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IDENTIFICATION OF AN ARYLESTERASE AS THE ENZYME HYDROLYSING DIACETYLMORPHINE (HEROIN) IN HUMAN PLASMA

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Abstract—The enzyme hydrolysing diacetylmorphine (heroin) in human plasma has been characterized as an arylesterase. The enzyme was shown to be identifiable with ARE_3 (according to the Burlina notation) by cellulose acetate electrophoresis and gel filtration. The evidence was further consolidated by the use of enzyme kinetics. A scheme for the partial purification of the enzyme (sixty fold) is presented. Additional information is reported on the status of arylesterases in relation to the hydrolysis of organophosphates (E-600).

Diacetylmorphine (heroin, diamorphine) is a morphine analogue in which the phenolic 3-hydroxyl and the alcoholic 6-hydroxyl functions have been converted to the diacetyl derivatives; the phenolic acetyl group will be more labile than the alcoholic acetyl group. The tissues of most species are reported to rapidly deacetylate diacetylmorphine to 6-monoacetylmorphine (MAM) and more slowly to morphine [1].

There has been considerable discussion as to whether diacetylmorphine and/or MAM and/or morphine are the pharmacologically active compounds responsible for analgesic activity. Heroin's increased potency over morphine has been ascribed to drug latentiation [3]: the blocking of the hydroxyl groups by biologically removable lipophilic groups [2, 4] thereby facilitating rapid entry into the central nervous system [5, 6].

Diacetylmorphine is stated to be metabolised to MAM and morphine by human blood [1, 2], however, in this laboratory, while heroin was rapidly deacetylated to MAM by rabbit, dog and human blood [7], only rabbit blood further appreciably deacetylated MAM to morphine; a result confirmed by the use of codeine acetate which contains only the alcoholic acetyl group when used as substrate. Codeine acetate was metabolised to codeine by rabbit blood only (unpublished observation).

Little work has been done previously to identify the enzyme(s) involved in diacetylmorphine hydrolysis. Doubt as to the enzymic nature of the hydrolysis has been raised with reference to dog's blood [8].

Cholinesterases are probably not significantly involved in diacetylmorphine hydrolysis [9, 10, 11], however several non specific esterases are known to occur in mammalian blood, principally arylesterases [12] (E.C. 3.1.1.2) and aliesterases [12, 13] (E.C. 3.1.1.1). Arylesterases hydrolyse phenolic esters only, aliesterases hydrolyse both aliphatic and phenolic esters. Aliesterase has been found to be absent in the human plasma, although it is the major esterase in the plasma of lower vertebrates [13]. The arylesterases present in human plasma have been separated into two major and one minor component by cellulose acetate electrophoresis [14]. Arylesterases in contrast to most other esterase enzymes are reported to metabolise, and not be inhibited by, organophosphorus esters (E-600) [16, 17].

The work in this paper was directed towards the elucidation of the enzymic metabolism of diacetylmorphine by human plasma.

MATERIALS AND METHODS

Plasma and red blood cell samples. Venous blood was withdrawn from the forearm of healthy volunteers of both sexes into heparinised syringes; and the plasma separated from the red blood cells by centrifugation at 4000g for 15 min at 4°C. The plasma was removed by aspiration. The red blood cells were washed twice by resuspending in 0·15M NaCl–0·05M phosphate pH 7·5 buffer (Buffer I) and were recovered by centrifugation (details as above).

Substrate chemicals. The following chemicals were used in substrate studies: diacetylmorphine–HCl (May and Baker), *p*-nitrophenol and *p*-nitrophenylacetate (B.D.H.), naphthyl acetate, *p*-nitrophenyl laurate and E-600 (diethyl *p*-nitrophenol phosphate, paraoxon) (Koch-Light).

Enzyme assay. All reactions were carried out in a shaking water bath (Gallenkamp) at 37°C. The enzyme (plasma, plasma fraction (I), or partly purified enzyme) was diluted with 0·05M phosphate pH 7·5 buffer (Buffer II) and preincubated for 5 min. In inhibition experiments the buffer contained the inhibitor. After preincubation the substrate was added to give a final volume of 1 ml. All reactions were run in parallel with blanks containing buffer and substrate. Hydrolysis products were detected by the gas chromatographic method of Smith and Cole [7] in the case of diacetylmorphine, and the absorbance increase in the case of *p*-nitrophenyl esters. The *p*-nitrophenol

formed was measured directly in the incubation medium without prior extraction at 400 nm. Buffer I was used when incubating separated red blood cells or whole blood. For pH's above 8·0, glycine/NaOH (0·05M) buffer was used. The graphic representation of inhibition studies were the result of triplicate experiments.

Protein estimation. Protein was estimated by the method of Lowry [18].

Cellulose acetate electrophoresis. Electrophoresis was performed on whole plasma (or purified fractions). The sample (5–10 μl) was applied at the negative electrode end of cellulose acetate membranes. The membranes were developed in a Shandon Universal electrophoresis tank containing 0·05M Barbitone pH 8·7 buffer [15] at a voltage of 16V/cm for 90 min. Localization of esterase bands was by the staining method of Burlina [14] using naphthyl acetate as substrate. For determination of the substrate specificity of individual bands, unstained strips, following electrophoretic development, were cut widthways into 1·0 or 0·5 cm wide strips. Each strip was then placed, according to mobility, into 0·1M phosphate pH 7·5 buffer (2 ml) for 24 hr to elute enzymes present prior to assay. Multiple preliminary experiments were performed initially to localize substrate specificity. Experiments depicted graphically were the result of four membranes run concurrently and sectioned.

Purification of enzyme. All procedures were carried out at 4°C. Initial experiments were conducted with plasma, or a plasma fraction (I) purified 1·5-fold by $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to diluted blood plasma (15 ml in 25 ml buffer II) until 50% saturation was attained. After standing for 4 hr the solution was centrifuged at 10,000 *g* for 15 min. The supernatant was then brought to 70% saturation by further addition of solid $(NH_4)_2SO_4$. After 4 hr the precipitate was removed by centrifugation (10,000 *g* for 15 min) and dialysed against three changes of buffer II for 30 hr.

A partially purified enzyme was prepared by concentrating blood plasma with the addition of solid Sephadex G-25 and centrifuging (5000 *g* for 5 min) to give a supernatant protein concentration of 128 mg/ml. The supernatant (2 ml) was then subjected to gel filtration on a Sephadex G-100 column (75 × 2 cm, bed volume 65 ml). Fractions (4 ml) were collected after the bed volume had passed through the column. The fractions containing diacetylmorphine hydrolysing activity were combined and solid $(NH_4)_2SO_4$ added to bring the saturation level to 50%. After standing for 4 hr the solution was centrifuged (10,000 *g* for 15 min), the supernatant removed and dialysed against three changes of buffer II for 36 hr. The dialysed preparation was termed partially purified enzyme. Gel filtration results were obtained in duplicate.

RESULTS

Whole plasma and plasma fraction (I). Incubations of blood plasma, red blood cells, and whole blood with diacetylmorphine (1 mM) showed blood plasma responsible for 30–35% (activity 22–32 μg diacetylmorphine hydrolysed/ml/min) of the diacetylmorphine hydrolysis activity in whole blood (63–110 μg/ml/min). The remaining 65–70% activity (41–78 μg/ml/min) was present in the red blood cells. When the red blood cells were lysed, by conducting the incubations in buffer II, there was no change in activity from those conducted in buffer I (containing unlysed cells).

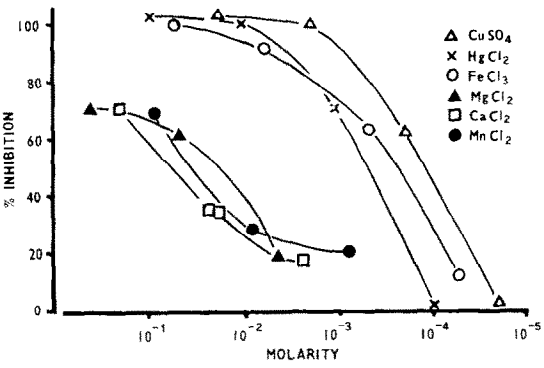

Fig. 1. Inhibition of plasma fraction (I) hydrolysis of diacetylmorphine with different concentrations of metal ions.

The plasma fraction (I) contained 90% of the diacetylmorphine hydrolysing activity of whole plasma. The plasma fraction (I) lost no activity upon dialysis. Preincubation with metal ions at various concentrations (Fig. 1) or at high temperature (65–85% loss of activity after preincubation at 60°C for 6 min) caused loss of diacetylmorphine hydrolysis activity. The effect of pH on the hydrolysis reaction by plasma fraction (I) is exhibited in Fig. 2. The pH optimum for the enzyme is 7·4–7·6. Addition of eserine to the plasma fraction (I) to give a concentration of 10^{-4}M had no effect upon the hydrolysis.

When diacetylmorphine (3·7–0·37 mM) was incubated with the plasma fraction (I) or with whole plasma in the presence of E-600 ($7{\cdot}2 \times 10^{-7}$M), the E-600 acted as a competitive inhibitor of the reaction (Fig. 3). There was an appreciable difference however between the K_i value (approx. $3{\cdot}4 \times 10^{-5}$) for E-600 as a competitive inhibitor of diacetylmorphine hydrolysis (Fig. 4, top) and the K_m value for its hydrolysis to *p*-nitrophenol by the plasma fraction (I), or whole plasma ($5{\cdot}0 \times 10^{-3}$M). This indicated that the enzyme responsible for diacetylmorphine hydrolysis

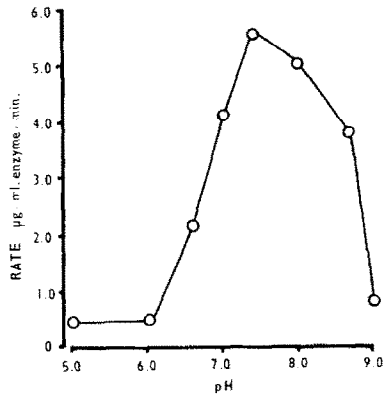

Fig. 2. Plasma fraction (I) hydrolysis of diacetylmorphine versus hydrogen ion concentration.

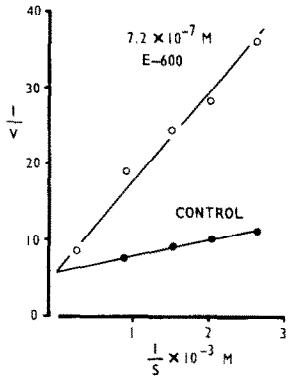

Fig. 3. Effect of E-600 on the kinetics (Lineweaver–Burke plot[22]) of diacetylmorphine hydrolysis by plasma fraction (I).

was probably only partly responsible for E-600 metabolism.

Cellulose acetate electrophoresis. Separation of blood plasma etc. of blood plasma by cellulose acetate electrophoresis and subsequent staining, using naphthyl acetate as substrate demonstrated two major bands corresponding to hydrolytic enzymes (ARE_3 and ARE_2 according to Burlina [14]), with a third fast moving indistinct band (ARE_1) (Fig. 5). This was in agreement with published observations. The plasma fraction (I) gave an identical electrophoretic pattern. Assay of fractions prepared from the sectioned membranes showed diacetylmorphine hydrolysis activity to be coincident with the slower moving major esterase band (ARE_3) (Fig. 5). E-600 hydrolytic activity was associated mainly with the faster moving major esterase (ARE_2). Both esterase bands readily hydrolysed *p*-nitrophenyl acetate. When 0·6% sodium deoxycholate was added to plasma 1 hr prior to electrophoresis the faster moving major band was split into four finer zones, while the slower diacetylmorphine hydrolysing esterase still moved as a discrete single band but with lowered mobility.

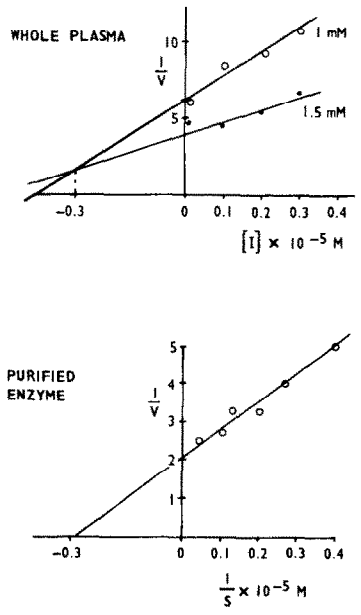

Fig. 4. (Top) Dixon[23] plot of diacetylmorphine hydrolysis (at 1 mM and 1·5 mM concentration) against inhibitor (E-600) concentration for whole plasma. (Bottom) Lineweaver and Burke [22] plot for hydrolysis of E-600 by partially purified enzyme.

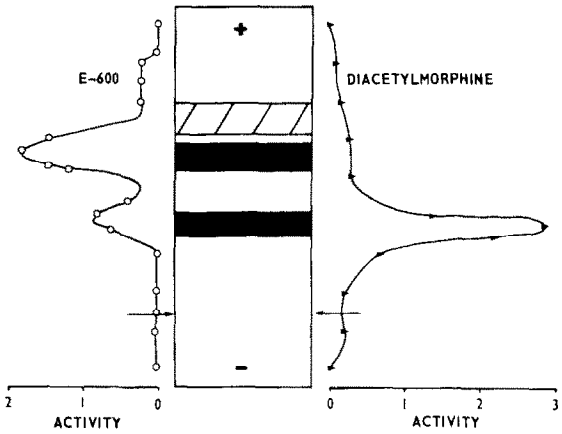

Fig. 5. Cellulose acetate electrophoretic pattern of human plasma esterases. (centre). The substrate characteristics of individual esterases determined by membrane section experiments (arbitary units) are depicted graphically.

Partially purified enzyme preparation. The results of gel filtration of concentrated plasma are illustrated in Fig. 6. Cellulose acetate electrophoresis of the two peaks between 0–35 ml (peak A) and 36–75 ml (peak B) showed the slower moving major esterase confined to peak A, and the faster moving major esterase confined to peak B. As illustrated, diacetylmorphine is hydrolysed only by the esterase present in peak A, *p*-nitrophenyl acetate is hydrolysed by the enzymes present in both peak A and B.

Details of the purification achieved by gel filtration and subsequent ammonium sulphate fractionation are listed in Table 1.

Kinetic studies with partly purified enzyme. The K_m value of the partly purified enzyme $3{\cdot}5 \times 10^{-5}$ for the hydrolysis of E-600 was the same as the K_i value previously calculated for the inhibition of diacetylmorphine hydrolysis by E-600 with the plasma fraction (I) or whole plasma (Fig. 4 bottom). Table 2 lists the K_m and V_{max} values of the partly purified enzyme for E-600, diacetylmorphine, *p*-nitrophenyl acetate and *p*-nitrophenyl laurate; *p*-nitrophenyl phosphate was not metabolised.

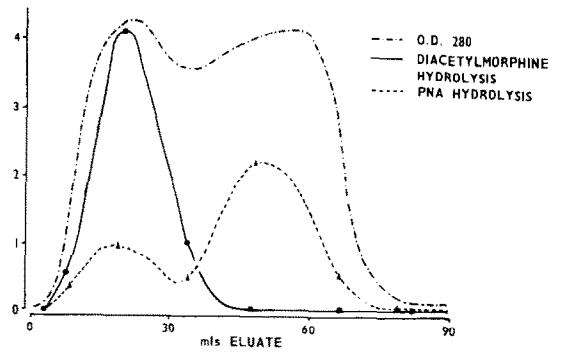

Fig. 6. Gel filtration (G-100 Sephadex) of concentrated human plasma illustrating separation of the two major esterases on a molecular weight basis (PNA = *p*-nitrophenyl acetate) (arbitary units).

Table 1. Partial purification of diacetylmorphine hydrolysing esterase

	Protein	Activity	Sp. act
Plasma (concentrate)	128	26·32	0·205
G-100 fraction A	6·66	7·93	0·95
50% $(NH_4)_2 SO_4$	0·160	1·98	12·4

Protein concentration is expressed in mg/ml activity in μg. diacetylmorphine hydrolysed/min/ml. sp. act. as μg. diacetylmorphine hydrolysed/min/mg protein.

Table 2. Kinetic constants of the partly purified enzyme for various esters.

	K_m	V_{max}
Diacetylmorphine	0·66	38
p-Nitrophenyl laurate	0·71	43
p-Nitrophenyl acetate	20·00	1980
p-Nitrophenyl phosphate	-	0
E-600	0·034	0·75

K_m is expressed as mM; V_{max} as nmoles/min/mg protein.

DISCUSSION

The enzyme in human blood plasma is responsible for 30–35% of diacetylmorphine hydrolysis. The remainder is associated with the red blood cells. Since there was no change in activity when the red blood cells were lysed it was concluded that the esterases are present on their outer surface.

Experiments utilising the plasma fraction (I) showed the enzyme to parallel some of the reported properties of arylesterases: pH optimum [16, 19, 20] temperature lability [19,20], and inhibition by metal ions [16, 19, 21]. The arylesterase enzymes hydrolyse E-600 in contrast to other esterase enzymes [16, 17], thus evidence of competitive inhibition by E-600 of diacetylmorphine hydrolysis is indicative that the same enzyme is involved in their metabolism. Competitive inhibition was demonstrated by the techniques of Lineweaver–Burke [22] (Fig. 3) and Dixon [23] (Fig. 4).

Cellulose acetate separation revealed two major esterases which could also be separated on a molecular weight basis by gel filtration. Both methods showed diacetylmorphine to be hydrolysed exclusively by the slow moving esterase band (ARE_3). When partially purified this esterase had an identical K_m for E-600 hydrolysis, to the K_i calculated for competitive inhibition of diacetylmorphine hydrolysis by E-600 using whole plasma or plasma fraction (I). This is indicative that E-600 and diacetylmorphine are hydrolysed by the same enzyme at the same active site [24], and that, this enzyme is wholly responsible for diacetylmorphine hydrolysis in human plasma.

These results clarify somewhat the properties of arylesterases. Recently there has been doubt as to whether the esterases in mammalian serum hydrolysing phenyl esters and the activity towards organophosphorus esters are the result of the same enzyme [25]. Main [26, 27] has purified an E-600 hydrolysing enzyme from sheep serum which did not hydrolyse phenyl acetate. We have found that the purified esterase described here (ARE_3) has all the characteristics of an arylesterase (including slow hydrolysis of E-600), but is not the principal esterase involved in E-600 metabolism. The fractionation by mild detergent conditions and electrophoresis show the fast moving esterase band (responsible for the major part of E-600 hydrolysis in human plasma) to be composed of more than one enzyme (or isoenzyme); and therefore it is plausible that E-600 may be hydrolysed by enzymes other than arylesterases in human plasma. It is possible that the results of the summation experiments of Aldridge [19], utilising E-600 and other esters, may have been due to E-600 acting as a substrate to some, or as a competitive inhibitor to other enzymes present.

The characterization and identification of the diacetylmorphine hydrolysis enzyme as an arylesterase explains the stability of MAM in human plasma: arylesterases will catalyse the hydrolysis of only the 3-phenolic acetyl group hence the stability of the 6-alcoholic acetyl group.

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