Biochemical Pharmacology, 1960, Vol. 5, pp. 173–186. Pergamon Press Ltd., Printed in Great Britain.

ARYLESTERASES IN BLOOD: EFFECT OF CALCIUM AND INHIBITORS

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(*Received* 11 *April* 1960)

Abstract—(1) The normal functioning of human serum arylesterase requires the presence of calcium. An enzyme in human plasma fraction IV-1 is probably identical with serum arylesterase. The activity of arylesterase is accelerated by calcium, inhibited by various chelating agents, metal ions, SH-reagents, and competitively and reversibly inhibited by the ionized form of 1-phenyl-2-hydrazinopropane. The D-isomer of this compound is more effective than the L-isomer. In contrast to results with arylesterase, the racaemic form of this agent accelerated the hydrolysis of phenyl acetate by human plasma cholinesterase. The best inhibitors of arylesterase were the rare earths, some of them active in the order of 10^{-8} M. Under the experimental conditions used calcium chloride protected the enzyme partially or fully against the inhibition and against loss of activity during dialysis.

(2) Swine serum contains an arylesterase with properties similar to those of human serum.

(3) The human red blood cell and serum arylesterase are not identical.

ALDRIDGE[1, 2] described in 1951–1953 the existence of an A esterase in mammalian blood sera. This enzyme hydrolyzes *p*-nitrophenyl acetate and diethyl *p*-nitrophenyl phosphate. It is probably identical with the aromatic esterase (Ae)* of Mounter and Whittaker[3] which is mainly responsible for the hydrolysis of PA in human plasma. The aryl (aromatic) esterase and the cholinesterase, the other enzyme in human plasma which can use the same substrate, were separated electrophoretically by Augustinsson[4] and by Marton and Kalow.[5] The dependence on calcium of the Ae as well as its inhibition by various agents was described by Erdös *et al.*[6] Some of the experiments given here in detail were summarized in the same article.

MATERIALS AND METHODS

The majority of the experiments were carried out in a Cary no. 11 recording u.v. spectrophotometer using a modification of Zeller's[7] method. The instrument was equipped with a 0–2·4 and with a 0–0·1 slide wire assembly, which increased the extent of registration on the chart paper twenty-four-fold. Four-milliliter absorption cells were used with a 10-mm light path. The temperature was kept constant at 27 °C and the concentration of PA was 1×10^{-3} M. Generally, activators or the inhibitors were pre-incubated for 5 min at 27 °C with the enzyme. The recording of the increase

* Abbreviations: Ae = aryl (aromatic) esterase; PA = phenyl acetate; tris = tris-hydroxymethyl-aminomethane; Å = Ångström; EDTA, Ca-EDTA, Mg-EDTA, Zn-EDTA = sequestrene: disodium ethylenediamine tetraacetic acid and its Ca, Mg, and Zn complexes; JB516 = dl-1-phenyl-2-hydrazinopropane HCl; DFP = diisopropylfluorophosphate.

in optical density started 30 sec after mixing the substrate and the enzyme. A 0·025 M tris buffer of pH 7·3 was employed. (The activity of the serum was markedly less in a phosphate buffer.) The hydrolysis was followed for 5–10 min; the results were calculated from the initial steady rates. Fig. 1 shows the changes in optical density during the hydrolysis of 1×10^{-3} M PA by the Ae in fraction IV-1 of human plasma. The u.v. range of the spectrophotometer was scanned at a rate of 10 Å/sec. The initial (PA) and the final (phenol) absorption spectra are marked A and B, respectively. In most of the routine studies a wavelength of 2800 Å was used.

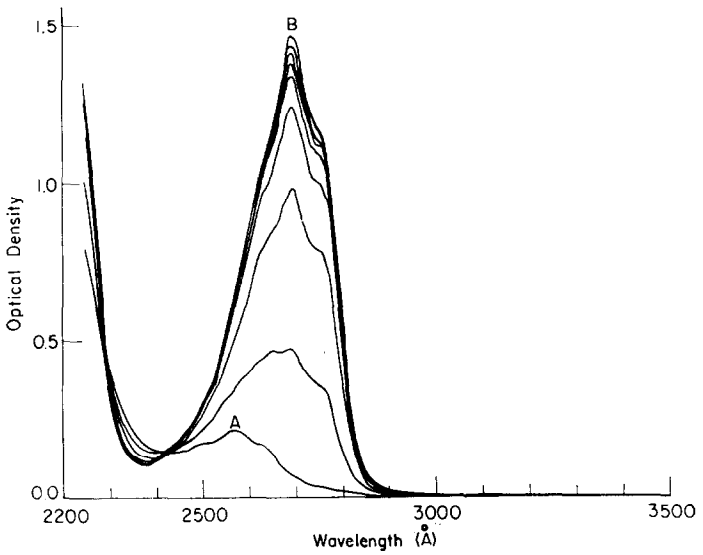

FIG. 1. Continuous scanning of the hydrolysis of phenyl acetate by arylesterase. A is the initial, B the final absorption spectrum.

Unlike the spectrophotometric technique, which measured the rate of hydrolysis of PA by determining the amount of phenol liberated, control studies assayed the amount of acid released by the enzymes. Here a Warburg manometric apparatus or a Radiometer automatic recording titrator was employed at 27 °C. With the conventional manometric method a $NaHCO_3$ solution of 0·031 M and a substrate concentration of 4×10^{-3} M were used. The time of pre-incubation was 20 min.

In the titrator the enzyme was assayed in 10 ml of NaCl solution, 0·025 M, by maintaining a constant pH (7·4), with 0·01 N, or in the red blood cell experiments; with 0·02 N NaOH. The concentration of substrate was 1×10^{-3} M.

In the earlier part of the investigation, human plasma, later pooled normal human serum, was used with similar results. During the experiments with the spectrophotometer a stock solution of serum in tris (1 : 100 v/v) was kept in an ice bath; subsequent dilutions were made 5–10 min before starting the reaction. The final dilution of serum was 1 : 2000 v/v with this technique; it was 1 : 1000 v/v with the manometric method. At this dilution only a negligible amount of PA was hydrolyzed by the cholinesterase present in the sera of healthy donors. The results with some sera were discarded because the preparation of the enzyme became unstable.

Human plasma fractions were obtained from the Protein Foundation, Inc. and from Cutter Laboratories. Swine serum was pooled from ten pigs (Colorado Serum Company). Swine plasma fractions were purchased from Pentex Corporation. The activity of plasma fractions was determined in a manner similar to that of human serum. The source of purified human plasma cholinesterase was fraction IV-6-1-2 (Cutter Laboratories), which is equivalent to Cohn fraction IV-6-3. Human red blood cells were washed three times in physiological saline and haemolyzed in distilled water. The final dilution of packed red blood cells was 1 : 60 v/v.

Dialysis experiments were carried out in an Oxford multiple dialyzer with Visking seamless cellulose tubing. The tubes contained 5 ml of enzyme solution which was dialyzed against 4 l. of tris of pH 7·4 at 2–4 °C. When indicated in the tables, EDTA or Ca-EDTA was added to this solution of enzyme. The activity of dialyzed Ae was compared with a sample stored in the refrigerator. During the 40-hr dialysis run, three changes of buffer were used. The dilutions of enzymes in tris in the dialysis tubes or during storage were: human serum, 1 : 10 v/v; fraction IV-1, 5 mg/ml; swine serum, 1 : 10 v/v. The final dilutions in the spectrophotometer were: human serum, 1 : 2000 v/v; fraction IV-1, 0·05 mg/ml; swine serum, 1 : 400 v/v.

When the enzyme was dialyzed against calcium, the same concentration of calcium chloride was used inside and outside the dialysis bags.

RESULTS

Human serum Ae

1. *Effect of chelating agents and calcium.* Our interest in the role of calcium in Ae stems from the observation that EDTA, added to prevent the coagulation of blood samples, inhibited Ae without affecting the activity of cholinesterase. EDTA at a concentration of $2{\cdot}5 \times 10^{-5}$ M inhibited the enzyme completely in the spectrophotometer. On the other hand, EDTA in concentrations less than 10^{-5} M enhanced the enzymic activity. We attribute this activation to the binding of an inhibitor present in our system. The character of the inhibition was further explored with the help of various sequestering agents. It is known that, to an extent largely determined by their respective stability constants (K),* cations can replace each other in the EDTA complex. Thus, for example, Ca^{2+} (log $K = 10{\cdot}6$) can replace Mg^{2+} (log $K = 8{\cdot}7$). Accordingly, in our experiments Mg-EDTA inhibited to approximately the same degree as EDTA, possibly through exchanging Mg^{2+} for Ca^{2+} in the system. Conversely, Zn-EDTA (log $K = 16{\cdot}6$) was an ineffective inhibitor. Calcium-EDTA also did not inhibit, but accelerated about 50 per cent in the 10^{-4}–10^{-5} M range. Chel DP (ethylenediamine di (O-hydroxyphenyl acetic acid)) which has little tendency to co-ordinate with alkaline earth ions (log K for $Ca^{2+} = 1{\cdot}6$) was similar to Ca-EDTA. Chel DM (hydroxyethyl ethylenediamine triacetic acid) is, on the other hand, a good inhibitor. Its log K for Ca^{2+} is 8·0, and it inhibits Ae 90 per cent at a 5×10^{-5} M concentration. Sodium citrate (see Fig. 2) also inhibited at a somewhat higher concentration ($I_{50} = 7 \times 10^{-4}$ M).

The inhibition of the enzyme by EDTA could be reversed by the addition of gelatin, ashed gelatin, or calcium to the system. These agents also accelerated the

* The stability constants of the chelating agents do not show the actual values under the present experimental conditions, but were taken from the literature to compare them against each other under identical conditions.

hydrolysis of PA. The various sera or plasma samples were accelerated by Ca^{2+} to varying extents. Experiments with nineteen different enzyme preparations gave a mean value of 83 per cent (s.e. = ± 9) acceleration by 1×10^{-4} M $CaCl_2$.

When a 1×10^{-4} M $CaCl_2$ solution was rapidly added into the absorption cell of the spectrophotometer, which already contained 5×10^{-5} M EDTA, enzyme and substrate, the inhibition gradually decreased and up to 70 per cent of the normal activity was restored. When $MgCl_2$ was used instead of $CaCl_2$, the reactivation was negligible.

2. *Effect of dialysis.* The importance of calcium was also seen in dialysis studies. Table 1 summarizes the results with human serum Ae. On the average, the activity of the enzyme decreased 65 per cent during the dialysis, but it was partially restored by adding calcium to the solution of enzyme. When the dialysis tube contained EDTA at the beginning of the dialysis, 94 per cent of the activity disappeared and calcium did not reactivate the enzyme. This indicates that the removal of calcium from the protein led to irreversible changes. Storing the enzyme with EDTA also abolished the activity. When the enzyme contained Ca-EDTA, the activity decreased during dialysis somewhat less than the control and increased during storage. Finally, the enzyme remained stable when dialyzed against 1×10^{-4} M $CaCl_2$.

TABLE 1. EFFECT OF DIALYSIS ON HUMAN SERUM ARYLESTERASE

	Activity in the presence of:					
	—		EDTA		Ca-EDTA	
Enzyme*		+ 1×10^{-4} M $CaCl_2$		+ 1×10^{-4} M $CaCl_2$		+ 1×10^{-4} M $CaCl_2$
I (stored)	100	177	1	16	153	204
II (dialyzed)	35	73	6	6	44	74
III (dialyzed)†	98	157	—	—	—	—

* Human serum was stored (I) or dialyzed (II, III) against tris for 17 hr. The concentration of EDTA or Ca-EDTA in the solution of the enzyme at the start of the experiments was 1×10^{-2} M, the final dilution in the non-dialyzed sample 5×10^{-5} M. The activity of the stored enzyme = 100.

† Dialyzed against 1×10^{-4} M $CaCl_2$ in tris.

3. *Effect of inhibitors.* The results with calcium prompted us to investigate the effect of metal ions and other agents on this enzyme which has been reported to be insensitive to a number of commonly used inhibitors.[4] Figs. 2 and 3 show the inhibition of serum Ae. Salts of the rare earth series and yttrium had the lowest I_{50} values. These trivalent cations inhibited the enzyme under our experimental conditions in the 10^{-7}–10^{-8} M range. Heavy metals like $CdSO_4$, $HgCl_2$, $AgNO_3$, etc., were also very active. It is of interest that even in tris buffer, Cu^{2+} was a good inhibitor. The sensitivity of the enzyme to the metal ions varied from one serum sample to another. Figs. 2 and 3 represent the mean values of the results. The I_{50} values in M were as follows: $GdCl_3$, $CeCl_3$, 4×10^{-8}; $LaCl_3$, 6×10^{-8}; $SmCl_3$, $Y(NO_3)_3$, 2×10^{-7}; $CdSO_4$, 3×10^{-7}; $HgCl_2$, $AgNO_3$, 7×10^{-7}; $PbCl_2$, 2×10^{-6}; $ZnSO_4$, 1×10^{-6}; $NiSO_4$, $CoSO_4$, 3×10^{-6}; $CuCl_2$, 8×10^{-6}; $MnSO_4$, 1×10^{-5}; $BaCl_2$, 6×10^{-5}; $SrCl_2$, 9×10^{-5}; $MgCl_2$, 4×10^{-4}.

The inhibition by the less basic metals, with some exceptions, follows their affinity toward sulfide, a circumstance which indicates that the inhibition might be due to attachment to free SH-groups of the protein. In addition to metals, the enzyme was also inhibited by the SH-reagent, *p*-chloromercuriphenylsulfonic acid, $I_{50} = 3 \times 10^{-6}$, and by the amine oxidase inhibitor,[8, 9] JB516, $I_{50} = 2 \times 10^{-4}$.

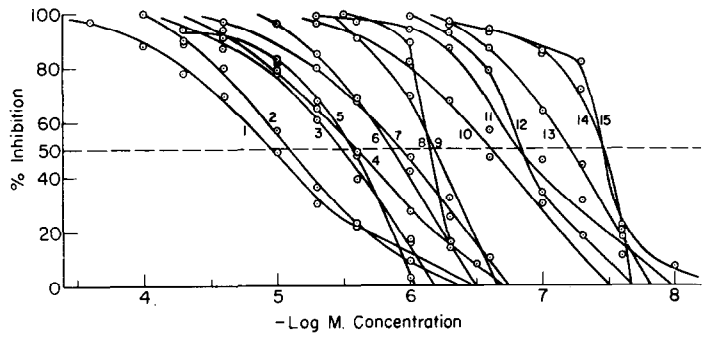

FIG. 2. Inhibition of the arylesterase of human serum. (1) $MnSO_4$; (2) $CuCl_2$; (3) $CoSO_4$; (4) *p*-chloromercuriphenylsulfonic acid; (5) $NiSO_4$; (6) $ZnSO_4$; (7) $PbCl_2$; (8) $AgNO_3$; (9) $HgCl_2$; (10) $CdSO_4$; (11) $Y(NO_3)_3$; (12) $SmCl_3$; (13) $LaCl_3$; (14) $GdCl_3$; (15) $CeCl_3$.

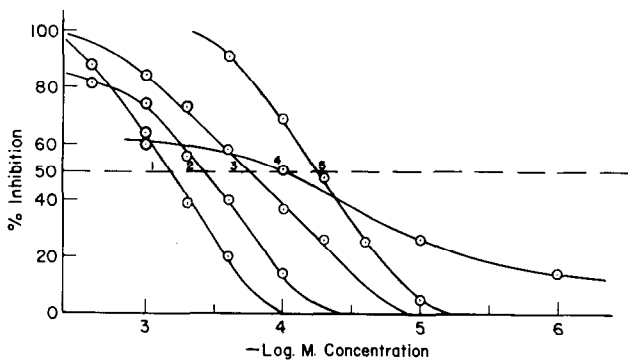

FIG. 3. Inhibition of the arylesterase of human serum: (1) sodium citrate; (2) $MgCl_2$; (3) JB516; (4) $SrCl_2$; (5) $BaCl_2$.

Because some enzyme preparations became unstable after a longer period, only 5 min were allowed for pre-incubation in the spectrophotometric studies. Experiments using longer periods of pre-incubation, with basically similar results, were done with the manometric method or with the more stable purified enzyme.

The inhibition by $AgNO_3$ was determined only with the manometric technique. With this method $CuCl_2$, $NiSO_4$, $HgCl_2$ and EDTA inhibited to about the same extent as in the spectrophotometric experiments.

Because $HgCl_2$ and *p*-chloromercuriphenylsulfonic acid are among the agents which are considered to react with the SH-groups of enzymes, an attempt was made to reverse inhibition of human Ae by adding glutathione. The experiments, resembling those of Aldrige[1] with rabbit plasma, show that glutathione partially restored the activity of Ae.

TABLE 2. REACTIVATION OF HUMAN SERUM ARYLESTERASE INHIBITED BY SH-REAGENTS*

Inhibitor	Rate†
1×10^{-6} M $HgCl_2$	27
1×10^{-6} M $HgCl_2$ + 1×10^{-3} M glutathione	72
1×10^{-5} M CMPS‡	9
1×10^{-5} M CMPS + 1×10^{-3} M glutathione	55

* Experiments were done in a Warburg manometric apparatus; 15 min after the inhibitor had been added to the enzyme, glutathione was dropped from a side arm into the main compartment of the vessels; then 15 min later the substrate was dropped from a second side arm.
† Uninhibited rate = 100.
‡ *p*-Chloromercuriphenylsulfonic acid.

Ae in fractions of human plasma

The Ae content of various fractions of human plasma was investigated in the hope of finding a source of more stable enzyme than that represented by diluted serum. Fraction IV-1 contained an enzyme with properties very similar to those of human serum Ae. Experiments with accelerators and inhibitors yielded qualitatively the same results. The highest sensitivity to activator or inhibitors was encountered in human serum, but the stability of the enzyme in fraction IV-1 exceeded that of other sources. EDTA inhibited the enzyme in fraction IV-1 completely at 5×10^{-5} M concentration. $CaCl_2$ reactivated the enzyme inhibited by EDTA. To a solution of Ae in fraction IV-1, 5×10^{-5} EDTA was added and 5 min later 1×10^{-4} M $CaCl_2$. After another 5 min the substrate was mixed with the enzyme. In contrast to the control, calcium completely abolished the inhibition. Furthermore, dialysis studies showed that the dependence of the enzyme on calcium had not changed during the purification.

TABLE 3. EFFECT OF DIALYSIS ON ARYLESTERASE IN HUMAN PLASMA FRACTION IV-1

	Activity in the presence of:					
			EDTA		Ca-EDTA	
Enzyme*		+ 2×10^{-4} M $CaCl_2$		+ 2×10^{-4} M $CaCl_2$		+ 2×10^{-4} M $CaCl_2$
I (stored)						
17 hr	100	144	0	26	133	165
40 hr	100	142	0	15	137	170
II (dialyzed)						
17 hr	57	73	7	7	67	75
40 hr	30	36	5	5	26	39
III (dialyzed)†						
17 hr	97	140	—	—	—	—
40 hr	91	136	—	—	—	—

* The arylesterase was stored (I) or dialyzed (II, III) against tris for 17 and 40 hr, respectively. The concentration of EDTA or Ca-EDTA in the solution of enzyme at the start of the experiments was 1×10^{-2} M, the final dilution in the non-dialyzed sample 1×10^{-4} M. The activity of the stored enzyme = 100.
† Dialyzed against 3×10^{-4} M $CaCl_2$. The activity of the stored enzyme which contained $CaCl_2$ = 100.

Table 3 shows that the Ae activity of fraction IV-1 decreases during dialysis; however, it can be partially reactivated with calcium. When the enzyme was stored in the presence of EDTA, the activity disappeared, but it was restored in part by calcium. The removal of calcium with EDTA during dialysis led to irreversible changes. Like that of serum Ae, the activity of the enzyme increased during storage in the presence of Ca-EDTA and behaved during dialysis similarly to the untreated control. The activity of fraction IV-1 remained constant when dialyzed against 3×10^{-4} M $CaCl_2$.

A hydrolytic enzyme for PA was also found in fractions IV-4 and IV-6-3. Others have reported[10] that this enzyme is identical with plasma cholinesterase. In spectrophotometric studies, traces of Ae activity were also seen in the clear supernatant of fractions III and III-0. When the soluble portions of the fractions were not separated in the centrifuge, but were tested as a homogenate in the automatic titrator, fractions III and III-0 showed more activity than previously. But even then the enzyme content of fraction III was less than 15 per cent of that of IV-1.

Inhibition of purified Ae

The inhibition of the enzyme in fraction IV-1 was comparable to that of human serum. It was sensitive to cations of rare earths and other metals. JB516 also inhibited the enzyme. To decide whether the charged or the uncharged form of this amine oxidase inhibitor ($pK_a = 7{\cdot}31$)[11] is responsible for the inhibition, the activity of the enzyme was measured in the presence and absence of the inhibitor at various pH values, within the range of the tris buffer. The degree of inhibition was calculated from the uninhibited control rates at each pH value and it seemed to parallel roughly the percentage ionization[12] of this compound.

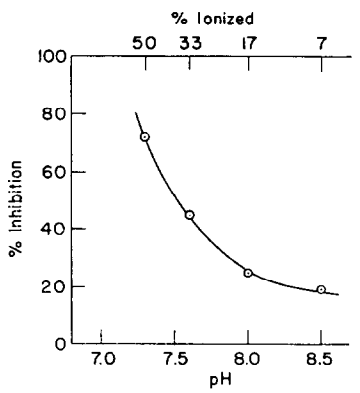

FIG. 4. Dependence of the inhibition of arylesterase in fraction IV-1 by JB516 (1×10^{-3}) on the pH and on the ionization of this inhibitor.

In contrast to this, $LaCl_3$ inhibited at pH 7·3 and 8·5 to about the same extent. The accelcrative effect of JB516 on plasma cholinesterase also depends upon the cationic form[13].

The importance of the hydrazine group in the side chain of this compound is shown by the fact that the arylalkylamine congeners of JB516, D- or L-amphetamine (1-phenyl-2-aminopropane) inhibited less than 10 per cent at 10^{-3} M concentration. In

the experiments described above the racemic form of the inhibitor was used. When D- and L-isomers were tested individually, a small but significant difference was found. D-1-phenyl-2-hydrazinopropane was a better inhibitor than the L analog. Fig. 5 compares the D- and L-forms; the I_{50} values were 4×10^{-4} and 2×10^{-4} M, respectively. The I_{50} for the longer homolog of the series D, L-1-phenyl-3-hydrazinobutane is also 2×10^{-4} M. The activity of racemic JB516 falls between those of its D- and L-derivatives.

In the present studies the Ae was inhibited reversibly by JB516. When experiments were carried out with the Ae in fraction IV-1, analogously to those described above above with calcium and EDTA, 1×10^{-3} M calcium fully reversed the inhibition of the enzyme by 1×10^{-3} M JB516.

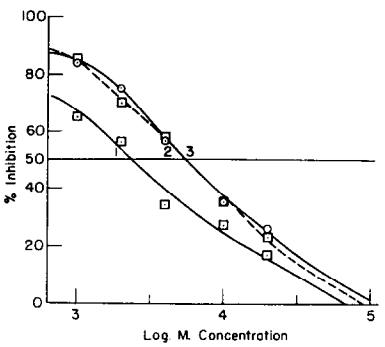

FIG. 5. Inhibition of arylesterase in fraction IV-1 by L-1-phenyl-2-hydrazinopropane (1); D, L-1-phenyl-3-hydrazinobutane (2); and by D-1-phenyl-2-hydrazinopropane(3).

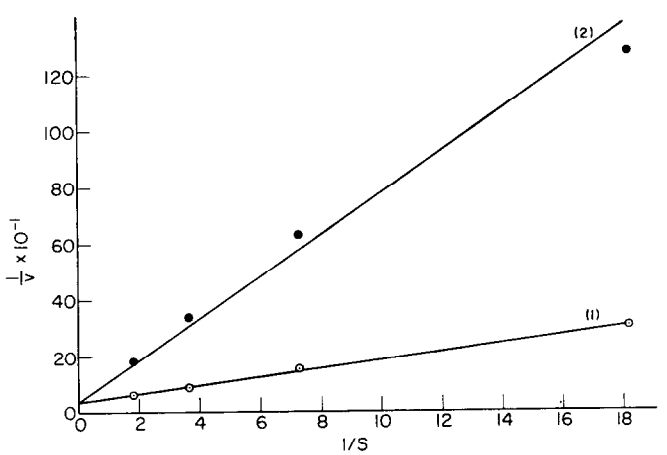

FIG. 6. Competitive inhibition of the hydrolysis of phenyl acetate by arylesterase in fraction IV-1 (1). Inhibitor: JB516 (2). Concentration of substrate and rates were expressed in optical density units.

The type of inhibition was further investigated by the method of Lineweaver-Burk. Figure 6 shows the results. The K_m of Ae from this curve is 8×10^{-4} M and JB516 seems to be a competitive inhibitor of the enzyme.

The inhibition of Ae by $HgCl_2$ or $LaCl_3$ could be clocked by calcium. In Fig. 7 the

reciprocal values of the M concentrations of calcium, 1/Ca, were plotted, according to Nishi,[14] against the reciprocal values of velocity, $1/V$, in the presence and in the absence of the inhibitors, $LaCl_3$ and $HgCl_2$. The inhibition decreased with increasing calcium concentration, but the rate of hydrolysis of the uninhibited, activated enzyme was not reached even in the presence of 3×10^{-3} M $CaCl_2$. The intercept, V_{max}, was different for the inhibited and uninhibited reaction.

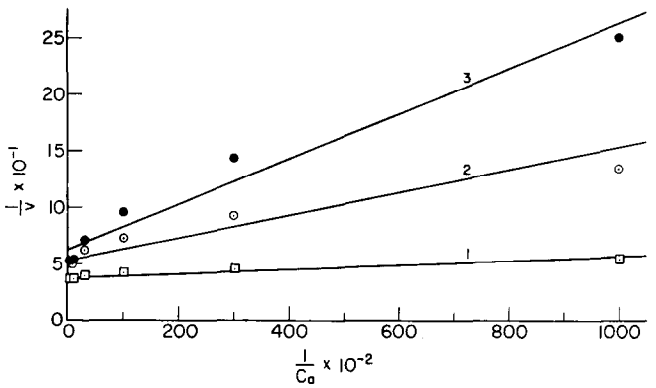

FIG. 7. Effect of increasing the concentration of $CaCl_2$ (1) on the inhibition of arylesterase in fraction IV-1 by 1×10^{-6} M $HgCl_2$ (2) and $LaCl_3$ (3). Uninhibited reaction (1). Regression lines 2 and 3 were calculated according to Finney[15]. Ordinate: Reciprocals of rate in optical density units. Abscissa: Reciprocals of M concentration of $CaCl_2$.

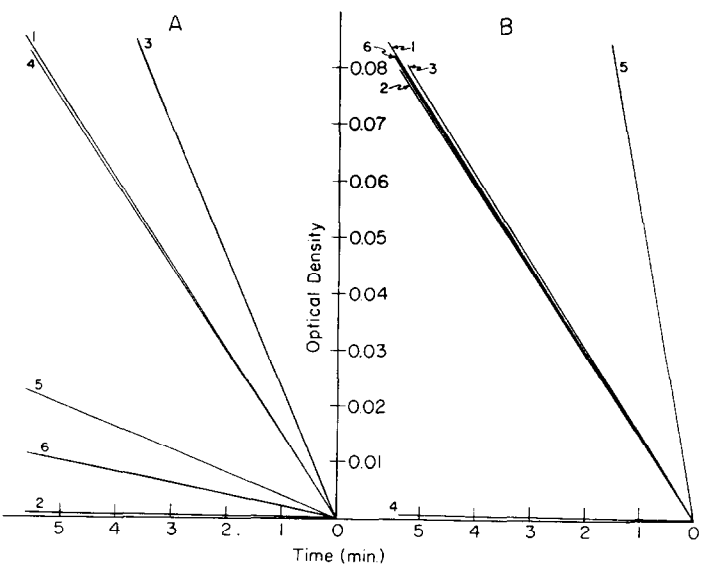

FIG. 8. Inhibition and acceleration of the hydrolysis of phenyl acetate by arylesterase in fraction IV-1 (A) and cholinesterase in fraction IV-6-3 (B) of human plasma: (1) control; (2) EDTA 5×10^{-5} M; (3) $CaCl_2$ 1×10^{-4} M; (4) eserine $2{\cdot}5 \times 10^{-6}$; (5) JB516 1×10^{-3}; (6) $LaCl_3$ 1×10^{-6}.

In a few control studies with various inhibitors, the time of pre-incubation was increased from 5 to 60 min without a significant increase in the percentage inhibition.

Ae belongs to the SH-enzymes which are sensitive to inhibition by urea. Calcium protects the enzyme against this type of inhibition also: the problem will be dealt with in detail elsewhere.

The differences between the Ae in fraction IV-1 and the cholinesterase in IV-6-3 are summarized in Fig. 8. The enzymes were assayed with the same u.v. spectrophotometric technique. Fraction IV-6-3 was diluted 1 : 2000 v/v from a 35 mg/ml stock solution; fraction IV-1, 1 : 100 v/v from a 5 mg/ml stock solution. In this figure the increase in optical density is plotted against time in minutes. Part A shows that the Ae was inhibited by EDTA, JB516 and $LaCl_3$, activated by $CaCl_2$ and unaffected by eserine. The activity of fraction IV-6-3 with PA substrate was accelerated by JB516, unchanged by $CaCl_2$ (1×10^{-4} M), EDTA or $LaCl_3$ and, as expected, inhibited by eserine.

Ae in swine plasma

Like human serum, swine plasma contains only two enzymes which hydrolyze PA: a cholinesterase and an Ae.[16] The Ae was also very sensitive toward the various inhibitors tried here. At 1 : 400 v/v dilution, the swine serum was inhibited 95 per cent by 1×10^{-4} M EDTA, 98 per cent by 1×10^{-4} M Mg-EDTA, 62 per cent by 1×10^{-3} M JB516; Ca-EDTA did not inhibit. Other inhibitors of human Ae were also active. At 1 : 1000 v/v dilution at a wavelength of 2700 Å the following inhibition was observed: $2{\cdot}5 \times 10^{-7}$ M $GdCl_3$ and $LaCl_3$, 68 and 76 per cent, respectively; 5×10^{-5} M *p*-chloromercuriphenylsulfonic acid, 50 per cent.

TABLE 4. EFFECT OF DIALYSIS ON ARYLESTERASE IN SWINE SERUM

	Activity in the presence of:					
	—		EDTA		Ca-EDTA	
Enzyme*		+ 1×10^{-3} M $CaCl_2$		+ 1×10^{-3} M $CaCl_2$		+ 1×10^{-3} M $CaCl_2$
I (stored)						
17 hr	100	97	4	81	108	110
40 hr	100	94	3	58	109	102
II (dialyzed)						
17 hr	86	90	46	49	95	90
40 hr	83	78	30	29	81	80

* Swine serum was stored (I) or dialyzed (II) against tris for 17 and 40 hr, respectively. The concentration of EDTA or Ca-EDTA in the solution of enzyme at the start of the experiments was 4×10^{-3} M, the final dilution in the non-dialyzed samples 1×10^{-4} M. The activity of the stored enzyme = 100.

In variance to that with human Ae, the observed rate of hydrolysis of PA in swine plasma did not increase in the presence of added calcium. Therefore, the role of calcium

was also investigated in dialysis studies. Even during the 40-hr run, the swine Ae level decreased but little. The presence of Ca-EDTA had no significant effect; on the other hand, 97 per cent of the activity disappeared during the storage with EDTA. Depending on the length of storage, the enzyme can be reactivated in part by adding calcium to the system. The dialyzed sample which contained EDTA at the beginning of the experiment lost 70 per cent of its activity in 40 hr. Here calcium did not reactivate. Thus, the experiments suggest that the inhibition of the enzyme is reversible in the presence of bound calcium. The removal of calcium from the medium leads to irreversible changes.

The distribution of the enzyme in the fractions of swine serum was less well defined than in human. The activity of the various samples was assayed with the manometric technique. EDTA (1×10^{-3} M) and eserine (1×10^{-5} M) were used as inhibitors. Ae was found in fractions I and III and cholinesterase in fractions II and IV.

Red blood cell Ae

The existence of three enzymes in human red blood cells, all capable of hydrolyzing PA, was described by Mounter and Whittaker.[3] The first enzyme, a cholinesterase, is inhibited by eserine, the second one, an aliesterase, by DFP. Lacking a specific inhibitor, these authors assumed that the third enzyme would be identical with plasma Ae. We have studied this enzyme in the presence of eserine, 1×10^{-5} M, in the automatic recording titrator. DFP achieved only 5 per cent additional inhibition, a finding which indicates that at the 1×10^{-3} M substrate level, the aliesterase did not play an important role in the overall hydrolysis rate. After 5 min of pre-incubation the activity of the third enzyme was remarkably resistant to inhibitors of serum Ae. Both JB516 and EDTA were ineffective at 1×10^{-3} M concentration; $CaCl_2$ did not activate the enzyme. 1×10^{-3} M *p*-chloromercuriphenylsulfonic acid inhibited three samples less than 25 per cent but the Ae in the red blood cells of the fourth individual was inhibited 90 per cent. Sodium fluoride, 1×10^{-2} M, inhibited only 20 per cent the hydrolysis of PA. As with phosphatases, $MgCl_2$ in the same concentration accelerated the enzyme by 31 per cent; 1×10^{-3} M or lower concentrations of $LaCl_3$, $GdCl_3$ and $CeCl_3$ instead of inhibiting, increased the acid release. As the liberation of acid in the titrator also increased in the absence of substrate, it was neither due to an enhancement of Ae activity nor to the rare earth alone,[17] but probably to the catalytic effect of rare earth ions. Trapmann[18] indicated that rare earth cations catalyze the hydrolysis of phosphoric acid esters, carboxylic acid esters, etc. The release of acid from haemolyzed red blood cells could reflect a similar catalytic activity.

The experiment suggests that Ae in serum differs from the Ae in red blood cells.

DISCUSSION

Human serum contains at least two enzymes which hydrolyze PA. One is a cholinesterase, the other one an aryl (aromatic) esterase (Ae). Our previous communication[6] has already indicated that calcium is indispensable for the functioning of the Ae. Independently, and a short time later, Marton and Kalow[5] came to similar conclusions. These authors also suggested that in addition to Ca^{2+}, other bivalent metal ions like Cu^{2+}, and Mn^{2+} might be of some importance. The present experiments do not seem to support this theory.

In the experiments described here, the role of calcium was explored with dialysis

studies and other procedures and by using various sequestering agents. During dialysis, the human enzyme remained stable only when it was dialyzed against calcium. Furthermore, EDTA and Mg-EDTA inhibited the Ae while Ca-EDTA was inactive. Thus, the inhibition by Mg-EDTA could mainly be due to the binding of a cation with an affinity to EDTA equal to or less than that of calcium, and higher than that of magnesium. Except for Ca^{2+}, only Sr^{2+} would approach this requirement, but $SrCl_2$ inhibited only. The experiments with other sequestering agents also point to calcium.

Recently, Bernsohn *et al.*[19] confirmed an earlier observation[20] that calcium increased the hydrolysis rate of PA in human serum. Our experiments contradict their failure to observe an activity loss on dialysis.

The best inhibitors of Ae are rare earth cations and yttrium, all of them active at a very low concentration. This effect is probably one of the strongest biological activities known for rare earths.[21] The inhibition caused by $LaCl_3$—and also by some other agents—is antagonized by calcium. It is of interest that calcium and rare earths have the opposite effect on the coagulation of blood[18] also.

The Ae is inhibited by reagents which are known to combine with the SH- groups of proteins. This inhibition is reversed in part by glutathione, as first shown in rabbit serum by Aldridge[1, 2] who also has noticed the inhibition of this enzyme by heavy metals and magnesium. Later, Underhay[22] and Hobbiger[23] mentioned the sensitivity of human Ae to SH-reagents and metals.

A current publication of Marton and Kalow[24] describes several inhibitors of Ae. The most active agents, like chlorpromazine and naphazoline, inhibited in the 10^{-4} M concentration. Chlorpromazine is also a potent inhibitor of cholinesterases.[25] In contrast to JB516, these inhibitors are not antagonized by calcium.

JB516 is also a useful agent for distinguishing the esterases in fractions IV-6-3 and IV-1. While the observed cholinesterase activity is enhanced by the ionized form of this agent, the Ae in fraction IV-1 is inhibited selectively. Some aspects of this acceleration were discussed briefly previously by ERDÖS *et al.*[26]; the details of this effect will be published elsewhere. It has already been shown by one of us that JB516 inhibits the "true" cholinesterase in snake venom.[27]

The Ae activity of human plasma was found mainly in Cohn fraction IV-1. This was briefly mentioned in an earlier communication by Erdös and Debay.[28] The inhibition and acceleration of the enzyme is very similar to that of human serum Ae. The behavior of this enzyme during dialysis is also comparable to that of serum Ae, but it loses activity somewhat slower than does the serum enzyme.

Augustinsson and Heimbürger[29] described the existence of an enzyme in fraction IV-1 capable of hydrolyzing an organophosphorus inhibitor, tabun. This enzyme is activated by Sr^{2+} and Ba^{2+} and unaffected by Ca^{2+}, La^{3+} or dialysis.[30] Augustinsson and Heimbürger[29] have already indicated that the same fraction can also hydrolyze *p*-nitrophenyl acetate.

After finishing the experimental part of the work, we noticed a recent publication mentioning the existence of Ae in fraction IV-1.[31]

Swine serum contains an aryl and a cholinesterase.[16] The Ae was inhibited by the inhibitors of human Ae. Ca^{2+} did not increase the observed hydrolysis rate, but it reactivated the enzyme which was inhibited by EDTA even after prolonged storage.

This effect was much more pronounced with swine enzyme than with Ae from human sources. The activity of Ae decreased but slightly after 40 hr of dialysis, a finding which indicates that the calcium is more firmly bound to the swine Ae than to the human, but it could be removed and the enzyme irreversibly inhibited by dialyzing the Ae with EDTA.

A recent report[16] indicated that the Ae in swine serum is a gene-controlled enzyme and is to be regarded as one of the first examples of a direct relation between genes and enzymes in higher animals. Naturally, the authors of the publication could only assay the active form of Ae. Our data suggest that Augustinsson and Olson's[16] results could just as well involve genetic factors having to do with the metal binding as with the total synthesis of the enzyme.

The Ae in red blood cells was remarkably resistant to most of the inhibitors of Ae. It was sensitive to *p*-chloromercuriphenylsulfonic acid, but less than to *p*-chloromercuribenzoic acid.[22] While Mounter and Whittaker[3] assumed the identity of the Ae in plasma and red blood cells, Underhay[22] already indicated some differences between the two enzymes. This observation was based on the different rate of hydrolysis of various substrates by the two Ae. Our experiments with selective inhibitors show that the two enzymes are probably not identical.

Acknowledgements—The writers are thankful for the advice and interest of Dr. T. H. Davies in their work and for the many helpful discussions with Drs. A. J. Cohen, J. A. Laswick and N. H. Sloane. The assistance of Miss L. Boggs is greatly appreciated.

The various materials used in these experiments were donated by the following individuals: Fractions of human plasma by Dr. J. H. Hink, Cutter Laboratories and by Dr. R. B. Pennell, Protein Foundation, Inc.; JB516 and its derivatives by Professor J. P. Buckley, University of Pittsburgh and by Drs. J. H. Biel and H. L. Friedman, Lakeside Laboratories; EDTA, its derivatives and the technical data by Dr. H. F. Harrison, Geigy Industrial Chemicals.

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