmacol. Rev.* **11**, 567 (1959).
37. R. D. HAWKINS and B. MENDEL, *Brit. J. Pharmacol.* **2**, 173 (1947).
38. J. H. AUSTIN and M. BUCHEL, *Blood* **17**, 216 (1961).
39. J. PAUL and P. FOTTRELL, *Biochem. J.* **78**, 418 (1961).
40. S. H. LAWRENCE, P. J. MELNICK and H. E. WEIMER, *Proc. Soc. exp. Biol. (N.Y.)* **105**, 572 (1960).
41. C. A. DUBBS, C. VIVIONA and J. M. HILBURN, *Science* **131**, 1529 (1960).
42. O. SVENSMARK, *Danish med. Bull.* **8**, 28 (1961).
43. E. G. ERDÖS and L. E. BOGGS, *Nature, Lond.*, **190**, 716 (1961).
44. B. H. J. HOFSTEE, *The Enzymes* 2nd ed, vol. 4, p. 485. Academic Press, New York (1960).
45. W. N. ALDRIDGE, *Biochem. J.* **53**, 110, 117 (1953).
46. D. S. ROBINSON and J. E. FRENCH, *Pharmacol. Rev.* **12**, 241 (1960).
47. G. A. OVERBEEK, *Clin. chim. Acta* **2**, 1 (1957).
48. G. GOMORI, *Microscopic Histochemistry*, p. 200. Univ. of Chicago Press (1958).
49. D. RICHTER and P. G. CROFT, *Biochem. J.* **36**, 746 (1942).
50. F. BERGMANN and M. WURZEL, *Biochim. biophys. Acta* **12**, 412 (1953).