

## STUDIES ON HUMAN SERUM PARAOXONASE/ARYLESTERASE

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### SUMMARY

The complete amino acid sequence of human serum paraoxonase/arylesterase and the DNA sequence coding for that protein have recently been determined in two independent laboratories. There is now considerable evidence that the esterase exists in two genetically determined allozymic forms, and these A and B allozymes possess both paraoxonase and arylesterase activities. The B-type esterase has relatively higher paraoxonase activity and is stimulated to a greater degree by 1 M NaCl than the A allozyme. The structural basis for the distinctive isozymic properties is a single nucleotide base at position 572. Codon 191 is CAA (for glutamine) in the A-type esterase, and CGA (for arginine) in the B-type enzyme. There is a second polymorphic site which affects amino acid 54; this can be either methionine or leucine, but these alternatives have not been found to affect either the level or the quality of the allozymes. Purified A or B-type esterases are stimulated by the addition of phosphatidylcholine. The latter addition increases the maximum velocity rate, but does not alter the  $K_m$  of the reaction with either paraoxon or phenylacetate. In serum, the esterase is tightly bound to the high density lipoproteins, particularly apo A-1, but the importance of this association as far as the stability and catalytic properties of the esterase is not clear, and still under study. No physiological role of the esterase has been established, but its ability to hydrolyze several potent organophosphates may be of some significance in protecting against organophosphate toxicity.

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*Key words:* Paraoxonase purification — Serum arylesterase characteristics — Organophosphate hydrolysis — Polymorphic isozymes

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## INTRODUCTION

Molecular biology studies on paraoxonase/arylesterase over the last two years in our laboratory [1] and by Furlong's group [2] have supported the model we proposed in 1983 [3]: that the human serum esterase exists in two closely related isozymic forms (A and B); that the two allozymes can hydrolyze both organophosphates and aromatic esters such as phenylacetate; and that isozyme B has a much higher turnover number for paraoxon than isozyme A. The affinity of the esterase in serum for binding tightly with other lipoproteins and to aggregate with such components may explain why another laboratory described several molecular forms of the esterase in serum, some of which appeared to have only paraoxonase or arylesterase activity [4]. However, the cloning and sequencing of the cDNA coding for the enzyme in the two laboratories leaves little doubt that two major isozymic forms (A and B) must be responsible in some way for what appear to be contrary observations from other laboratories (see La Du [5]).

The enzyme has been highly purified from human serum in our laboratory and has a molecular weight of about 43.0 kDa [6]. It requires calcium for stability as well as its catalytic activity with either phenylacetate or paraoxon. Our method of purification of the enzyme and the basic characteristics of the isozymes have recently been published [6,7]. Several laboratories have estimated the allelic frequencies of the paraoxonase polymorphism in ethnic groups in different parts of the world [8–11], but most of these estimates are based on measuring paraoxonase levels, alone, rather than by a method which distinguishes individual phenotypes (see La Du [5]). The recent progress in the molecular analyses of the polymorphism should make it simpler to relate phenotypes and genotypes, and estimate allelic frequencies more accurately. The molecular studies have not yet helped us to understand the possible physiological function of the esterase, its significance in endogenous metabolism, or its close association with the HDL complex.

This report will summarize some of the recent molecular and catalytic studies on human serum paraoxonase/arylesterase in our laboratory.

## MATERIALS AND METHODS

Paraoxonase/arylesterase was purified from human serum previously phenotyped so that individual units of the same A, AB, or B phenotype could be pooled. The purification method was developed in our laboratory [6], and we were able to obtain large quantities of enzyme of over 50% purity, with small amounts of albumin and apolipoprotein A-1 as impurities. Estimates of paraoxonase and arylesterase activities and the phenotyping of individual sera were carried out as described previously [3,12]. Ratios of paraoxonase activity in the presence of 1 M NaCl divided by the arylesterase activity were used to identify individual phenotypes, as follows: type A,  $1.21 \pm 0.19$ ; type AB,  $4.68 \pm 0.85$ , and type B,  $8.36 \pm 0.07$  (means  $\pm$  S.D.).

For sequencing of the DNA in the polymorphic regions, DNA was isolated

from blood samples (buffy coat) by the salt-chloroform extraction method of Mullenbach [13]. Both polymorphic sites were near intron-exon junctions, so PCR primers were used which bridged the junctions to amplify the regions corresponding to amino acids 54 and 191. These gave products of about 180 and 230 base pairs (bp), respectively. Sequencing primers synthesized by the University of Michigan DNA Sequencing Facility and Oligos Etc. (Wilson, OR) were then used for direct sequencing of the polymorphic segments after PCR amplification to determine individual genotypes, as we recently reported [14].

Phospholipids tested for their effects on arylesterase and paraoxonase activities with purified serum esterase were obtained from Sigma Chemical Co., St. Louis, MO. These were mixed in a buffer and dispersed by sonication, then premixed with preparations of the esterase at 37 °C for about 30 min before the enzymatic assays.

## RESULTS AND DISCUSSION

### *Amino acid sequence of human serum paraoxonase/arylesterase*

The amino acid sequence of the mature protein and the location of the two polymorphic sites of human serum paraoxonase/arylesterase are shown in Fig. 1. Although the amino acid sequence shown has been deduced from our analyses of genomic DNA and liver cDNA nucleotide sequences, nearly 45% of the total amino acid sequence was confirmed in our laboratory by direct sequencing of tryptic and peptic digest peptides derived from the purified esterase [14]. Evidence for the polymorphism at position 191 was also obtained from high pressure liquid chromatography analysis of unique peptides identified in the tryptic digest profiles from purified A- and B-type esterases [14,15].

One interesting feature of the esterase structure, as first noted by Hassett et al. [2], is the unusual concentration of hydrophobic amino acid residues at the amino terminal end of the protein. It appears to represent the retention of a modified leader sequence. Presumably, a mutation altered the leader sequence and prevented removal of this hydrophobic region. No doubt, its presence helps account for the tendency of this protein to bind to other proteins, and aggregate by itself.

There are 354 amino acids in the mature protein with only 3 cysteine residues. Two of these are near the ends of the protein; the third is residue number 283. In our sequencing of tryptic digest peptides from purified esterase without reduction, a disulfide bridge linking two tryptic peptides was found. One of these was at the amino terminal end, and the other was near the carboxyl terminal position (Fig. 2). These were subsequently sequenced after reduction and separation to verify their locations. These results suggest that the ends of the native protein are linked by a disulfide bond, and this would leave only the cysteine at position 283 as the likely candidate for being a key component in the active center of this esterase.

### *Analysis of the structural polymorphisms*

Variations in the amino acid residues at positions 54 and 191 were noticed by

Ala	Lys	Leu	Ile	5	Leu	Thr	Leu	Leu	10	Met	Gly	Leu	Ala	15	Phe	Arg	Asn	His	20	Gln	Ser	Ser	Tyr	Gln	Thr
GCC	AAG	CTG	ATT	GCG	CTC	ACC	CTC	TTC	GGG	ATG	GGA	CTG	GCA	CTC	TTC	AGG	AAC	CAC	CAG	TCT	TCT	TAC	CAA	ACA	
Arg	Leu	Asn	Ala	30	Leu	Arg	Glu	Val	35	Val	Glu	Leu	Pro	40	Asn	Cys	Asn	Leu	45	Gly	Ile	Glu	Thr	Gly	
CGA	CTT	AAT	GCT	CTC	CGA	GAG	GAA	CAA	CCC	GTA	GAA	CTT	CCT	AAC	TGT	AAT	TTA	GTT	AAA	GGA	ATC	GAA	ACT	GGC	
Ser	Glu	Asp	(Met)	55	Ile	Leu	Pro	Asn	60	Leu	Ala	Phe	Ile	65	Ser	Ser	Gly	Leu	70	Pro	Gly	Ile	Lys	Ser	
TCT	GAA	GAC	(ATG)	GAG	ATA	CTG	CCT	AAT	GGA	CTG	GCT	TTC	ATT	AGC	TCT	GGA	TTA	AAG	TAT	CCT	GGA	ATA	AAG	AGC	
Phe	Asn	Pro	Asn	80	Pro	Gly	Lys	Ile	85	Leu	Met	Asp	Leu	90	Asn	Glu	Glu	Asp	95	Thr	Val	Leu	Glu	Leu	
TTC	AAC	CCC	AAC	AGT	CCT	GGA	AAA	ATA	CTT	CTG	ATG	GAC	CTG	AAT	GAA	GAA	GAT	CCA	ACA	ACA	GTG	TTG	GAA	TTG	
Ile	Thr	Gly	Ser	105	Phe	Asp	Val	Ser	110	Phe	Asn	Pro	His	115	Gly	Ile	Ser	Thr	120	Phe	Thr	Asp	Glu	Asp	
ATC	ACT	GSA	AGT	AAA	TTT	GAT	GTA	TCT	TCA	TTT	AAC	CCT	CAT	GGG	ATT	AGC	ACA	TTC	ACA	GAT	GAA	GAT	AAT	CCC	
Met	Tyr	Leu	Leu	130	Val	Val	Asn	His	135	Pro	Asp	Ala	Lys	140	Ser	Thr	Val	Glu	145	Leu	Phe	Lys	Phe	Gln	
ATG	TAC	CTC	CTG	GTG	GTG	AAC	CAT	CCA	GAT	CCC	AAG	TCC	ACA	GTG	GAG	TTG	TTT	AAA	TTT	CAA	GAA	GAA	GAA	LAA	
Ser	Leu	Leu	His	155	Leu	Lys	Thr	Ile	160	Arg	His	Lys	Leu	165	Pro	Asn	Leu	Asn	170	Asp	Ile	Val	Ala	Val	
TCC	CTT	TTG	CAT	CTA	AAA	ACC	ATC	AGA	CAT	AAA	CTT	CTG	CCT	AAT	TTG	AAT	GAT	ATT	GTT	GCT	GTG	GGA	CCG	GAG	
His	Phe	Tyr	Gly	180	Asn	Asp	His	Tyr	185	Phe	Leu	Asp	Pro	190	Tyr	Leu	(Gln)	Ser	195	Met	Tyr	Leu	Gly	Leu	
CAC	TTT	TAT	GGC	ACA	AAT	GAT	CAC	TAT	TTT	CTT	GAC	CCC	TAC	TTA	(GAA)	TCC	TGG	GAG	TTT	ATG	TAT	TTG	GGT	TTA	
Trp	Ser	Tyr	Val	205	Val	Tyr	Tyr	Ser	210	Pro	Ser	Glu	Val	215	Arg	Val	Ala	Glu	220	Phe	Asp	Phe	Ala	Asn	
TGG	TCG	TAT	GTT	GTC	TAC	TAT	AGT	CCA	AGT	ACT	GAA	GTT	CGA	GTG	Val	Val	GCA	GAA	GGA	GGT	TTT	TTT	GCT	GGT	
Asn	Ile	Ser	Pro	230	Asp	Gly	Lys	Tyr	235	Val	Tyr	Ile	Ala	240	Glu	Leu	Ala	His	245	Lys	Ile	His	Val	Tyr	
AAC	ATT	TCA	CCC	GAT	GCC	AAG	TAT	GTC	TAT	ATA	GCT	GAG	TTG	CTG	GCT	CAT	AAG	ATT	CAT	GTC	TAT	GAA	AAG	CAT	
Ala	Asn	Trp	Thr	255	Leu	Thr	Pro	Leu	260	Lys	Ser	Leu	Asp	265	Phe	Asn	Thr	Leu	270	Val	Asp	Asn	1le	Ser	
GCT	AAT	TGG	ACT	TFA	ACT	CCA	TTG	AAG	TCC	CTT	GAC	TTT	AAT	ACC	CTC	GTG	GAT	AAC	ATA	TCT	TCT	GTG	GAT	CCG	
Thr	Gly	Asp	Leu	280	Trp	Val	Gly	Cys	285	His	Pro	Asn	Gly	290	Met	Lys	Ile	Phe	295	Phe	Tyr	Asp	Ser	Glu	
ACA	GGA	GAC	CTT	TGG	GTT	GGA	TGC	CAT	CCC	AAT	GGC	ATG	AAA	ATC	TTC	TTC	TAT	TAT	TCA	GAG	AAT	CCG	CCG	GCA	
Ser	Glu	Val	Leu	305	Arg	Ile	Gln	Asn	310	Ile	Leu	Thr	Glu	315	Pro	Lys	Val	Thr	320	Gln	Val	Val	Ala	Glu	
TCA	GAG	GTG	CTT	CGA	ATC	CAG	AAC	ATT	CTA	ACA	GAA	GAA	CCG	AAA	GTG	ACA	CAG	GTT	TAT	GCA	GAA	AAT	GGC	ACA	
Val	Leu	Gln	Gly	330	Ser	Thr	Val	Ala	335	Ser	Val	Tyr	Lys	340	Gly	Lys	Leu	Leu	345	Ile	Gly	Thr	Val	Phe	
GTG	TTG	CAA	GCC	ACT	ACA	GTT	GCC	TCT	GTC	TAC	AAA	GGG	AAA	CTG	CTG	ATT	GGC	ACA	GTC	TTT	CAC	AAA	GCT	CTT	
Tyr	Cys	Glu	Leu																						
TAC	TGT	GAG	CTC																						

Fig. 1. Amino acid sequence and codons for human serum paraoxonase/arylesterase. The locations of the two polymorphic sites at residues 54 and 191 are indicated. (modified from Ref. 14).

## N-Terminal Peptide

GLU VAL GLN PRO VAL GLU LEU PRO ASN CYS ASN LEU VAL LYS 45

|  
S  
|  
S  
|

ALA LEU TYR CYS GLU LEU 354

## C-Terminal Peptide

Fig. 2. Amino acid sequences of disulfide-linked peptides obtained by tryptic digestion of purified human serum paraoxonase/arylesterase.

Furlong's laboratory [2] and our laboratory [14,15]. At amino acid residue 54, it was methionine or leucine, and at position 191 it was glutamine or arginine. The two residues at one of these positions, alone, might explain the paraoxonase/arylesterase polymorphism, or certain combinations within the four isomers could be required. It was thus necessary to compare the genotypes obtained by direct sequencing of the PCR amplified genomic DNA at the two polymorphic sites with phenotyping results from serum of the same individuals.

Blood samples from twenty-seven unrelated volunteers were phenotyped by their ratios of paraoxonase activity in the presence of 1 M NaCl divided by their arylesterase activity (Fig. 3), and their DNA samples were used to sequence the two polymorphic regions after PCR amplification. The results of these analyses

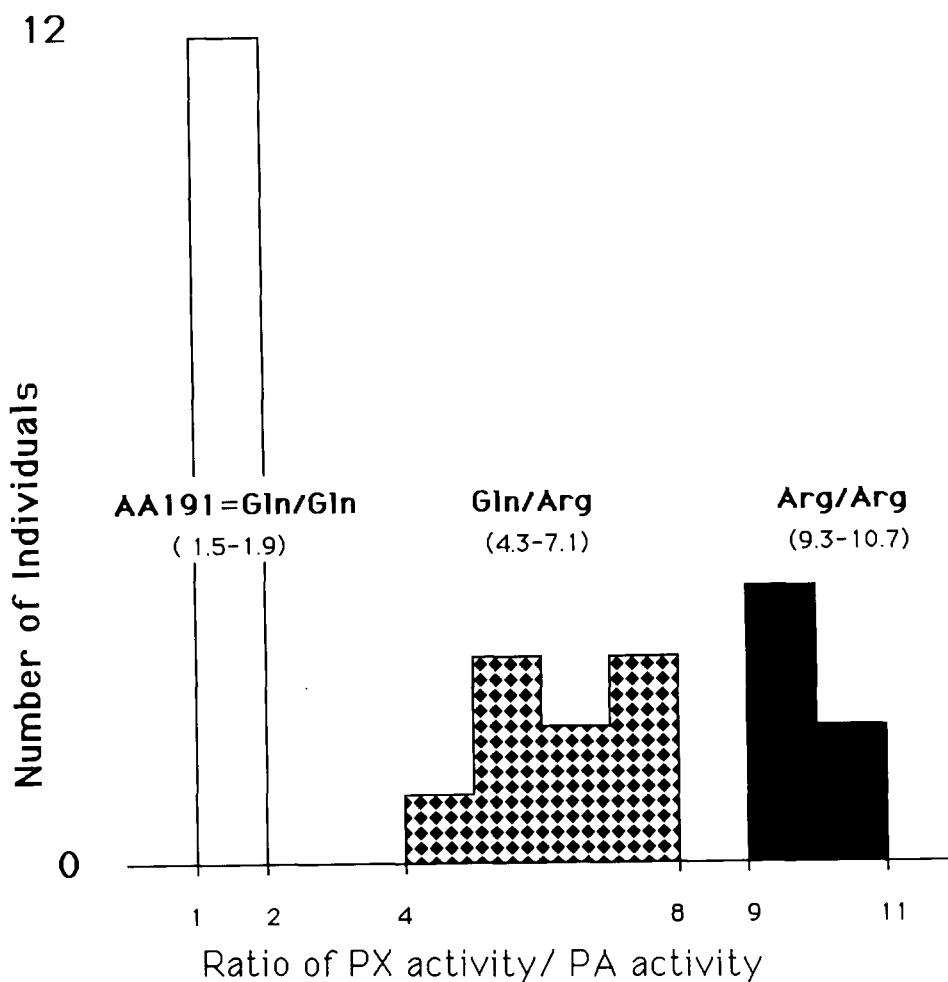


Fig. 3. Distribution of ratios of paraoxonase activity in the presence of 1 M NaCl divided by the arylesterase activity of 27 unrelated individuals. The three modes correspond to the A, AB and B phenotypes.

are shown in Table I. Comparison of the serum esterase phenotypes and the DNA analyses showed that the amino acid at position 191 coincided exactly with the serum enzyme phenotype in every individual. All those of phenotype A had glutamine, and those of phenotype B all had arginine at position 191; heterozygous individuals (AB) had both of these amino acids represented. On the other hand, the methionine/leucine polymorphism determined by the codon for position 54 did not affect the serum phenotype in any obvious way. It is of interest that all B-type individuals were homozygous for leucine at the latter site, but this may simply be the result of linkage disequilibrium since these genetic markers are very close. The perfect agreement between the amino acids at position 191 and the serum esterase phenotypes in the individuals indicates that gene frequencies estimated from either of these traits would be the same. In contrast,

TABLE I

CORRELATION OF SERUM PARAOXONASE PHENOTYPES AND DNA GENOTYPES IN 27 UNRELATED INDIVIDUALS

Serum phenotype	Individual	$\mu$ Moles PA <sup>a</sup>	nMoles PX <sup>b</sup>	PX/PA ratio	Amino acid 191	Amino acid 54
		Min $\times$ ml	Min $\times$ ml			
A	D.Li	95	155	1.6	GLN, GLN	MET, MET
A	L.S.	100	169	1.7	GLN, GLN	MET, MET
A	R.S.	100	191	1.9	GLN, GLN	MET, MET
A	S.V.	82	152	1.9	GLN, GLN	MET, MET
A	S.P.	98	169	1.7	GLN, GLN	MET, LEU
A	J.K.	62	105	1.7	GLN, GLN	MET, LEU
A	A.H.	122	212	1.7	GLN, GLN	MET, LEU
A	W.W.	89	157	1.8	GLN, GLN	MET, LEU
A	S.L.	134	223	1.7	GLN, GLN	LEU, LEU
A	D.La	86	162	1.9	GLN, GLN	LEU, LEU
A	P.C.	129	201	1.6	GLN, GLN	LEU, LEU
A	E.L.	118	176	1.5	GLN, GLN	LEU, LEU
AB	S.A.	58	321	5.6	GLN, ARG	MET, MET
AB	K.D.	100	627	6.3	GLN, ARG	MET, MET
AB	B.D.	81	496	6.1	GLN, ARG	MET, LEU
AB	J.De	141	784	5.6	GLN, ARG	MET, LEU
AB	T.I.	57	398	7.0	GLN, ARG	MET, LEU
AB	H.C.	51	355	7.0	GLN, ARG	MET, LEU
AB	K.C.	114	496	4.3	GLN, ARG	LEU, LEU
AB	L.Z.	129	758	5.9	GLN, ARG	LEU, LEU
AB	M.G.	110	780	7.1	GLN, ARG	LEU, LEU
B	M.S.	91	846	9.3	ARG, ARG	LEU, LEU
B	B.L.	86	890	10.3	ARG, ARG	LEU, LEU
B	A.T.	106	1006	9.5	ARG, ARG	LEU, LEU
B	J.Da	76	737	9.7	ARG, ARG	LEU, LEU
B	A.C.	100	1063	10.7	ARG, ARG	LEU, LEU
B	J.V.	106	1020	9.7	ARG, ARG	LEU, LEU

<sup>a</sup> Phenylacetate.

<sup>b</sup> Paraoxon in the presence of 1 M NaCl.

the frequencies of the methionine/leucine polymorphic choices at residue 54 did not show a similar agreement. It is, none the less, a very common polymorphism, and its importance must still be determined.

A schematic representation of the enzyme structure showing the free sulfhydryl group (cysteine), the single internal disulfide bridge, the two polymorphic sites, and the location of four possible carbohydrate chains based on the requisite amino acid sequence is shown in Fig. 4.

*Stimulation of esterase activity by phospholipids*

It has been observed that with higher purification of the serum paraox-

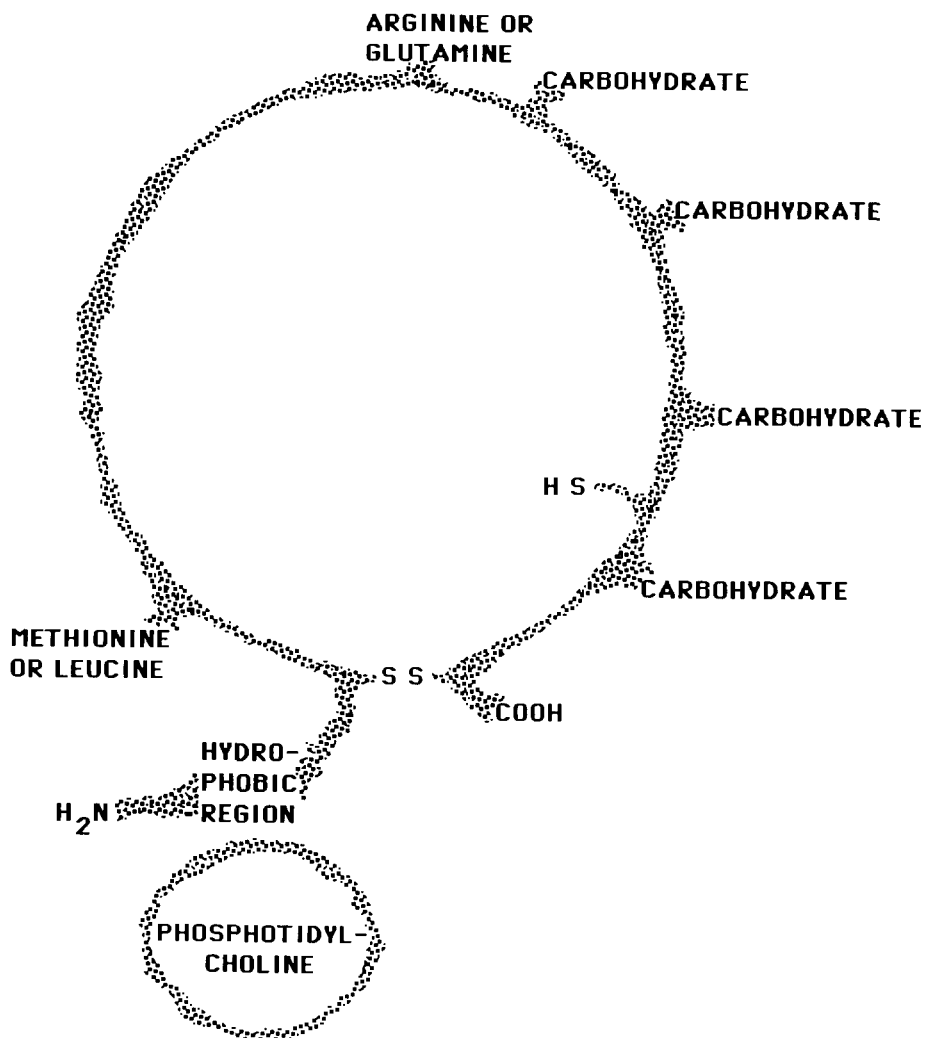


Fig. 4. Schematic representation of the paraoxonase/arylesterase enzyme showing the polymorphic sites, hydrophobic N-terminal segment, the disulfide bridge and single free sulfhydryl residue.

onase/arylesterase some loss of activity regularly takes place, but this loss can be largely regained by adding back certain fractions obtained during the DEAE column chromatography step. We analyzed these fractions and found that they were enriched in phospholipids. When we substituted purified dilauroyl phosphatidylcholine, a maximal degree of stimulation was obtained (Table II). Commercial lecithin preparations and most other phospholipids were somewhat less effective; phosphatidyl-serine was inhibitory. A comparison of the effectiveness of the compounds shows that there is some degree of specificity for this effect, and saturated medium length fatty acids were more effective than the other analogs. We now suspect that some of our earlier observations on protective or stimulatory effects obtained by combining apolipoprotein A-1 with the purified esterase were probably due to the phospholipid content of the mixture. We have yet to see a clear beneficial effect of apolipoprotein A-1 with the purified esterase when there is also an optimal concentration of choline-containing phospholipids. Among the compounds not effective in place of the phosphatidylcholine were tripalmitin, sphingomyelin, and phosphatidylinositol. Simple lipids, then, are not all stimulatory like selective phospholipids. Dilauroyl phosphatidylcholine does not change the  $K_m$  appreciably, but it increases the  $V_{max}$  of the reaction. Thus, the phosphatidylcholine is not simply facilitating the interaction of substrate with the enzyme, but is making the enzyme a more efficient catalyst, and presumably is increasing the turnover number of the esterase with either paraoxon or phenylacetate (Fig. 5).

The very close association of serum paraoxonase/arylesterase with apolipoprotein A-1 and with phosphatidylcholine may take place through the hydrophobic region at the amino terminus of the enzyme. However, a more exact understanding of the interactions of the esterase with these components will be possible after we obtain the three-dimensional characteristics of the molecule by crystallographic analysis.

TABLE II

## STIMULATION OF PHENYLACETATE HYDROLYSIS BY PHOSPHATIDYLCHOLINE AND ANALOGUES

Compound	% Inhibition	% Stimulation
Dilauroyl-PC		65.0
Dioleoyl-PC		56.3
Lecithin		41.1
Lysolecithin		31.6
Phosphatidylethanolamine		18.6
Phosphatidylglycerol		9.6
Phosphatidylserine	33.3	
6-palmityl-ascorbic acid	37.4	

No effect: Tripalmitin, cholesterylacetate, sphingomyelin, cardiolipid, phosphatidylinositol, vitamin K3, glycerophosphorylcholine. (Control activity: 64.4 U/ml.)



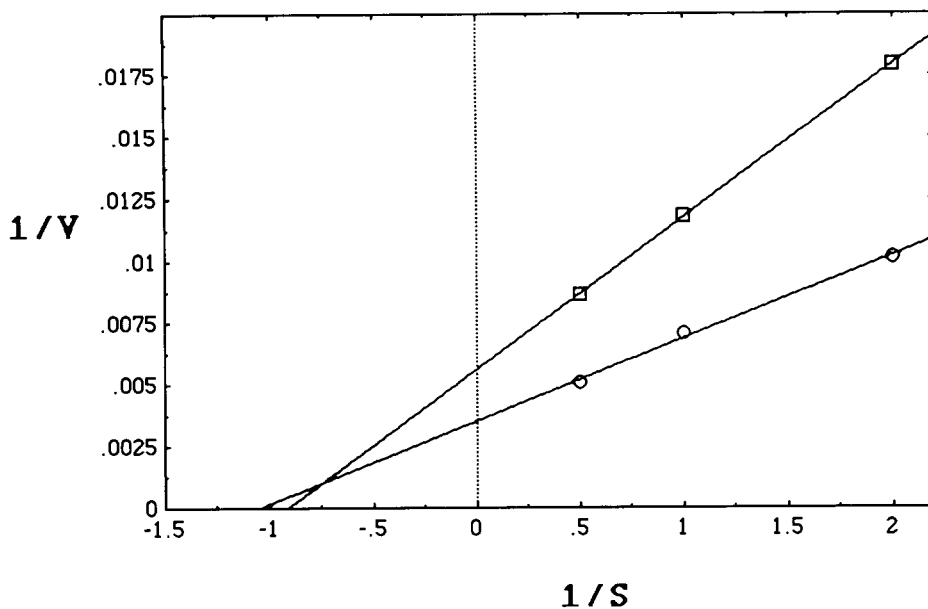


Fig. 5. Effect of dilauroyl phosphatidylcholine on arylesterase activity of purified human serum paraoxonase/arylesterase. Squares indicate enzyme alone and circles indicate enzyme with dilauroyl phosphatidylcholine.

#### ACKNOWLEDGEMENTS

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