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The Arylesterases of Human Serum

By C. E. WILDE AND R. G. O. KEKWICK
Department of Biochemistry, University of Birmingham

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It has been apparent for some time that the hydrolysis of phenyl esters by human serum, first observed by McGuire & Falk (1934), is due to more than one enzyme. Huggins & Lapides (1947) found that most of the activity was insensitive to concentrations of eserine sufficient to inhibit serum cholinesterase. Subsequently Mounter & Whittaker (1953) found that the hydrolysis of phenyl acetate by plasma was only slightly sensitive to inhibition by DFP.* In addition, the ratio of the rate of DFP- and eserine-sensitive hydrolysis of phenyl acetate by purified human serum cholinesterase to the rate of hydrolysis of acetylcholine was much lower than that of the total rate of hydrolysis of phenyl acetate to that of acetylcholine catalysed by whole serum, suggesting that a DFP- and eserine-insensitive phenyl acetate esterase had been removed by purification. Aldridge (1954) showed that the enzyme catalysing the DFP-insensitive hydrolysis of phenyl acetate by rabbit serum also catalysed the hydrolysis of diethyl *p*-nitrophenyl phosphate (E600) and showed that the enzyme

* The abbreviation used is: DFP, di-isopropyl phosphorofluoridate.

was distinct from cholinesterase. He also found that E600 was hydrolysed by human plasma.

A clear resolution of the different arylesterase activities in plasma was first obtained by Augustinsson (1959), who assayed the components of human serum, separated by electrophoresis on a cellulose column, for esterase activity against several esters. He obtained a separation of the eserine- and organophosphorus-sensitive phenyl acetate-hydrolysing activity, which migrated with the cholinesterase between the α_2 - and β -globulins, and the eserine- and organophosphorus-insensitive activity, which travelled with the albumin. The latter fraction contained by far the major part of the phenyl acetate-hydrolysing activity. Augustinsson (1959) thought that some human plasmata could contain an esterase 'running close to, but more rapidly than, the main peak fraction since this esterase peak was not uniform'.

When the arylesterase activity of human serum was separated by starch-gel electrophoresis and detected with β -naphthyl acetate as substrate, Dubbs, Vivonia & Hilburn (1960) found that most of this activity was separated from the albumin and

migrated with the haptoglobins. Preliminary experiments in this Laboratory with a similar technique confirmed this observation and suggested that the main factor might have more than one component.

An attempt was therefore made to separate the main arylesterase activity from the albumin and to compare its characteristics with those of serum cholinesterase when hydrolysing phenyl acetate.

MATERIALS AND METHODS

Source of enzymes. The enzymes were obtained from the serum of either one of us. Blood was drawn by venipuncture and was allowed to clot for 6 hr. at room temperature. Preliminary experiments showed that a portion of the arylesterase activity of whole serum was Ca^{2+} ion-dependent. Assays were therefore carried out in the presence of 10 mM- CaCl_2 , the concentration found to give maximal activity.

When fresh serum was stored at either -2° or -10° there was an initial loss of about 8% of the original arylesterase activity over the first 24 hr. Subsequent storage at -10° for 7 weeks produced no further loss of activity, but storage at 2° for the same period caused a further loss of about 14% of the original activity. Samples were therefore stored at -10° ; immediately before use each sample was thawed once, as repeated freezing and thawing also caused loss of enzyme activity.

Substrates. Phenyl esters were purified by the method of Aldridge (1954). Free phenol was removed by passing a solution of the ester in light petroleum through an alumina column, the light petroleum was distilled off, and the esters were redistilled (phenyl acetate, b.p. $192-194^\circ$; phenyl propionate, b.p. 208° ; phenyl butyrate, b.p. 222°).

Acetylcholine perchlorate and benzoylcholine chloride were obtained from British Drug Houses Ltd.

Measurement of enzyme activity. Two methods were used: in one phenol liberated by the arylesterases was measured colorimetrically, in the other the acid liberated was measured titrimetrically. In both cases the enzyme reaction was carried out at pH 7.4. Although this was lower than the pH optimum for any of the enzymes studied it was found that the correction for non-enzymic hydrolysis, which was always applied, became very high at more alkaline pH values.

The arylesterase activity of the column eluate was assayed by the colorimetric method first described by Aldridge (1954). Fractions were incubated for 30 min. at 37° with 30 mM-phenyl acetate in 0.05 M-tris-maleic acid buffer, pH 7.4, instead of the phosphate buffer used by Aldridge. The phenol liberated was assayed by measuring the extinction at $510\text{ m}\mu$ of the complex formed with 4-aminoantipyrine. The blanks arising from non-enzymic hydrolysis were kept to a minimum by redistilling the ester immediately before use and by keeping it as cold as possible before the incubation.

For kinetic studies, arylesterase activity was measured titrimetrically at 25° , normally at pH 7.4, with a glass electrode and a pH-meter. The incubation medium was unbuffered but contained NaCl (0.15 M); the pH was maintained constant at pH 7.4 by the addition of 0.02 N-NaOH from an Agla micrometer syringe. Initial velocities were

measured over the first 10–15 min., readings of added alkali being taken at intervals of 1 min. The specific activity of the esterases was expressed in $\mu\text{moles of ester hydrolysed/10 min./mg. of protein nitrogen}$. All assays were corrected for non-enzymic hydrolysis of the substrate at the given temperature and pH. For the selective inhibition of cholinesterase, eserine and DFP were used at concentrations of $10\text{ }\mu\text{M}$.

Determination of protein concentration. The protein content of column eluate was assayed by measurement of the extinction at $280\text{ m}\mu$ in a Unicam SP. 500 spectrophotometer. Nitrogen determinations for the measurement of the specific activities of purified fractions were carried out by the micro-Kjeldahl procedure.

Electrophoretic separation. Preparative electrophoresis was carried out in a jacketed column (100 cm. $\times$ 4 cm.) with an apparatus similar to that described by Porath (1954) and by Flodin & Porath (1954). The columns were packed with ethanolyzed cellulose obtained from Munktel.

Starch-gel electrophoresis was carried out in an apparatus similar to that described by Smithies (1955) with the discontinuous buffer system of Poulik (1957). Gels were prepared from hydrolysed starch (Connaught Laboratories, Toronto) used at the strength recommended for the batch; and the electrophoresis was carried out for $4\frac{1}{2}$ hr. at an initial potential gradient of 6.5 v/cm. The gels were then sliced longitudinally as described by Smithies (1955). One half was stained for protein with a freshly prepared saturated solution of Naphthalene Black in methanol-water-acetic acid (5:5:1, by vol.). The region of arylesterase activity in the other half of the gel was located by an adaptation of Gomori's (1952) modification of the method of Nachlas & Seligman (1949), in which the gel was treated with a solution of 0.02% β -naphthyl acetate and 0.04% tetrazotized o-dianisidine (Brentamine Blue B; British Drug Houses Ltd.) in tris buffer, pH 7.2, containing 2% (v/v) of acetone. The bands of arylesterase activity reached maximal intensity after incubation for 15 min. at room temperature. The substrate solution was washed away with water and the gels were stored in the same solvent as was used for staining protein.

Chromatography. Serum proteins were separated on commercial DEAE-cellulose (as first described by Peterson & Sober, 1956) obtained from W. and R. Balston Ltd. The columns used were 1.5 cm. $\times$ 6 cm. The protein was dialysed against the starting buffer before application to the column and the elution was carried out at 2° .

RESULTS

Separation of the arylesterases

Electrophoretic separation. The distribution of arylesterase activity in the fractions eluted from a cellulose column after electrophoresis in barbiturate buffer, pH 8.6 and $I\ 0.02$, is shown in Fig. 1. The main peak of arylesterase activity migrated somewhat more rapidly than the albumin but there was a slight shoulder on the trailing side of the main esterase peak, suggesting that there is some activity associated with the albumin itself. Behind the main peak there was another peak of phenyl acetate-hydrolysing activity between the α_2 - and

β -globulin peaks and associated with cholinesterase. The discontinuous form of the main arylesterase peak suggests that it arises from several components. This is seen more clearly in Fig. 2, which shows the results of an electrophoretic separation carried out in phosphate-borate buffer, pH 8.4 and I 0.065. In this experiment the activity of the main arylesterase peak was less than that of the cholinesterase, owing to the removal of Ca^{2+} ions by the phosphate of the buffer.

The tubes comprising each of the three arylesterase fractions marked in Fig. 1 were combined into three fractions for analysis by starch-gel electrophoresis. Fraction A, the pre-albumin fraction, which accounted for 58 % of the arylesterase activity initially applied to the column, contained, in addition to the two pre-albumins described by Smithies (1955), albumin and a small amount of protein migrating behind the albumin in the region of the haptoglobins. This last minor component contained the entire arylesterase activity of the fraction. Fraction B, the albumin fraction from the column, containing 12 % of the arylesterase activity applied to the column, gave a single albumin band on starch-gel electrophoresis, the arylesterase activity being coincident with this band. Fraction C, the cholinesterase fraction from the column, had 11 % of the initial activity which

was associated with the slow α_2 -globulin on starch-gel electrophoresis; staining for proteins showed the presence of albumin and a number of α - and β -globulins in this fraction.

Separation on diethylaminoethylcellulose. Whole serum was subjected to chromatography on DEAE-cellulose to fractionate the arylesterase activities. As the pre-albumin fraction from the electrophoresis column was unstable in solutions more acid than pH 5.5, chromatography was carried out in 50 mM-acetate buffer, containing calcium chloride (10 mM) at this pH; the proteins eluted with increasing concentrations of sodium chloride are shown in Fig. 3. Three arylesterase fractions were again separated, two of which were insensitive to 10 μM - eserine; the third was sensitive to 10 μM - eserine and was therefore due to cholinesterase. The arylesterase eluted with the buffer alone, which contained 5 % of the activity applied to the column, was found on starch-gel electrophoresis to be albumin; the arylesterase activity was again coincident with the albumin band, and the preparation contained no cholinesterase and no pre-albumin arylesterase. The second arylesterase peak, eluted with 0.05M-sodium chloride, accounted for 8.2 % of the initial activity, and was found on starch-gel electrophoresis to contain cholinesterase migrating in the slow α_2 -globulin region; staining

Fig. 1. Separation of arylesterases by the electrophoresis of human serum on a 4 cm. $\times$ 100 cm. cellulose column containing barbiturate buffer, pH 8.6 and I 0.02, for 54 hr. at 600v and 21 ma. $\circ$, Protein measured by $E_{280}^{1\text{cm.}}$; $\bullet$, arylesterase activity assayed colorimetrically by measuring $E_{510}^{1\text{cm.}}$.

with Naphthalene Black revealed the presence of a large number of other protein components in this fraction. The arylesterase eluted by a gradient from 0.05M- to 0.1M-sodium chloride contained 46 % of the initial arylesterase activity; this fraction was almost entirely eserine-insensitive and was partially resolved into two components by the chromatography. On starch-gel electrophoresis the arylesterase migrated as a broad band in the haptoglobin region and could not be clearly resolved into more than one component.

After this experiment the three arylesterase fractions obtained by column electrophoresis in barbiturate buffer were, as shown in Fig. 1, each subjected to chromatography on DEAE-cellulose under the same conditions to remove the contaminating arylesterases from each fraction. As shown in Fig. 4, the procedure provided a means of removing albumin-fraction arylesterase from pre-albumin-fraction arylesterase, and of albumin-fraction arylesterase from cholinesterase.

Further attempts were made to resolve the components of the pre-albumin-fraction esterase by chromatography both on CM-cellulose and on DEAE-Sephadex at pH 5.5, but these were not successful.

To obtain more evidence on the nature of the arylesterase activity of the albumin fraction, the activities of two preparations obtained by different methods were assayed.

Albumin, prepared by the acid-alcohol technique of Michael (1962), gave an albumin precipitin line on immunoelectrophoresis and a single band on starch-gel electrophoresis; the arylesterase activity was coincident with this band in the starch gel.

A preparation of albumin (AP₃) obtained by the ether-fractionation technique of Kekwick & Mackay (1954) which contained 97 % of albumin on moving-boundary electrophoresis contained arylesterase which again migrated with the albumin band on starch-gel electrophoresis. This albumin was also subjected to chromatography on a hydroxyapatite column by the procedure of Tiselius, Hjerten & Levin (1956) for the fractionation of bovine serum albumin. Three fractions of albumin were eluted at 0.075M-, 0.1M- and 0.4M-phosphate buffer, pH 6.8, respectively. All these three peaks had arylesterase activity, but the specific activity of the first peak was 1.5 times that of the other two. This first peak was found by Tiselius *et al.* (1956) to contain only albumin

Fig. 2. Separation of arylesterases by the electrophoresis of human serum on a 4 cm. $\times$ 100 cm. cellulose column in phosphate-borate buffer, pH 8.4 and I 0.065, for 54 hr. at 600v and 21 ma. $\circ$, Protein measured by $E_{280}^{1\text{cm.}}$; $\bullet$, arylesterase activity assayed colorimetrically by measuring $E_{510}^{1\text{cm.}}$.

monomer when bovine serum albumin was thus fractionated, and the products were examined in the ultracentrifuge.

Properties of the arylesterases

The kinetic characteristics of the pre-albumin-fraction esterase, prepared by column electrophoresis, and of albumin-fraction arylesterase and cholinesterase, both prepared by column electrophoresis followed by chromatography on DEAE-cellulose, were compared.

After the observation that Ca^{2+} ions had a marked activating effect on the arylesterase activity of whole serum, as noted above, the effect of other group II α metal ions and Mn^{2+} ions on this activity was studied.

The requirement for Ca^{2+} ions was absolute, the optimum concentration being 10 mM, and the other metal ions had no effect. Both Mg^{2+} and Ba^{2+} ions (0.1 mM) incubated with serum in the presence of sub-optimum amounts of Ca^{2+} ions decreased the activating effect of Ca^{2+} ions, but this concentration of these ions did not decrease the stimulation produced by the optimum concentration of Ca^{2+} ions. From this it appeared that the other ions were competing with Ca^{2+} ions for the active site of the enzyme.

The removal of Ca^{2+} ions from whole serum by reagents known to combine with Ca^{2+} ions caused a decrease in arylesterase activity. When trisodium citrate was added to whole serum to give a final concentration of $14\mu\text{M}$ there was an initial loss of about 25 % of the total arylesterase activity, followed by the loss of a further 40 % of the initial activity after storage at 4° for 2 weeks.

To determine which of the arylesterase fractions was Ca^{2+} ion-sensitive whole serum was subjected to starch-gel electrophoresis. After the run the gel was cut into segments and the protein eluted from the segments was assayed for activity in the presence and in the absence of 10 mM- Ca^{2+} ion. The results of this experiment (Fig. 5) show that the arylesterase activity migrating behind the albumin, in the region of the haptoglobins, and which on the cellulose electrophoresis column migrates faster than albumin, was the only Ca^{2+} ion-dependent arylesterase activity.

The effect of the removal of Ca^{2+} ions from the purified preparations of the three arylesterase fractions by dialysis for 40 hr. against two changes of phosphate buffer, pH 7.4, was studied. The results (Table 1) show that the pre-albumin-fraction esterase is markedly sensitive to the Ca^{2+} ion concentration and that activity lost on dialysis can

Fig. 3. Separation of the arylesterases by chromatography of serum on DEAE-cellulose. The protein (5 ml. of serum) was applied in 0.05M-acetate buffer, pH 5.5, containing CaCl_2 (0.05M) and was eluted with increasing concentrations of NaCl. ○, Protein measured by $E_{280\text{ m}\mu}^{1\text{cm}}$; ●, total hydrolysis of phenyl acetate assayed colorimetrically by measuring $E_{510\text{ m}\mu}^{1\text{cm}}$; ■, esterase-sensitive hydrolysis of phenyl acetate assayed colorimetrically by measuring $E_{510\text{ m}\mu}^{1\text{cm}}$; ▲, concn. of Cl^- ions.

only be partially restored with 10 mM- Ca^{2+} ion. The fraction is also almost completely inhibited by 0.1 mM-EDTA. Of the other two fractions albumin had no requirement for Ca^{2+} ions and was insensi-

tive to EDTA, and cholinesterase lost a small amount of activity reversibly on dialysis and was slightly sensitive to 0.1 mM-EDTA.

The bivalent metal ions Cu^{2+} , Zn^{2+} and Hg^{2+} at 0.01 mM inhibited all the arylesterase activity of dialysed serum, and the arylesterase activity could not be restored by the addition of Ca^{2+} ions to a concentration of 10 mM.

Inhibition by reagents for thiol groups. In view of the inhibition of all the arylesterase activity of serum by Cu^{2+} and Hg^{2+} ions, the effect of reagents, known to react with the thiol groups of proteins, on the arylesterases was studied.

Iodoacetamide had very little effect on the arylesterase activity of whole serum except at relatively high concentrations; 1 mM-iodoacetamide

Fig. 4. Chromatography on DEAE-cellulose of fractions from the electrophoretic run shown in Fig. 1. The combined fractions A, B and C (Fig. 1) were used in (a), (b) and (c) respectively. ○, Protein measured by $E_{280}^{1\text{cm}}$; ●, arylesterase activity assayed colorimetrically by measuring $E_{510}^{1\text{cm}}$.

Fig. 5. Activation of serum arylesterases, separated by starch-gel electrophoresis at pH 8.9, by 10 mM- CaCl_2 . The histogram shows arylesterase activity in each slice measured colorimetrically at pH 7.4: □, in the presence of CaCl_2 ; ■ in the absence of CaCl_2 .

Table 1. *Effect of dialysis and subsequent addition of calcium ions and of ethylenediaminetetra-acetate on the arylesterase activities of three serum fractions measured titrimetrically*

The pre-albumin fraction was isolated by preparative electrophoresis, and the albumin and cholinesterase fractions by preparative electrophoresis followed by chromatography on DEAE-cellulose. Experimental details are given in the text.

Fraction	Dialysis	10 mM- Ca^{2+} ion	0.1 mM-EDTA	Arylesterase activity (percentage of maximum hydrolysis)
Pre-albumin fraction	—	+	—	100
	—	—	+	2
	+	—	—	36
	+	+	—	56
Albumin fraction	—	+	—	100
	—	—	+	91
	+	+	—	100
Cholinesterase fraction	—	—	—	100
	—	—	+	98
	+	—	—	91
	+	+	—	100

produced an inhibition of about 2% whereas 10 mM-iodoacetamide caused a 99% inhibition.

The inhibitory action of some reagents for thiol groups on the purified enzyme fractions was investigated. The results in Table 2 show that the pre-albumin-fraction arylesterase is highly sensitive to all reagents for thiol groups but that, of those studied, sodium arsenite was the least inhibitory. The albumin-fraction arylesterase was sensitive only to Cu^{2+} and to Hg^{2+} ions, and to these only at high concentration. Cholinesterase behaved somewhat similarly to the albumin-fraction arylesterase but was slightly more sensitive to Cu^{2+} and Hg^{2+} ions.

Effect of anticholinesterases. The only enzyme fraction inhibited by either eserine or by DFP was cholinesterase.

Substrate specificity. The purified arylesterase fractions were assayed against phenyl acetate, phenyl propionate and phenyl butyrate at a concentration of 10 mM. The results are shown in Table 3, where the rates of hydrolysis of the three esters are given as percentages of the rate of hydrolysis of phenyl acetate by each enzyme fraction. The pre-albumin-fraction esterase showed a marked preference for the acetyl group, whereas the preferred group for cholinesterase was butyryl, as has been found for alkyl esters by Adams & Whittaker (1949). No marked acyl-group specificity could be discerned for the albumin-fraction arylesterase when hydrolysing these three esters.

The qualitative histochemical technique, used to demonstrate the arylesterases separated by starch-gel electrophoresis, showed a difference in specificity

of the enzymes with respect to α - and β -naphthyl acetate. Cholinesterase was the only enzyme fraction that could hydrolyse both these esters to any appreciable extent; the other two acted only on β -naphthyl acetate. Cholinesterase was also the only enzyme fraction that hydrolysed *O*-acetylindoxyl.

Relation between initial velocity and substrate concentration. The effect of the substrate concentration, s , on the initial velocity, v , for the hydrolysis of phenyl acetate by the three enzyme fractions at pH 7.4 and 25° was investigated. The relation between $1/v$ and $1/s$ (Lineweaver & Burk, 1934) for the albumin-fraction arylesterase was linear, K_m for the hydrolysis of phenyl acetate being 0.92 mM. This relation between $1/v$ and $1/s$ was, however, not linear for the pre-albumin-fraction arylesterase, suggesting the presence of two or more enzymes having different K_m values, and confirming the enzymic heterogeneity of the fraction observed in electrophoresis. This reciprocal Lineweaver-Burk plot was linear for the hydrolysis of phenyl acetate by cholinesterase, giving K_m 4.3 mM, but when the similar results were plotted for the hydrolysis of either acetylcholine or benzoylcholine the relation was not linear (Fig. 6). Fig. 7 shows the result of plotting against $-\log s$, for the two substrates, from which it can be deduced that two enzymes were involved in the hydrolysis of acetylcholine: one was active at low substrate concentrations and inhibited by excess of substrate, and had K_m 0.16 mM, and the other was active at high substrate concentration and had K_m 3.9 mM. Somewhat similar results were obtained with benzoylcholine, but with this

Table 2. *Inhibition of arylesterase activity by reagents for thiol groups*

The substrate used was phenyl acetate and the enzymes were assayed titrimetrically. The fractions were isolated by the same procedures as those given for Table 1.

Inhibitor	Inhibition of arylesterase activity (%)		
	Pre-albumin fraction	Albumin fraction	Cholinesterase fraction
0.1 mM-Cupric chloride	96	56	81
1 mM-Cupric chloride	—	73	89
0.1 mM-Mercuric chloride	96	—	—
1 mM-Mercuric chloride	97	19	40
0.1 mM- <i>p</i> -Chloromercuribenzenesulphonic acid	98	—	0
1 mM- <i>p</i> -Chloromercuribenzenesulphonic acid	98	4	7
0.1 mM-Sodium arsenite	25	1	0
1 mM-Sodium arsenite	93	1	0

Table 3. *Specificity of the arylesterases of human serum*

The fractions were isolated by the same procedures as those given for Table 1.

Substrate	Relative rates of hydrolysis		
	Phenyl acetate	Phenyl propionate	Phenyl butyrate
Fraction			
Pre-albumin fraction	100	25	7
Albumin fraction	100	75	80
Cholinesterase fraction	100	190	210

substrate one enzyme had a very low optimum concentration (K_m less than 0.1 mM), and the other, active at higher substrate concentrations, had K_m 0.56 mM.

Fig. 6. Relation between $1/v$ and $1/s$ for the hydrolysis of phenyl acetate and acetylcholine by the three arylesterase fractions measured titrimetrically at 25° and at pH 7.4. (a) Pre-albumin fraction; (b) albumin fraction; (c) cholinesterase fraction. Substrates: ○, phenyl acetate; ●, acetylcholine.

Optimum pH. The dependence on pH of the rates of hydrolysis of phenyl acetate and phenyl butyrate by the three enzyme fractions is shown in Fig. 8. The albumin-fraction and pre-albumin-fraction arylesterases seem to have very similar pH-activity curves, both fractions having pH optima at about 7.9 for the hydrolysis of either substrate. In contrast with this, the cholinesterase fraction had a pH optimum at 8.5 for the hydrolysis of phenyl acetate, phenyl butyrate or acetylcholine; the pH optimum for the hydrolysis of benzoylcholine was, on the other hand, pH 7.5.

Fig. 7. Relation between $-\log s$ and v for the hydrolysis of (a) acetylcholine; (b) benzoylcholine and (c) phenyl acetate, measured titrimetrically at pH 7.4, and at 25°.

Effect of temperature. The variation in the rate of hydrolysis of phenyl acetate (30 mM) with temperature by the three enzymes was studied. The pre-albumin-fraction arylesterase was the least stable, having an optimum temperature of about 32°; the optimum temperature of cholinesterase was 47°, but little of the activity of the albumin-fraction arylesterase was lost at 55°.

As both the cholinesterase and the albumin-fraction arylesterase were saturated with the substrate at all the temperatures studied, the observed velocity, v , was maximal and proportional to the velocity constant for the breakdown of the enzyme-substrate complex to give the reaction products. The relation between $\log v$ and $1/T$ could therefore

be plotted according to the Arrhenius (1889) equation. From the slope of the line it was found that the activation energy for the hydrolysis of phenyl acetate by albumin-fraction arylesterase was 10.3 kcal./mole, whereas for the cholinesterase fraction it was 5.3 kcal./mole. These values of the energy of activation were obtained for measurements made below 34°, because, as had previously been observed with cholinesterase (Kekwick, 1960), the relation between $\log v$ and $1/T$ was not linear above this temperature.

DISCUSSION

Of the three fractions of human serum obtained which had arylesterase activity, the albumin-fraction enzyme, though being so far the least active, was enzymically the only homogeneous fraction obtained.

Since three different preparations of albumin, each obtained by different methods, and two of which were very pure, all had arylesterase activity, it is likely that this activity is due either to the albumin itself or to a component very closely associated with albumin. The behaviour of the arylesterase on hydroxyapatite columns suggests that it may be associated with albumin monomer.

This association of weak esterolytic activity with serum albumin is in accordance with the work of Cassida & Augustinsson (1959) and of Tove (1962). Cassida & Augustinsson (1959) found that purified human serum albumin, obtained by the cold-ethanol technique (Cohn fraction V), could catalyse the hydrolysis of α -naphthyl acetate and p -nitrophenyl acetate; Tove (1962), who used a crystalline commercial preparation of human serum albumin, obtained hydrolysis of the β -naphthyl esters of aliphatic acids ranging from acetic acid to palmitic acid, of which β -naphthyl acetate was the most rapidly hydrolysed, but, like the present authors, he found the arylesterase to be relatively unspecific. Harris, Hopkinson & Robson (1962) found that arylesterase activity could be detected, with β -naphthyl acetate as substrate, in albumin separated by subjecting human serum to electrophoresis on paper in one direction and in starch gel in a second.

The electrophoretic behaviour of the main arylesterase peak is unusual in that it migrates more rapidly than albumin on starch and cellulose columns in barbitone buffer, pH 8.6, but less rapidly than albumin in starch gel containing tris-citrate buffer, pH 8.9. Most of the pre-albumin fraction, obtained from the cellulose column, moved ahead of albumin in starch gel, and the arylesterase activity actually was present in a very minor component of this fraction which migrated behind the albumin in the starch gel.

Fig. 8. Effect of pH on the hydrolysis of phenyl acetate (0.03M), measured titrimetrically at 25°, by the three purified arylesterase fractions. (a) Pre-albumin fraction; (b) albumin fraction; (c) cholinesterase fraction.

The kinetic and electrophoretic observations presented leave no doubt that there are several enzymes present in this pre-albumin fraction from the electrophoretic columns, and the results from starch-gel electrophoresis are in agreement with those of Dubbs *et al.* (1960), who observed several esterases migrating in the haptoglobin region of human serum.

The metal ion requirement for this pre-albumin-fraction esterase is similar to that found by Marton & Kalow (1962) for the arylesterase activity present in the fraction IV-I obtained by the Cohn cold-ethanol technique. These authors also found that Mg^{2+} and Mn^{2+} ions competed with Ca^{2+} ions for the active sites.

The kinetic evidence presented for the existence of more than one enzyme in serum catalysing the hydrolysis of choline esters is similar to that of Berry (1960), who studied whole human serum, and of Heilbronn (1958), who used a preparation of cholinesterase purified by ethanol fractionation (Cohn fraction IV-6-4). There is also considerable electrophoretic evidence for the existence of more than one cholinesterase in serum. Dubbs *et al.* (1960) detected two bands of cholinesterase activity, with β -naphthyl acetate as substrate, after human serum had been subjected to electrophoresis in starch gel, and Harris *et al.* (1962) have demonstrated the presence of four cholinesterase components in normal human serum by electrophoresis on paper in one direction followed by electrophoresis in starch gel in a second, and by using α -naphthyl acetate as substrate.

The observation that the plot of $1/v$ against $1/s$ for the hydrolysis of phenyl acetate by this fraction, shown to contain more than one cholinesterase, was linear needs some explanation. Possibly only one of the cholinesterases present in this fraction and separated by starch-gel electrophoresis can hydrolyse phenyl acetate. This seems unlikely, as two regions of cholinesterase activity have been demonstrated with β -naphthyl acetate. Alternatively, the two esterases separated by starch-gel electrophoresis have different charges, different K_m values for the hydrolysis of choline esters, but similar K_m values for the hydrolysis of phenyl acetate. The observation of Svensmark (1961) that the electrophoretic mobility of cholinesterase depends on its sialic acid content, and of Augustinsson & Ekedahl (1962) that the K_m for the hydrolysis of acetylcholine at high substrate concentration remains constant after the sialic acid has been removed, may have some bearing on this point.

The spectrum of serum arylesterase activities revealed in this is slightly at variance with that described by Uriel (1961) based on observations of electrophoresis and immunoelectrophoresis in agar gel at pH 8.2. He described five precipitin lines

associated with arylesterase activity. Although one of these was given by a protein migrating between α_2 - and β -globulin, having similar properties to those of cholinesterase described above, the activity associated with the fastest arylesterase component, which migrated ahead of the albumin and which was activated by Ca^{2+} ions, was unlike the pre-albumin-fraction arylesterase described in the present paper in being insensitive to heat. He also found activity migrating in the albumin region but the precipitin line was different from that of albumin, and unlike the albumin-fraction arylesterase of the present paper this enzyme was heat-sensitive.

SUMMARY

1. Preparative electrophoresis on cellulose columns at pH 8.0 revealed three distinct forms of arylesterase activity. The leading esterase zone migrated ahead of albumin on a cellulose electrophoresis column but behind the albumin on starch-gel electrophoresis. The second arylesterase zone was associated with albumin, and the third with cholinesterase.

2. The pre-albumin and cholinesterase fractions each contained more than one arylesterase but the albumin fraction contained only one arylesterase.

3. The pH optima of the pre-albumin-fraction and albumin-fraction arylesterases were both 7.9, whereas the optimum pH for the hydrolysis of both phenyl acetate and acetylcholine by cholinesterase was 8.5.

4. In experiments with phenyl esters, the preferred acyl group for the pre-albumin-fraction arylesterase was acetyl, the albumin-fraction enzyme showed no marked specificity and that for cholinesterase was butyryl.

5. The pre-albumin-fraction esterase, unlike the other two, was sensitive to reagents for thiol groups and required Ca^{2+} ions for activation.

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The Effect of Adrenalectomy on the Metabolism of the Mammary Glands of Lactating Rats

By A. L. GREENBAUM AND F. J. DARBY

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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The mammary glands of lactating rats extensively utilize glucose as an energy source and as a source of metabolic precursors for the synthesis of milk constituents. It is well established that the pentose phosphate pathway of glucose utilization is very active in milk-producing mammary-gland tissue (Glock & McLean, 1953; Abraham, Hirsch & Chaikoff, 1954; Peeters, Debackere & Sierens, 1957), and that there is a close correlation between lactational ability and the extent of operation of this pathway (see Glock & McLean, 1958; McLean, 1958*a*; Abraham & Chaikoff, 1959). It is probable that an intimate relation exists between the pentose phosphate pathway and the systems for the synthesis of lipids, mediated via NADP⁺ and NADPH, in mammary gland (McLean, 1960; see Folley & Greenbaum, 1960), as well as in liver (Siperstein & Fagan, 1958), since both the possible mechanisms for lipogenesis (the malonyl-CoA pathway and the reversed Lynen-Green system with crotonyl-CoA reductase) require NADPH (Wakil, 1962; Langdon, 1957). The presence of the malonyl-CoA pathway in the mammary gland has been reported (Ganguly, 1960; Abraham, Matthes & Chaikoff, 1961; Dils & Popják, 1962).

Adrenalectomy is known to cause a diminished milk yield from the mammary glands of lactating

rats (Cowie & Folley, 1948; see Mayer & Klein, 1949), and it was decided to study its effects on pathways of intermediary metabolism that are closely associated with the synthesis of the constituents of milk, in an endeavour to establish the nature of the primary block caused by the loss of adrenal hormones, and with particular reference to the relationships between the several pathways of glucose catabolism. It was hoped to find a preferential loss of activity of one of the pathways and to clarify the role of the nicotinamide nucleotides in the regulation of the intermediary metabolism of the mammary gland. Thus investigations have been made: into the relationships between the Embden-Meyerhof and the pentose phosphate pathways in the condition of diminished lactation produced by the loss of the adrenal hormones and induced at a time when both pathways are fully established in the mammary gland; into lipogenesis associated with these systems; and into the limitations that the availability of the nicotinamide nucleotides imposes on them. That the activities of the enzymes in the pentose phosphate pathway in mammary-gland tissue were decreased by adrenalectomy has been shown by Willmer (1960*b*), and it is shown below that the activity of the Embden-Meyerhof pathway is also decreased, but that lipogenesis from acetate is unaffected.