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Effects of Lipid Removal on the Molecular Size and Kinetic Properties of Bovine Plasma Arylesterase

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A purified arylesterase preparation from bovine plasma was characterized to the extent that it has a partial specific volume of 0.91 ml/g and an apparent *z*-average molecular weight of 440000. The relatively large magnitude of the former reflects the presence of phospholipids, cholesterol, triglycerides and β-carotene, the last-named being responsible for the pronounced yellow colour of the preparation. Removal of the lipid material is accompanied by a decrease in the apparent *z*-average molecular weight to 120000, the size of the smallest species detected by high-speed sedimentation equilibrium being in the vicinity of 70000 daltons: denaturation of the lipid-free preparation with 6M-guanidine hydrochloride caused essentially complete breakdown into subunits of this size. In kinetic studies on the enzyme the maximal velocity for the hydrolysis of phenyl acetate was found to increase by 60% on addition of 1 mM-Ca^{2+}, with the K_m showing a concomitant decrease from 6.6 to 2.1 mM. Removal of lipid had no detectable effect on $V_{max.}$ or K_m in either the presence or the absence of Ca^{2+}. It is concluded that the bovine plasma arylesterase preparation is either a lipoprotein or an enzyme–lipoprotein complex with properties very similar to those of the α_1-lipoprotein or high-density lipoprotein (HDL_2) fraction of serum.

The enzymic characteristics of arylesterases have in the main been documented on the basis of results obtained with components of standard serum fractions (Erdös *et al.*, 1959, 1960; Augustinsson, 1961; Marton & Kalow, 1962) rather than with purified enzyme preparations. Choi & Forster (1967*a*,*b*), however, have reported a procedure for isolating bovine plasma arylesterase, the properties of which were noteworthy in two respects. (i) The gel-chromatographic behaviour of the arylesterase on Sephadex G-200 was characteristic of a protein with a much higher molecular weight than that of most other mammalian esterases (Kingsbury & Masters, 1970). (ii) On precipitation of the arylesterase from plasma by addition of $(NH_4)_2SO_4$ the protein and a yellow-coloured material floated on the surface, a factor that suggests the existence of the enzyme as a lipoprotein complex; a similar conclusion has also been derived from immunological studies (Talal *et al.*, 1963; Hermann *et al.*, 1963). The purpose of the present paper is to investigate further these two features of the bovine plasma arylesterase preparation, particularly with regard to the effect of lipid removal on its molecular and kinetic properties.

Experimental

Materials

Special enzyme-grade $(NH_4)_2SO_4$ was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A., as were the samples of apoferritin (horse), ovalbumin (twice crystallized), cytochrome *c* (horse heart) and crystalline bovine serum albumin used to calibrate a Sephadex G-200 column. Additional standard proteins used for the latter purpose were ox liver catalase (twice crystallized) and soybean lipoxidase, obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., who also supplied eserine sulphate, Triton X-155, Tris (Trizma base) and β-carotene. Phenyl acetate, a product of Eastman Organic Chemicals, Rochester, N.Y., U.S.A., was redistilled before use. Other chemicals were of reagent grade.

Methods

Isolation of the enzyme. Blood was collected from individual animals and processed by a method based on that described by Choi & Forster (1967*b*), which

entails fractionation with $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose and Sephadex G-200. In accordance with their procedure, Ca^{2+} (1mM) and EDTA (5μM) were added as a routine to the 10mM-Tris–HCl buffer (pH8.0) used, but their inclusion of Triton X-155 was discontinued because of its inhibitory effect on arylesterase activity (Fig. 1). This result seemingly contrasts with those of Choi & Forster (1967*a*), who found it necessary to add Triton X-155 for reproducibility in their assay procedure. Since Choi & Forster (1967*a*,*b*) used emulsified phenyl acetate as substrate, it now seems likely that the detergent exerted a stabilizing effect on the emulsion rather than on the enzyme. In the present investigation a 60-fold purification of bovine plasma arylesterase, with 30% recovery, was achieved. Omission of the ion-exchange chromatography step led to a 25-fold increase in specific activity over the arylesterase activity in bovine plasma, the molecular-size properties of this partially purified preparation being indistinguishable experimentally from those of the material with a higher specific activity: with phenyl acetate as substrate, this partially purified material had a specific activity of 12.5μmol/min per mg of protein. Arylesterase preparations from the blood of four animals were indistinguishable enzymically or physicochemically.

Assay procedure. Enzymic activity was determined with phenyl acetate as substrate (Zeller, 1956). Enzyme solution (0.05–0.10ml) was incubated with 2.7ml of the 10mM-Tris–HCl buffer (pH8.0) containing Ca^{2+} (1mM), EDTA (5μM) and eserine sulphate (40μM) for 5min at 25°C, the last being added to inhibit cholinesterase activity. The reaction was initiated by adding 0.3ml of phenyl acetate (10mM) and its progress followed by monitoring the increase in absorbance at 270nm on a Unicam SP. 800 recording spectrophotometer. One unit of activity was defined as the production of 1μmol of phenol/min per ml of enzyme solution. In the conversion of these values into specific activities (μmol of phenol/min per mg of protein) protein concentrations were based on absorbance measurements at 260nm and 280nm (Warburg & Christian, 1942). Alternatively, the biuret procedure of Gornall *et al.* (1949) was used for the measurement of protein concentrations, which were then based on colour yields for bovine serum albumin. Values obtained by the two procedures agreed to within 5%.

Gel chromatography. Samples (2ml) of arylesterase and of each standard protein were applied separately to a column (1.5cm×70cm) of Sephadex G-200, equilibrated with 10mM-Tris–HCl, pH8.0, containing Ca^{2+} (1mM) and EDTA (5μM): the flow rate in these experiments, conducted at 5°C, was adjusted to 7.5ml/h. A fraction collector operating on a time basis was used to divide the column eluate into 1ml fractions, which were analysed spectrophotometrically and, where relevant, for arylesterase activity. The elution volume of each solute was as a routine converted into a partition coefficient, K_D (Gelotte, 1960). In the construction of calibration plots for the estimation of molecular size, the molecular weights of the standard proteins were taken from Table 1 of Andrews (1965), as were the diffusion coefficients used for the calculation of Stokes radii.

Solvent extraction of arylesterase. Purified enzyme solution (20ml, containing 70mg of protein) was extracted with 200ml of acetone at −10°C for 15min and then centrifuged for 5min at −10°C and 4000*g*. The resulting precipitate was redissolved in 20ml of cold (5°C) 10mM-Tris–HCl (pH8.0) containing 1mM-Ca^{2+} and 5μM-EDTA and dialysed at 5°C against 100vol. of the same buffer for 16h: any undissolved material was removed by centrifuging. Re-extraction at −10°C of this acetone-extracted preparation with more acetone followed by diethyl ether yielded a lipid-free sample of the bovine plasma arylesterase preparation (63mg of protein). Based on protein content the specific activities of the solvent-treated preparations were indistinguishable from that of the isolated lipoprotein fraction (12.7 and 12.5μmol/min per mg of protein respectively).

After each extraction the organic solvent was evaporated to dryness in a rotary film evaporator, after which the fatty material was redissolved in a minimum amount of chloroform and sealed in glass-stoppered vials under O_2-free N_2 until required for lipid analysis.

Lipid and carbohydrate analysis. The extracted lipid material was subjected to t.l.c. on silica gel in a hexane–diethyl ether (25:2, v/v) solvent system (Gloster

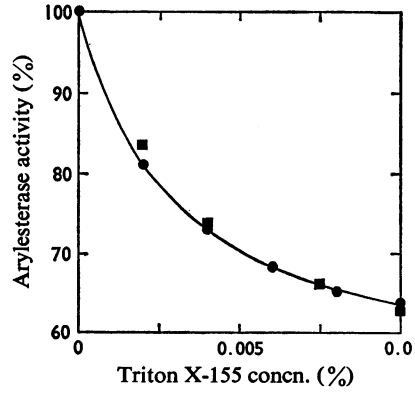

Fig. 1. *Effect of Triton X-155 on the arylesterase activity of bovine plasma*

● and ■ refer to plasma from different animals. For details see the text.

& Fletcher, 1966). Lipids were detected by spraying plates with aq. 50% (v/v) H_2SO_4 followed by heating to 160°C for 30min. Spots were identified by comparison of R_F values with those of standard lipids. β-Carotene was measured separately by the method of McLaren *et al.* (1969). Enzyme solutions were assayed for the presence of sialic acid and neutral hexoses by the methods of Aminoff (1961) and Dubois *et al.* (1956) respectively.

Ultracentrifugal analysis. Before ultracentrifugation, solutions of the native enzyme (lipoprotein) and the lipid-free preparation were dialysed for 16h against 10mM-Tris–HCl (pH8.0) containing 1mM-Ca^{2+}, 5μM-EDTA and 100mM-KCl. Samples were then subjected to sedimentation at 20°C in a Spinco model E ultracentrifuge. Protein concentrations of approx. 5mg/ml were used in velocity experiments, from which sedimentation coefficients were calculated by standard methods and corrected to water at 20°C. From photographic records of schlieren profiles it was found that 2mm columns of esterase solutions (2mg/ml initially) had attained sedimentation equilibrium after rotation for 16h at either 11272 or 20410rev./min, the latter speed being sufficient to yield patterns of the Yphantis (1964) type: a similar situation applied to high-speed (33450rev./min) runs on lipid-free enzyme that had been dialysed for 16h against buffer in which the KCl had been replaced by 6M-guanidine hydrochloride. Patterns were measured on a Nikon two-dimensional comparator fitted with a projection screen and accurate to 2μm. Results were analysed either by method 2 of Van Holde & Baldwin (1958) or by the Lamm (1929) procedure: the former yields $\bar{M}_z^{app}$ the apparent z-average molecular weight of the sample, whereas the latter gives $(M_z^{app})_r$, the apparent z-average molecular weight at a point distance r from the centre of rotation (Marler *et al.*, 1964). A value of 0.73 was assumed for the partial specific volume ($\bar{v}$) of the lipid-free arylesterase preparation in both buffer environments; $\bar{v}$ of the native enzyme preparation was taken as 0.91 on the basis of the hydrated density obtained from velocity sedimentation of 0.5mg/ml of solutions of the enzyme in which the density was varied by adding KCl (0.25–1.25M) to the buffer.

The diffusion coefficient ($D_{20,w}$) of the native arylesterase preparation was estimated by the conventional height–area analysis of a synthetic-boundary experiment performed at low speed (approx. 5000rev./min) in the ultracentrifuge: a portion of the enzyme solution used for the velocity run was used in this experiment.

Kinetic studies. To investigate the effect of Ca^{2+} on the enzymic hydrolysis of phenyl acetate, 3ml samples of the arylesterase preparation (either native or lipid-free material) were first subjected to gel chromatography on a column (1.5cm×20cm) of Sephadex G-25, equilibrated with 0.05M-Tris–HCl, pH8.0, to remove the Ca^{2+} ions already present: good separation was achieved between the protein and Ca^{2+} peaks. Fractions containing protein were pooled and used as the source of enzyme. The method adopted for kinetic studies differed from that described in the assay procedure by virtue of (i) the use of stronger (0.05M) Tris–HCl buffer, (ii) omission of the eserine sulphate, (iii) addition of Ca^{2+} only to the relevant reaction mixtures and (iv) variation of the amount of phenyl acetate added so that its initial concentration in reaction mixtures ranged from 0.66 to 3.0mM. Each reaction mixture contained the same amount (0.5mg) of protein.

Results

The gel-filtration behaviour of the purified arylesterase on Sephadex G-200 is shown in Fig. 2, which shows that the enzymic activity, u.v.-absorbing and coloured material co-chromatograph as a single, symmetrical peak. Indeed, the enzyme preparation was deep yellow, owing to the presence of 0.3g of β-carotene/100g of protein. β-Carotene was identified by spectral studies and by t.l.c., which also indicated the presence of phospholipids, cholesterol and triglycerides: the method of detection precluded quantitative analysis in terms of these lipids, which must,

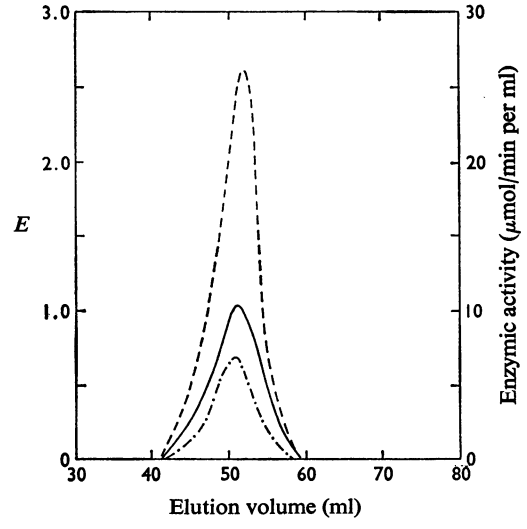

Fig. 2. *Elution profiles obtained in gel chromatography of purified bovine plasma arylesterase*

A column (1.5cm×70cm) of Sephadex G-200 equilibrated with 10mM-Tris–HCl, pH8.0, containing 1mM-Ca^{2+} and 5μM-EDTA, was used. ——, E_{280}; -·-·-·-, E_{465}; ----, esterase activity towards phenyl acetate (right-hand ordinate).

however, comprise 40–50% of the lipoprotein complex to account for its hydrated density (1.09_5 g/ml). Carbohydrates detected in the lipid-free arylesterase preparation were sialic acid (0.1%) and neutral hexoses (0.9%): these carbohydrate contents are slightly lower than those reported for human (Scanu, 1966) and rat (Marsh & Fritz, 1970) serum lipoproteins.

The effect of acetone and ether extraction on the gel-chromatographic behaviour of the enzyme is summarized in Fig. 3, where the circles (●) refer to the activity results presented in Fig. 2: a Stokes radius of 5.7nm (57Å) and hence an apparent molecular weight in excess of 350000 are obtained for the native arylesterase on the basis of the Andrews (1964, 1965) method of molecular-weight estimation. After one treatment with acetone the enzyme exhibited greater heterogeneity on gel chromatography (■ in Fig. 3), owing to appearance of active material with larger distribution coefficient K_D (Gelotte, 1960) and hence smaller apparent molecular weight. Further extraction with acetone and finally with ether increased the proportion of more slowly migrating material, to the extent that the elution volume of the major peak of activity corresponded to a Stokes radius of 4.5nm (45Å), or an apparent molecular weight of 140000 (▲ in Fig. 3): esterase activity corresponding to material with even larger K_D (smaller apparent molecular weight) is also evident.

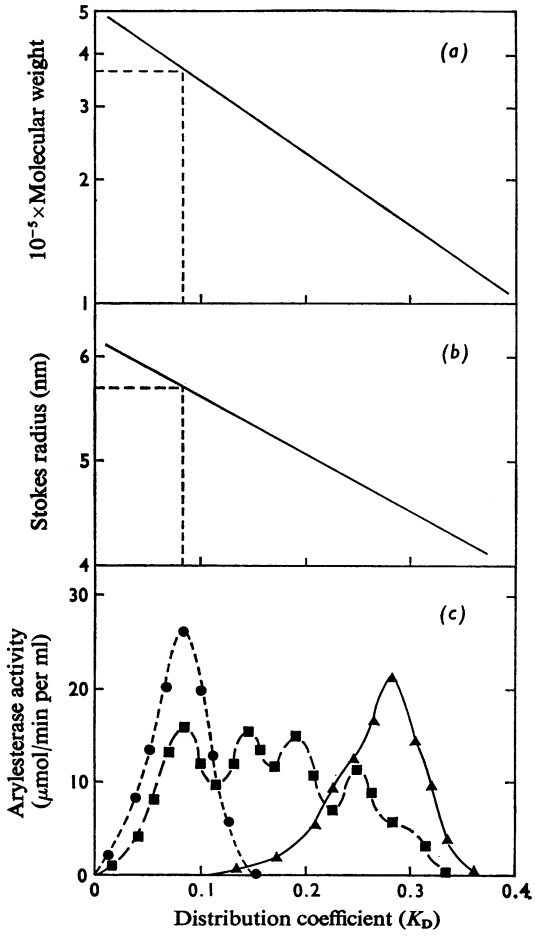

Fig. 3. *Effect of lipid removal on the gel-chromatographic behaviour and hence apparent molecular size of bovine plasma arylesterase*

Experimental conditions are as described in Fig. 2. ●, Native enzyme; ■, enzyme extracted once with acetone; ▲, enzyme extracted twice with acetone and once with ether (for further details see the text).

Greater heterogeneity and also dissociation was also observed in sedimentation-velocity experiments on the lipid-free enzyme: Fig. 4 presents patterns obtained with identical protein concentrations (5mg/ml) of the native (upper pattern) and lipid-free (lower pattern) enzyme. In this connexion it is noted that the single boundary obtained with the lipoprotein does not signify homogeneous sedimentation behaviour, since analysis of boundary spreading by the Van

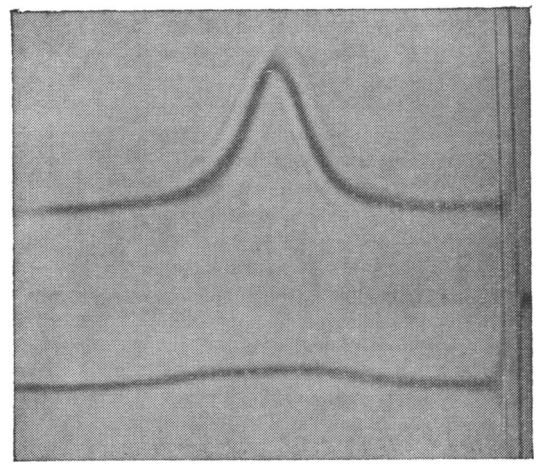

Fig. 4. *Typical sedimentation-velocity patterns for native (upper trace) and lipid-free (lower trace) bovine plasma arylesterase preparations*

Preparations were centrifuged in 10mM-Tris–HCl, pH8.0, containing 100mM-KCl, 1mM-Ca^{2+} and 5μM-EDTA. In this exposure, taken after centrifugation for 60min at 59780rev./min, sedimentation is from right to left.

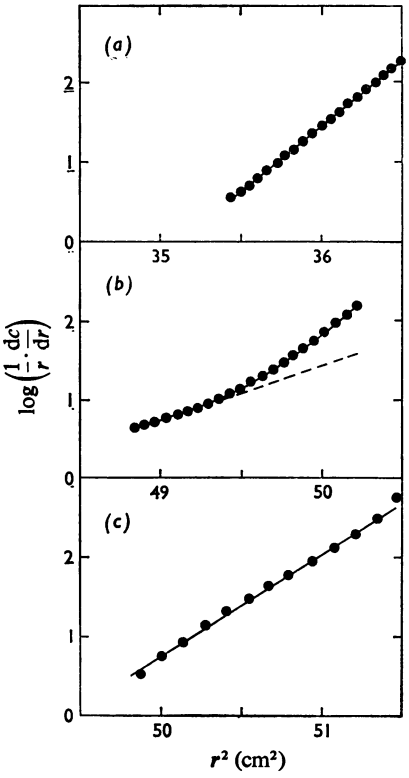

Fig. 5. *Lamm (1929) plots derived from schlieren patterns obtained in high-speed sedimentation-equilibrium experiments on bovine plasma arylesterase*

(*a*) Native enzyme in 100mM-KCl–1mM-Ca^{2+}–5μM-EDTA–10mM-Tris–HCl, pH8.0; (*b*) lipid-free enzyme in the same medium; (*c*) lipid-free preparation in the same buffer but with the KCl replaced by 6M-guanidine hydrochloride. The nominal rotor speed was 33450rev./min in (*c*) and 20410rev./min in (*a*) and (*b*). For details see the text.

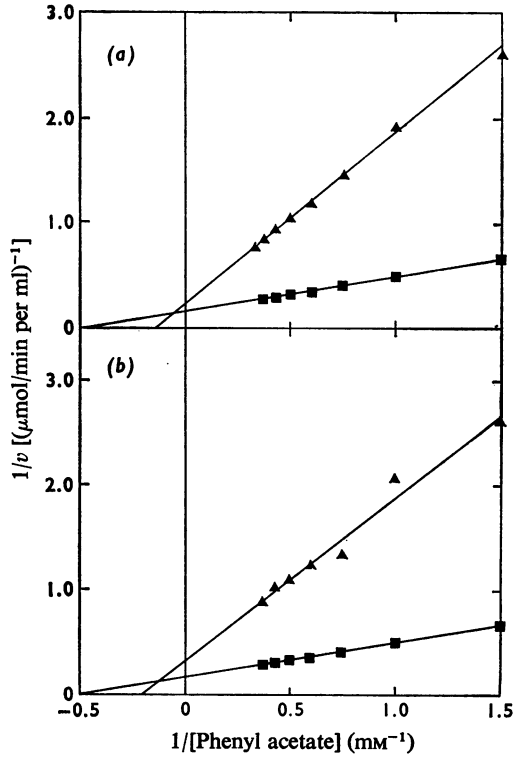

Fig. 6. *Double-reciprocal plots of kinetic data for the hydrolysis of phenyl acetate by bovine plasma arylesterase in 50mM-Tris–HCl, pH8.0, containing 5μM-EDTA*

(*a*) Native enzyme; (*b*) a lipid-free preparation. ▲, Reaction mixtures without Ca^{2+}; ■, mixtures supplemented with 1mM-Ca^{2+}.

Holde (1960) adaptation of the Fujita (1956) procedure yielded an estimate of the diffusion coefficient much greater than that obtained from a low-speed synthetic-boundary run. The value of 3.8×10^{-7}cm$^2\cdot$s^{-1} obtained for $D_{20,w}$ correlates well with Fig. 3 in the sense that a Stokes radius of 5.7nm (57Å) is again indicated, its combination with the estimate of 5.8S for $s_{20,w}$ yielding an apparent molecular weight of 440000 for the native arylesterase preparation.

To confirm the size of the native enzyme preparation and to obtain similar information on the lipid-free material, solutions were subjected to low-speed sedimentation equilibrium. Application of method 2 of Van Holde & Baldwin (1958) to results from a low-speed equilibrium experiment substantiated the gel-chromatographic results inasmuch as it yielded values of 430000 and 120000 for $\bar{M}_z^{app}$, the apparent *z*-average molecular weights of the native and lipid-free arylesterase preparations respectively. Lamm (1929) plots of results from a high-speed run are shown in Fig. 5, which shows that the results for the native enzyme (Fig. 5*a*) conform reasonably well to a straight line, the slope of which yields a value of 450000 for $(M_z^{app})_r$. The equivalent plot for the lipid-free enzyme is non-linear (Fig. 5*b*), an estimate of approx. 70000 being obtained for $(M_z^{app})_r$ in the region of lowest solute concentration. The heavier material present in this lipid-free preparation could also be dissociated to a subunit of similar size by denaturation with 6M-guanidine hydrochloride.

From Fig. 5(*c*), which presents the Lamm (1929) plot from a high-speed run on a solution made 6M with respect to guanidine hydrochloride, a value of 67000 is obtained for (M_z^{app}), throughout almost the entire column length in which macromolecular solute is distributed.

Results of kinetic studies of the hydrolysis of phenyl acetate by the native and lipid-free arylesterase preparations are summarized in Fig. 6, about which the following points are noted. First, addition of Ca^{2+} produces marked activation of the native arylesterase activity (Fig. 6*a*). Not only is $V_{max.}$ increased from 4.1 (±0.4) to 6.6 (±0.1) μmol/min per ml, but K_m also decreases from 6.5 (±0.8) to 2.3 (±0.1) mM: values in parentheses are based on the standard errors of slopes and intercepts obtained by subjecting the results to least-squares calculations. Secondly, these results find qualitative parallels in studies of arylesterase activity in fraction IV-1 (Cohn *et al.*, 1946) of human serum inasmuch as Marton & Kalow (1962) also report a 60% increase in $V_{max.}$ and a threefold decrease in K_m on addition of 1 mM-Ca^{2+} to their system. Thirdly, removal of lipid material from the present preparation had no detectable effects on either $V_{max.}$ or K_m (Fig. 6*b*). The values of 6.4 (±0.3) μmol/min per ml for $V_{max.}$ and 2.1 (±0.2 mM) for K_m obtained with lipid-free material in the presence of 1 mM-Ca^{2+} are clearly in excellent agreement with the corresponding figures for native enzyme, and the respective values in the absence of Ca^{2+}, i.e. 3.3 (±1.0) μmol/min per ml and 5.3 (±2.0) mM, are also indistinguishable experimentally from the results obtained with the native arylesterase preparation.

Discussion

As noted above, the kinetic results obtained with the present bovine plasma arylesterase preparation bear a marked similarity to those reported for the arylesterase activity of fraction IV-1 of human serum (Marton & Kalow, 1962). In view of the presence of lipid in our arylesterase preparation, it seems plausible to correlate their findings with the existence of α_1-lipoprotein in fraction IV-1. Indeed, the molecular properties of the present preparation bear a striking resemblance to those of α_1-lipoprotein or high-density lipoprotein $(HDL)_2$ from human serum (see Table 1): the decrease in size of the latter lipoprotein on lipid removal is also well established (Scanu *et al.*, 1958; Scanu, 1966). This similarity raises the question as to whether arylesterase is a lipoprotein, or whether it is a protein (or glycoprotein) with pronounced hydrophobic character and which therefore is found in association with the α_1-lipoprotein complex of plasma. To this end it is noted that apart from β-carotene the non-protein constituents of the present arylesterase preparation are also those found in fraction HDL_2 of human serum (Scanu, 1966). The fact that the β-carotene of human serum is associated with the β-lipoprotein (or LDL) fraction (Gurd *et al.*, 1949; Oncley *et al.*, 1950) and not the fraction IV-1 studied by Marton & Kalow (1962) greatly decreases the likelihood of the pigment being an essential part of the bovine plasma arylesterase. Further evidence for the concept of the bovine plasma enzyme being a protein or glycoprotein in association with a lipoprotein complex rather than a lipoprotein *per se* is the similar hydrolytic characteristics of the native and lipid-free preparations towards phenyl acetate (Fig. 6), particularly in the event that the enzyme exhibits similar kinetic properties with its biological substrate (Aldridge, 1953). It is noteworthy also that a recent immunological investigation of animal sera led to the conclusion of non-identity between the high-density lipoproteins and esterase activity (Kaminski & Dubois, 1972).

From the above discussion it is evident that the molecular weight determined for the native enzyme preparation does not necessarily pertain to bovine plasma arylesterase itself. Further, the minimal value (approx. 70000 daltons) for the lipid-free material would refer to the enzyme only in the event of bovine plasma arylesterase being (a) the smallest macromolecule present in the α_1-lipoprotein complex and (b) a substantial contributor to its protein content. Thus, this investigation of bovine plasma arylesterase has succeeded only in identifying the Choi & Forster (1967*b*) preparation as either a lipoprotein or an enzyme–lipoprotein complex with molecular properties very similar to those of the α_1-lipoprotein (or HDL_2) fraction of serum. Because of the relatively low purification achieved (approx. 60-fold) and the lack of any known specific biological role for large amounts of relatively inactive enzyme, the second proposition seems the more likely, in which case

Table 1. *Comparison of the molecular properties of bovine plasma arylesterase with those of α_1-lipoprotein and high-density lipoprotein (HDL_2) of human serum*

Preparation	Mol.wt.	$\bar{v}$ (ml/g)	$s_{20,w}$ (S)	Reference
Bovine enzyme	440000 (M_z^{app})	0.91	5.8	The present paper
α_1-Lipoprotein	435000 (M_w^{app})	0.91 (5)	5.5	Shore (1957)
Fraction HDL_2	400000 (M_w^{app})	0.90 (5)	5.4 (5)	Hazelwood (1958)

α_1-lipoprotein or high-density lipoprotein could well prove a more convenient source of arylesterase for further purification.

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