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Beef Liver Esterase as a Catalyst of Acyl Transfer to Amino Acid Esters*

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ABSTRACT: Purified beef liver esterase has been shown to be an efficient catalyst in the transfer of acyl groups from the methyl esters of suitable amino acids (*e.g.*, L-phenylalanine) and fatty acids (*e.g.*, β-phenylpropionic acid and hexanoic acid) to the α-amino group of L-phenylalanine methyl ester and of other amino acid esters at pH 7.2–8.5. Although D-phenylalanine methyl ester is readily hydrolyzed by the enzyme, it is relatively ineffective as an acceptor of acyl groups from the methyl esters of L-phenylalanine and β-phenylpropionic acid. With L-phenylalanine methyl ester as the acceptor amine, the acylamino acid ester initially formed (Phe-Phe-OMe, β-phenylpropionyl-Phe-OMe, or hexanoyl-Phe-OMe) is hydrolyzed to the acylamino acid (Phe-Phe, β-phenylpropionyl-Phe, or hexanoyl-Phe), in accordance with the side-chain specificity of the enzyme with respect to the acyl donor in hydrolytic or transfer reactions. On the other hand, with L-isoleucine methyl ester or glycine methyl ester as the acceptor amine, the acylamino acid ester that is formed is not converted to a detectable extent to the corresponding acylamino acid; this finding is consistent with the relative resistance of these two amino acid esters to the hydrolytic action of beef liver esterase. For the quantitative estimation of the reaction components that contain an acyl group derived from a fatty acid ester, analytical gas–liquid partition chromatography was employed. A detailed study has been made of the reaction in which L-phenylalanine methyl ester serves both as the acyl donor and the acceptor amine, with respect to the influence of initial substrate concentration, enzyme concentration, pH, and the presence of an organic solvent (dioxane). By quantitative estimation of the components of the reaction mixture, using automatic spectrophotometric monitoring of effluents from a Sephadex G-10 column, it has been possible to define the effect of these variables on the partition of the phenylalanyl units of L-phenylalanine methyl ester between hydrolysis to free amino acid and the synthesis of Phe-Phe-OMe and Phe-Phe. In connection with these studies, the kinetic parameters were determined for the action of beef liver esterase at pH 7.2–8.5 on an extensive series of fatty acid methyl esters. These data served as a basis for the selection of methyl β-phenylpropionate and of methyl hexanoate as potential acyl donors to amino acid esters. Comparison of the kinetic data with those reported by other investigators for purified liver esterase preparations provides additional evidence for the view that the ability to transfer suitable acyl units to the α-amino group of L-amino acid esters is an intrinsic property of beef liver esterase.

In a previous communication from this laboratory, it was reported that an enzyme preparation (from beef liver) effective in the hydrolysis of esters such as methyl butyrate and L-leucine methyl ester also is an efficient catalyst in the synthesis of peptides from suitable amino acid esters (Krenitsky and Fruton, 1966). Indeed, in its action on 50 mM L-phenylalanine methyl ester at pH 7.5, the enzyme preferentially catalyzes the formation

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of Phe-Phe[1] (with Phe-Phe-OMe as an intermediate) rather than the hydrolysis of Phe-OMe to the free amino acid. For this reason, the enzymic activity responsible for the hydrolytic and transfer reactions undergone by amino acid esters was termed "aminoacyl transferase," although attention was drawn to the strong possibility that the transferase activity leading to peptide synthesis is an intrinsic property of the long-known carboxyl esterases. This possibility was supported by the parallel inhibition of hydrolytic and transfer reactions by treatment of the enzyme with DFP, and by the parallel concentration of activity toward methyl butyrate and L-leucine methyl ester during the course of extensive purification. In subsequent work, Benöhr and Krisch (1967) purified beef liver esterase by a different method, and have shown that their apparently homogeneous preparation catalyzes the synthesis of Phe-Phe from Phe-OMe. This confirmation of the findings of Krenitsky and Fruton (1966) makes it more probable that aminoacyl transferase activity is indeed an intrinsic property of beef liver esterase. In what follows, therefore, the enzyme under study will be termed beef liver esterase.

In the present communication data are presented on the time course of the transformation of L-phenylalanine methyl ester by our beef liver esterase preparation, and on the effect of variation in pH, enzyme concentration, initial substrate concentration, and the presence of organic solvents on the relative extent of hydrolysis and peptide synthesis. The quantitative determination of the components (Phe-OMe, Phe, Phe-Phe-OMe, and Phe-Phe) of the reaction mixtures was performed by automatic recording of the absorbance of effluents from Sephadex G-10 columns.

The previous data (Krenitsky and Fruton, 1966) on the specificity of beef liver esterase as a hydrolase indicated that the principal determinant is the nature of the carbon skeleton attached to the α-carbon of the acyl group of the substrate. The nature of the other group at the α-carbon (in addition to the α-H and COOR groups) appears to be less decisive, and may be H (as in fatty acid esters), NH_2 (as in amino acid esters), or RCONH (as in acylamino acid or peptide esters). In keeping with this conclusion was the finding that the enzyme does not exhibit absolute stereospecificity as a hydrolase, since esters of suitable D-amino acids (*e.g.*, D-phenylalanine methyl ester) are hydrolyzed rapidly.

Krenitsky and Fruton (1966) found, however, that the stereochemical discrimination lacking in the hydrolytic process was clearly evident in the transfer reaction leading to peptide synthesis. With D-phenylalanine methyl ester as the substrate, the predominant reaction is hydrolysis to the free amino acid, and relatively little Phe-Phe is formed. This observation suggested that when an amino acid ester acts as an acceptor of an acyl group derived from a suitable substrate, the specificity requirements are more demanding than when an amino acid ester serves as an acyl donor. To characterize more precisely the specificity of beef liver esterase as a transferase with respect to the nature of the acceptor amine, experiments were undertaken in which a suitable fatty acid ester was used as an acyl donor and various amino acid esters were used as acceptors. The choice of the fatty acid esters for such experiments was based on studies of the kinetics of the hydrolysis of a series of fatty acid methyl esters, and the most suitable of these in terms of reactivity were selected as potential acyl donors. In the present communication, data are presented on the products formed when the methyl esters of β-phenylpropionic acid and hexanoic acid are the sources of acyl groups in esterase-catalyzed transfer reactions. For the determination of the reaction components containing such acyl groups, analytical gas–liquid partition chromatography was employed, thus providing quantitative data on the partition of the acyl groups between transfer to water and transfer to the acceptor amine. In the development of suitable conditions for the analysis of acylamino acid esters by gas–liquid partition chromatography, the studies of Gehrke and Shahrokhi (1966) and of Stalling *et al.* (1967) on the quantitative determination of the *n*-butyl esters of trifluoroacetylamino acids served as valuable guides.

Experimental Section

Materials. The methyl esters of the following acids were used: butyric acid, bp 102°; 3-methylbutyric acid, bp 115–116°; pentanoic acid, bp 126–128°; 3-methylpentanoic acid, bp 140–142°; 4-methylpentanoic acid, bp 140–142°; hexanoic acid, bp 149–150°; heptanoic acid, bp 170–172°; β-phenylpropionic acid, bp 87–89° (2.5 mm); and β-phenyl-L-lactic acid, mp 48–49°. Methyl butyrate was an Eastman product, and methyl β-phenyl-L-lactate was obtained from Pierce Chemical Corp. The other esters were prepared from commercial samples of the corresponding acids by esterification in the presence of H_2SO_4, with CH_2Cl_2 as the solvent (Clinton and Laskowski, 1948). The final products were checked for identity and purity by determination of the refractive index (Vogel, 1948) and by gas–liquid partition chromatography. The properties of the amino acid methyl ester hydrochlorides used in this work have been described previously (Krenitsky and Fruton, 1966). L-Phenylalaninol was prepared in the manner described by Inouye and Fruton (1967).

β-Phenylpropionyl-L-phenylalanine was prepared by the addition of β-phenylpropionyl chloride (5.1 g) to a solution of L-phenylalanine (5.0 g) in 50 ml of 1.5 N NaOH. After being shaken for 1 hr, the solution was acidified with HCl, and the precipitate was collected. After two recrystallizations from ethanol–petroleum ether (bp 30–60°), the product melted at 161–162°, yield 5.4 g (60%). *Anal.* Calcd for $C_{18}H_{19}NO_3$ (297.3): N, 4.72. Found: N, 4.80.

β-Phenylpropionyl-L-phenylalanine methyl ester was prepared by the reaction of β-phenylpropionyl chloride (1.97 g) with L-phenylalanine methyl ester hydrochloride (2.5 g) in the presence of triethylamine (3.2 ml), with ether as the organic solvent. The resulting product was recrystallized twice from ethanol–petroleum ether: yield 2.9 g (53%), mp 73–74°. *Anal.* Calcd for $C_{19}H_{21}NO_3$

[1] The abbreviated designation of amino acid derivatives used in this paper is that listed in *Biochemistry* 5, 2485 (1966).

(311.4): N, 4.50. Found: N, 4.55. The enantiomer of this compound was prepared in 68% yield (mp 73–74°) by the above method, with D-phenylalanine methyl ester hydrochloride as the starting material. This method was also used to prepare β-phenylpropionylglycine methyl ester (mp 72–74°) in 50% yield of the pure compound.

Efforts to obtain a crystalline sample of β-phenylpropionyl-L-isoleucine methyl ester were unsuccessful. Esterification of the corresponding acid (mp 119–121°) (prepared as described above for the L-phenylalanine derivative) with BF_3–methanol (Analabs Inc.) yielded an oil with the expected molar absorbance at 257.5 mμ. *Anal.* Calcd for $C_{16}H_{23}NO_3$ (277.4): N, 5.05. Found: N, 4.85.

Hexanoyl-L-phenylalanine was prepared in a manner analogous to that used to make the β-phenylpropionyl derivative. After recrystallization from benzene–petroleum ether, it melted at 133°. *Anal.* Calcd for $C_{15}H_{21}NO_3$ (263.3): N, 5.30. Found: N, 5.12. Esterification of this product with BF_3–methanol in the usual manner gave hexanoyl-L-phenylalanine methyl ester, mp 40°. *Anal.* Calcd for $C_{16}H_{23}NO_3$ (277.4): N, 5.05. Found: N, 5.15.

Dioxane was purified by the procedure of Inagami and Sturtevant (1960), except for the omission of the initial treatment with NaOH. The other organic solvents were purified and distilled before use. Analytical grade reagents were used throughout.

Enzyme Preparation and Assay. The enzyme preparation used in this work was made in the manner described previously (Krenitsky and Fruton, 1966). It was found that storage of lyophilized samples of the "0.44–0.61 AS Fraction," the final product of this procedure, for periods up to 1 year at 0° permitted retention of over 90% of the original activity. Dilute solutions of the enzyme are not stable upon storage, and were prepared from lyophilized material immediately before use. One unit of enzymic activity is defined as the amount of enzyme (in 1 ml of incubation mixture) that produces 1 μequiv of H^+/min, with 50 mM L-leucine methyl ester as the substrate at pH 7.0 and 37°. The specific activity of the enzyme preparation used in this work was 30 units/mg of protein, as determined by the biuret method (Gornall *et al.*, 1949).

A sample of purified pig liver esterase was generously provided by Dr. W. P. Jencks, Brandeis University. It was stored at a concentration of 6.25 mg/ml in 0.02 M potassium phosphate buffer (pH 7.4) at −28° and a portion was diluted for test of its aminoacyl transferase activity. At an enzyme concentration of 4.0 μg/ml of incubation mixture, the pig liver enzyme hydrolyzed methyl β-phenyl-L-lactate (22 mM) at pH 7.2 and 37° with a specific rate of 165 μmoles/min per mg of protein per ml of incubation mixture. Under the same conditions, the corresponding value for the beef liver enzyme was 200 μmoles/min per mg of protein per ml.

Kinetics of Enzymic Hydrolysis of Esters. The rate of enzymic hydrolysis of fatty acid esters was determined by automatic titration of the liberated H^+ with standard NaOH (0.002–0.01 N) at constant pH and 37.0 ± 0.1°, using a Radiometer TTT1 meter in conjunction with a TTA31 titration assembly and a SBR2 titrigraph. The substrate solution (5.0 ml) contained 0.2 M NaCl; it was kept at 37° for 1 hr, and its pH was adjusted to the desired value with the pH-Stat. The low solubility of the fatty acid esters in water made it necessary to check the substrate concentration; this was done by spectrophotometry at 257.5 mμ (for the aromatic compounds), by the hydroxamate reaction (Wiggans *et al.*, 1954), or by allowing the enzymic reaction to go to completion. Separate experiments showed that the three methods gave concordant results.

In the kinetic studies, after a base line had been established for the slow nonenzymic release of H^+ in the substrate solution, the enzyme solution (0.1 ml) was injected, and the initial linear rate of enzymic action (up to 10–20% hydrolysis) was determined. These data were used for v *vs.* v/s plots, and in all cases satisfactory Michaelis–Menten kinetics were observed over the range of substrate concentration in these studies. From these plots, values of K_M and of V_m were estimated. The above terms are defined by the expression $v = V_m es/(K_M + s)$, where v is the initial velocity (mM/min per enzyme unit), e is the enzyme concentration (units/ml), and s is the initial substrate concentration (mM). The kinetic data were subjected to computer analysis, using the FORTRAN IV program of Hanson *et al.* (1967), and the 95% confidence limits for K_M and V_m were calculated.

Gas–Liquid Partition Chromatography of Methyl Esters of Fatty Acids and of Acylamino Acids. A Hewlett-Packard Model 5750 instrument, equipped with dual-flame detector, Moseley recorder, and Disc Instruments integrator was used for all analyses. The dual stainless-steel columns (6 ft × 0.155 in. i.d.) were packed with 80–100 mesh acid-washed, silanized Diatoport S coated with 10% silicone gum rubber UC-W98 (F & M Division of Hewlett-Packard). The general operating conditions involved maintenance of the injector port and detector at 275–280°, and a flow of 480 cc/min of compressed air to the detector. The sensitivity of the instrument was set at $^1/_{256}$ or $^1/_{128}$, and the chart speed was 0.5 in./min. The samples (3–4 μl of an acetone solution) were injected by means of a Hamilton 10-μl fixed-needle syringe. In Table I are given the specific conditions used for several of the compounds of interest in this work and their retention times relative to that of ethyl stearate. A variety of operating conditions was employed to permit rapid analysis of multiple samples, in preference to a single program for the analysis of both the fatty acid ester and the acylamino acid ester present in a sample from an enzymic incubation mixture. The peaks obtained fell in a range corresponding to 400–700 counts on the Disc integrator (precision, *ca.* ±12 counts). Linear standard curves were prepared for methyl β-phenylpropionate, methyl hexanoate, and for each of the available synthetic acylamino acid esters (see Materials), relating amount injected (0.5–10 mμmoles) to the counts corresponding to the peak produced. A plot of counts per millimicromole against the number of carbon atoms per molecule for the β-phenylpropionyl derivatives of Gly-OMe, Ile-OMe, and Phe-OMe and for hexanoyl-Phe-OMe gave a linear relationship (an increment of 20 counts/mμmole per additional carbon atom), thus permitting an estimate of the appropriate conversion factor for the β-phenylpropionyl derivatives of Ala-OMe

TABLE I: Relative Retention Times of Methyl Esters of Fatty Acids and Acylamino Acids.[a]

Compound	Isothermal Temp (°C)	H_2 Flow (cc/min)	He Flow (cc/min)	Rel Retention Time
Methyl hexanoate	80	84	110	0.0041[b]
Methyl β-phenylpropionate	130	48	55	0.021[b]
Methyl β-phenyl-L-lactate	166	48	55	0.028[b]
β-Phenylpropionylglycine methyl ester	200	84	110	0.25[c]
β-Phenylpropionyl-L-isoleucine methyl ester	200	84	110	0.49[c]
β-Phenylpropionyl-L-phenylalanine methyl ester	230	84	110	1.86[d]
Hexanoyl-L-phenylalanine methyl ester	200	84	110	0.53[c]

[a] For details, see Experimental Section. [b] These values are based on runs at 166°; retention time for ethyl stearate, 170 min. [c] Retention time for ethyl stearate, 31 min. [d] Retention time for ethyl stearate, 9.5 min.

and Leu-OMe and for the hexanoyl derivatives of Gly-OMe and Ile-OMe. The analytical data obtained by gas–liquid partition chromatography are expressed in terms of microequivalents of acyl units per milliliter of enzymic incubation mixture.

Enzymic Synthesis of Phe-Phe-OMe and Phe-Phe from Phenylalanine Methyl Ester. Solutions of 5 ml, containing L- or D- phenylalanine methyl ester (2.5–50 mM), were incubated for 15 min at 37° and their pH was adjusted to the desired value in the pH-Stat. The enzyme solution (0.1 ml) was then injected, and the reaction was allowed to proceed, the pH being maintained by automatic titration with 0.02 N NaOH. At a specified time, the reaction was stopped by the addition of 0.1 ml of 10 N HCl, and the components of the reaction mixture (Phe-OMe, Phe, Phe-Phe-OMe, and Phe-Phe) were determined by passage through a Sephadex G-10 column, and monitoring of the effluent solution with a Gilford multiple sample absorbance spectrophotometer (Model 2000) at 257.5 mμ, in the manner described by Krenitsky and Fruton (1966). To facilitate comparison of the data, they are expressed in terms of microequivalents of Phe per milliliter of incubation mixture. Replicate analyses of solutions of known concentration indicated that the precision of this method is approximately ±3%. A representative elution diagram is shown in Figure 1.

Transferase Action of Enzyme Preparation with Fatty Acid Esters as Acyl Donors and Amino Acid Esters as Acceptors. The substrate solution (5 ml), containing both the fatty acid ester and the amino acid ester in the presence of dioxane (0.25%, v/v), was equilibrated at 37° and its pH was adjusted in the pH-Stat. The enzyme solution (0.1 ml) was injected, and the desired pH was maintained by automatic titration. At a specified time, the reaction was stopped by the addition of 0.1 ml of 10 N HCl, and the reaction mixture was diluted to 25 ml by the addition of 0.2 N HCl.

This solution was extracted four times with 10-ml portions of ether. In the experiments in which phenylalanine methyl ester was used, the aqueous layer was concentrated *in vacuo* to 5 ml, and a sample was analyzed for Phe-OMe, Phe, Phe-Phe-OMe, and Phe-Phe by passage through a Sephadex G-10 column as described above.

The combined ether extract was extracted four times with 10-ml portions of 2% $NaHCO_3$, and the ether layer was washed twice with 10-ml portions of water. After evaporation of the ether *in vacuo*, the residue was dissolved in acetone (volume of solution, 2 ml). Appropriate dilution of this solution with acetone gave concentrations suitable for analysis by gas–liquid partition chromatography in the manner described above. The data so obtained gave values for the amount of unreacted fatty acid ester and of the acylamino acid ester present in the incubation mixture.

The combined bicarbonate extract was acidified with 4 ml of 10 N HCl, and the resulting mixture was extracted four times with 10-ml portions of ether. The combined ether extract was dried with exsiccated Na_2SO_4 and filtered, and the volume of filtrate was brought to 50 ml with ether. This solution was divided into 25-ml portions for duplicate esterification. To each portion, 14% BF_3-methanol (2 ml) was added, and the solution was heated

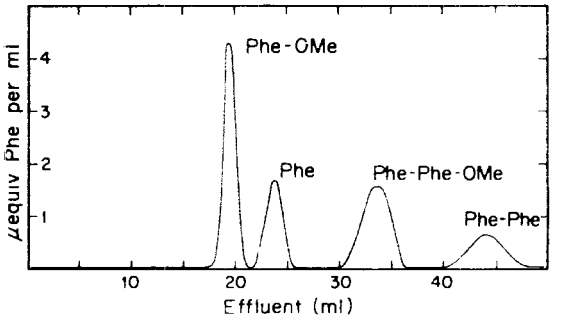

FIGURE 1: Representative elution diagram for the separation of the reaction components in the action of beef liver esterase on L-phenylalanine methyl ester. Initial substrate concentration, 50 mM; enzyme concentration, 0.24 unit/ml; volume of incubation mixture, 5.1 ml; pH 7.2; 37°; 15 min. After acidification, a sample (corresponding to 0.35 ml of the incubation mixture) was applied to a Sephadex G-10 column (122 × 0.5 cm), and the elution was performed with 0.2 N HCl at a flow rate of 2.5 ml/hr and at room temperature. The effluent was monitored at 257.5 mμ with the Gilford Model 2000 absorbance recorder; for the calculation of the concentration of phenylalanyl units (in microequivalents per milliliter), the molar absorbance was assumed to be 195/phenylalanyl unit. The areas under the peaks were determined by means of a planimeter.

TABLE II: Action of Beef Liver Esterase on L-Phenylalanine Methyl Ester.[a]

Initial Phe-OMe (μmoles/ml)	Phe-OMe Reacted[b] (μmoles/ml)	Products Formed (μequiv of Phe/ml)			
		Phe[c]	Phe-Phe-OMe	Phe-Phe	Total
1	1.0	0.82 (82)	0	0.14	0.96
2.5	2.5	2.0 (80)	0	0.50	2.5
5	4.8	2.7 (56)	0.3	1.6	4.6
10	8.7	4.3 (49)	0.2	4.1	8.6
15	12.3	4.9 (40)	1.8	5.2	11.9
25	18.6	6.5 (35)	4.3	7.9	18.7
50	33.3	8.9 (27)	15.3	8.3	32.5

[a] Enzyme concentration, 0.24 unit/ml of incubation mixture; pH 7.2; 37°; 15 min. [b] Initial Phe-OMe minus amount of Phe-OMe found. [c] Numbers in parentheses denote per cent hydrolysis of Phe-OMe.

for 20 min on a steam bath. The ether having evaporated, the residue was dissolved in 25 ml of ether, and the solution was washed twice with 10-ml portions of water. After evaporation of the ether *in vacuo*, the residue was taken up in acetone and, as above, appropriately diluted solutions were analyzed by gas–liquid partition chromatography. This portion of the procedure gave data on the amount of free fatty acid and of acylamino acid present in the incubation mixture.

Replicate experiments were performed in which the above procedure was applied to the analysis of a solution containing known concentrations (*ca.* 20 μmoles/ml) of β-phenylpropionic acid and its methyl ester, β-phenylpropionyl-L-phenylalanine and its methyl ester, as well as L-phenylalanine and its methyl ester. The recovery of the individual components determined either by gas–liquid partition chromatography or by gel filtration varied between 86 and 95%. In view of the numerous steps in the fractionation procedure, this recovery may be considered to be acceptable. It may be added that separate experiments on the efficiency of the esterification procedure were performed with β-phenylpropionic acid, β-phenyl-L-lactic acid, and hexanoyl-L-phenylalanine; analysis of the resulting mixtures by gas–liquid partition chromatography showed that 95–100% of the acid had been converted into its methyl ester under the conditions described above. In the case of β-phenyl-L-lactic acid, the methyl ester was the only product detectable by gas–liquid partition chromatography. An unfavorable feature of the above method was the loss of methyl hexanoate during the course of the evaporation of the ether; consequently, reliable analytical data could not be obtained for the amount of this compound and of hexanoic acid in incubation mixtures.

Results

Enzymic Synthesis of Phe-Phe from Phe-OMe. The data in Table II confirm and extend the previous findings of Krenitsky and Fruton (1966) on the efficiency of the transfer reaction leading to peptide synthesis. It will be noted that, at the highest concentration of Phe-OMe tested (50 mM), the fraction of reacted substrate that had undergone hydrolysis was about 27%, the remainder having been converted into Phe-Phe-OMe and Phe-Phe. The dependence of the partition between hydrolysis and peptide synthesis on initial substrate concentration is clearly evident. In the final column of Table II are given the total products accounted for by the analytical method employed; the agreement of these figures with the amount of substrate that had undergone reaction (initial substrate concentration minus that found) is reasonably satisfactory, and may be taken as an indication of the reliability of the analytical method.

Examination of the effect of changes in enzyme concentration at a fixed concentration of Phe-OMe (25 mM) showed that the partition of phenylalanyl units between hydrolysis and peptide synthesis increases somewhat in favor of peptide synthesis with increasing enzyme concentration (Table III). The data in Table III also show clearly that Phe-Phe-OMe is an intermediate in the formation of Phe-Phe, thus confirming and extending the results of Krenitsky and Fruton (1966).

The data in Table IV indicate that the fraction of phenylalanyl units going to peptide synthesis is less at pH 6.5 than at pH 7.2–8.5, and are consistent with the view that the conjugate base of Phe-OMe (pK_a = *ca.*7.0 at 25°; Almond *et al.*, 1959; Hay *et al.*, 1966) is the acceptor in peptide synthesis.

It was of interest to find that a purified sample of pig liver esterase also effected the synthesis of Phe-Phe-OMe from Phe-OMe at pH 7.2 and 37°, but with an efficiency much lower than that found for the beef liver enzyme preparation. At an initial substrate concentration of 200 mM and a concentration of the pig liver enzyme of 6.4 μg of protein/ml, the predominant reaction during 25 min was hydrolysis to phenylalanine. Of the 161 μequiv/ml of the phenylalanyl units of Phe-OMe that had reacted, only 4.4 μequiv/ml appeared in the form of Phe-Phe-OMe, and no Phe-Phe was evident upon gel filtration with Sephadex G-10. In the absence of enzyme, no Phe-Phe-OMe was formed under these conditions.

In connection with the studies on the transfer of fatty acyl units to amino acid esters, it was necessary to in-

TABLE III: Effect of Enzyme Concentration on Action of Beef Liver Esterase on L-Phenylalanine Methyl Ester.[a]

Enzyme Concn (unit/ml)	Time (min)	Phe-OMe Reacted[b] (μmoles/ml)	Products Formed (μequiv of Phe/ml)		
			Phe	Phe-Phe-OMe	Phe-Phe
0.016	15	4.8	2.8	1.5	0.7
	30	7.0	3.5	2.0	1.2
	45	7.6	3.5	2.7	1.7
	60	9.2	4.0	3.2	2.2
0.030	15	7.1	3.2	1.6	2.1
	30	10.6	4.2	2.5	4.5
	45	12.5	4.9	2.8	5.3
	60	14.5	5.5	3.0	6.9
0.064	15	9.5	4.3	1.7	4.9
	30	15.9	5.5	2.4	8.2
	45	18.5	6.3	3.6	8.8
	60	19.3	6.6	2.9	10.1
0.12	15	14.1	4.6	3.9	5.6
	30	19.5	5.8	5.2	8.5
	45	23.4	7.7	4.4	11.7
	60	24.5	7.7	3.4	12.8
0.28	15	20.7	6.3	7.6	7.3
	30	24.0	8.6	4.4	12.2
	45	25.0	8.9	1.9	14.6
	60	25.0	9.0	0.6	16.0

[a] Substrate concentration, 25 μmoles/ml; pH 7.2; 37°. [b] Initial Phe-OMe minus amount of Phe-OMe found.

troduce solutions of the fatty acid esters in an organic solvent. Various solvents (methanol, tetrahydrofuran, dioxane, and acetonitrile) were tested at concentrations of 0.5–10% (v/v) for their effect on the initial rate of hydrolysis of methyl butyrate (50 mM) by the enzyme (0.39 unit/ml) at pH 7.0 and 37°. Among these solvents, methanol and tetrahydrofuran were found to be most inhibitory (*ca.* 0.1 M solutions caused 40% inhibition). An effect of acetonitrile made itself evident only at 0.2 M (20% inhibition). On the other hand, no change in rate was observed in dioxane solutions up to 0.23 M (2% v/v), with only slight inhibition (16%) at 0.6 M (5% v/v) and stronger inhibition (37%) at 1.2 M (10% v/v). It may be added that storage of a sample of the enzyme in 10% dioxane for 15 min, and 20-fold dilution of this solution for test of its activity, showed no loss of potential esterase activity; such reversibility was not found with tetrahydrofuran or with methanol.

In Table V are given data on the effect of increasing concentration of dioxane on the partition of the phenylalanyl units of Phe-OMe between hydrolysis and peptide synthesis. Comparison of the data in Table V with those in Table II indicates that dioxane (2.5–10%, v/v) causes an apparent slight increase in the rate of disappearance of Phe-OMe with a larger fraction undergoing hydrolysis. It is clear, however, that the enzymic reaction proceeds at relatively high dioxane concentrations. Of special interest is the observation that, at all substrate concentrations tested, the addition of 2.5% dioxane causes a significant shift in the partition of the phenylalanyl units of Phe-OMe in favor of transfer to water. At the highest dioxane concentration tested (10%, v/v) in the experiments reported in Table V, there is a very marked decrease in the rate of conversion of Phe-Phe-

TABLE IV: Effect of pH on Peptide Synthesis from L-Phenylalanine Methyl Ester by Beef Liver Esterase.[a]

pH	Phe-OMe Reacted[b] (μmoles/ml)	Products Formed (μequiv of Phe/ml)		
		Phe[c]	Phe-Phe-OMe	Phe-Phe
6.5	7.9	5.4 (68)	0.4	2.7
7.2	9.1	4.7 (52)	1.1	3.2
7.8	10.0	4.9 (49)	0	4.8
8.5	10.0	4.4 (44)	0	5.4

[a] Substrate concentration, 10 μmoles/ml; 0.24 enzyme unit/ml of incubation mixture; 37°; 25 min. [b] Initial Phe-OMe minus amount of Phe-OMe found. [c] Numbers in parentheses denote per cent hydrolysis of Phe-OMe.

TABLE V: Effect of Dioxane on Action of Beef Liver Esterase on L-Phenylalanine Methyl Ester.[a]

Initial Phe-OMe (μmoles/ml)	Dioxane (%, v/v)	Phe-OMe Reacted (μmoles/ml)	Products Formed (μequiv of Phe/ml)		
			Phe	Phe-Phe-OMe	Phe-Phe
5	2.5	5	3.7	0.1	0.7
	5	4.8	3.6	0.3	0.5
	10	4.4	3.6	0.3	0.2
10	2.5	9.8	7.4	0.7	2.0
	5	9.6	6.8	1.2	1.5
	10	8.3	7.2	1.1	0.4
15	2.5	14.4	9.5	1.8	3.0
	5	14.1	8.2	3.1	2.7
	10	12.6	8.7	3.0	1.0
25	2.5	22.7	12.4	5.2	5.1
	5	22.5	10.4	7.6	4.3
	10	19.7	11.9	6.2	1.5
50	2.5	41.0	16.1	15.5	6.7
	5	41.6	13.0	19.8	5.4
	10	39.8	17.5	17.3	3.1

[a] Experimental conditions as in Table II.

OMe to Phe-Phe, as compared to the control (*cf.* Table II). A similar effect was noted upon the addition of chloramphenicol (Krenitsky and Fruton, 1966).

Enzymic Transfer of Fatty Acyl Units to Amino Acid Esters. In order to guide the selection of fatty acid methyl esters for experiments on the synthesis of fatty acylamino acid esters by beef liver esterase, the kinetics of the enzymic hydrolysis of a series of fatty acid methyl esters was performed. The data are collected in Table VI. It will be noted that, for each of the compounds studied, the value of V_m/K_M increased over the pH range 7.2–8.5, largely because of a decrease in K_M with increasing pH. Furthermore, for a given pH, the values of V_m/K_M for the methyl esters of the straight-chain aliphatic fatty acids increased in the manner previously reported by Hofstee (1954, 1967), and the introduction of a methyl group at the β-carbon of the fatty acid markedly reduced the rate of hydrolysis. These changes in V_m/K_M values largely reflect differences in V_m, as the K_M values at a given pH show relatively little variation for the esters of the branched and unbranched aliphatic fatty acids. It is relevant to the subject of this communication that the methyl esters of 3-methylbutyric acid and of 3-methylpentanoic acid are hydrolyzed very slowly; the carbon skeletons of these two acids are those of valine and isoleucine, respectively. As shown previously (Krenitsky and Fruton, 1966), the methyl esters of L-valine and of L-leucine are relatively resistant to hydrolysis by beef liver esterase under conditions that lead to the rapid hydrolysis of L-leucine methyl ester.

Because of their relatively rapid hydrolysis by beef liver esterase, the methyl esters of β-phenylpropionic acid and of hexanoic acid were selected as potential acyl donors in transfer experiments with amino acid esters as acceptors. The utility of these two fatty acid esters is severely limited, however, by their sparing solubility in aqueous solutions. At a dioxane concentration of 0.25% (0.03 M), used in the experiments to be described below, the maximum concentration of these two esters that could be attained was about 6 mM. As will be noted in Table VI, the presence of 0.25% dioxane alters the kinetic parameters for the enzymic hydrolysis of methyl β-phenylpropionate at pH 7.2 only slightly, the principal effect being to lower somewhat the value of K_M.

In addition to the sparingly soluble methyl esters of β-phenylpropionic acid and hexanoic acid, methyl β-phenyl-L-lactate also was tested as a substrate of beef liver esterase. It will be noted in Table VI that the values of V_m for methyl β-phenyl-L-lactate and for methyl β-phenylpropionate are very similar, and that K_M for the α-hydroxy acid ester is significantly greater than for the fatty acid ester. A determination of the kinetic parameters in the action of pig liver esterase on methyl β-phenyl-L-lactate (4–22 mM) at pH 7.2 and 37° gave a K_M value of 2.1 ± 0.5 mM. The value of V_m (expressed as micromoles per minute per milligram of protein per milliliter) for the pig liver enzyme was 175 ± 15, as compared with the value of 450 ± 50 for the beef liver enzyme. Further studies are needed to determine whether the two esterases exhibit similar divergence in their kinetic parameters in their action on other substrates.

When methyl β-phenylpropionate or methyl hexanoate was used as a potential acylating agent of a suitable amino acid ester, in the presence of beef liver esterase, analysis by gas–liquid partition chromatography readily showed the appearance of a product whose rel-

TABLE VI: Kinetics of Hydrolysis of Fatty Acid Methyl Esters by Beef Liver Esterase.[a]

Substrate	pH	s[b] (mM)	V_m (mM/min per enzyme unit per ml)	K_M (mM)	V_m/K_M
Methyl butyrate	7.2	1.3–6.5 (5)	1.4 ± 0.2	3.9 ± 0.1	0.36
	7.8	1.3–6.5 (5)	1.7 ± 0.1	2.4 ± 0.5	0.71
	8.5	1.3–6.5 (5)	1.7 ± 0.1	1.0 ± 0.2	1.7
Methyl 3-methylbutyrate	7.2	1.5–3.8 (4)	0.28 ± 0.07	2.8 ± 0.2	0.1
	7.8	0.9–2.3 (5)	0.27 ± 0.01	0.7 ± 0.04	0.39
	8.5	1.0–2.8 (7)	0.40 ± 0.02	0.7 ± 0.04	0.57
Methyl pentanoate	7.2	2.1–4.9 (5)	3.5 ±0.4	3.4 ± 0.7	1.0
	7.8	2.1–4.9 (5)	3.3 ± 0.3	1.5 ± 0.1	2.2
	8.5	1.4–4.8 (5)	3.3 ± 0.4	0.5 ± 0.1	6.6
Methyl 3-methylpentanoate	7.2	0.8–2.0 (5)	0.45 ± 0.03	1.5 ± 0.3	0.3
	7.8	0.8–2.0 (5)	0.65 ± 0.08	1.3 ± 0.3	0.5
	8.5	0.8–2.0 (5)	0.67 ± 0.06	0.8 ± 0.2	0.84
Methyl 4-methylpentanoate	7.2	1.0–4.3 (7)	3.8 ± 0.3	3.1 ± 0.1	1.2
	7.8	1.2–3.5 (6)	4.0 ± 0.6	1.8 ± 0.5	2.2
	8.5	1.5–3.8 (4)	4.1 ± 0.5	1.3 ± 0.4	3.2
Methyl hexanoate	7.2	1.2–4.6 (6)	5.6 ± 0.3	2.0 + 0.3	2.8
	7.8	1.2–4.6 (6)	6.9 ± 0.1	1.7 ± 0.1	4.1
	8.5	1.2–4.6 (6)	6.3 ± 0.3	0. 9± 0.1	7.0
Methyl heptanoate	7.2	1.2–2.1 (4)	4.9 ± 0.4	1.0 ± 0.1	4.9
	7.8	1.2–2.1 (4)	5.1 ± 0.1	0.8 ± 0.1	6.4
	8.5	1.2–2.1 (4)	5.7 ± 0.8	0.7 ± 0.1	8.2
Methyl β-phenylpropionate	6.5	1.1–3.2 (4)	11.3 ± 1.3	8.4 ± 0.8	1.3
	7.2	2.1–5.3 (5)	13.4 ± 0.6	5.1 ± 0.7	2.6
	7.2[c]	0.6–5.6 (10)	13.3 ± 1.0	2.1 ± 0.4	6.3
	7.8	2.1–5.3 (5)	13.3 ± 2.3	3.3 ± 0.2	4.0
	8.5	2.1–5.3 (6)	13.8 ± 0.5	1.8 ± 0.1	7.6
Methyl β-phenyl-L-lactate	7.2	5.6–55.5 (8)	16.5 ± 0.9	33.9 ± 0.6	0.49
	7.8	2.2–21.8 (7)	17.1 ± 0.3	22.8 ± 0.6	0.75
	8.5	4.4–21.8 (5)	10.3 ± 0.5	7.3 ± 0.3	1.4

[a] Enzyme concentration, 0.01–0.24 unit/ml of incubation mixture; 0.2 M NaCl; 37°. [b] Range of initial substrate concentration; the numbers in parentheses denote the number of points in the v *vs.* v/s plot. [c] Dioxane (0.25% v/v) also present.

ative retention time was identical with that of an authentic sample of the expected acylamino acid ester, or whose relative retention time accorded with the value predicted from the behavior of closely related compounds. Control experiments, in the absence of added enzyme, failed to reveal the formation of detectable amounts of such products under the conditions of these studies.

The data in Table VII, on the time course of the enzyme-catalyzed reaction between methyl β-phenylpropionate (4.84 mM) and L-phenylalanine methyl ester (10 mM) at pH 7.2, show that at these concentrations of the reactants, about 65% of the fatty acid ester undergoes hydrolysis, the remainder of the phenylpropionyl units appearing as phenylpropionyl-L-phenylalanine (Ppr-Phe), with the acylamino acid ester (Ppr-Phe-OMe) as an intermediate. Of the amino acid ester that had undergone reaction at 25 min, about 50% of the phenylalanyl units went to phenylalanine by hydrolysis, and the remainder was partitioned among Ppr-Phe-OMe, Ppr-Phe, Phe-Phe-OMe, and Phe-Phe. The data in Table VIII indicate that, at the above concentration of the reactants, a change in pH from 7.2 to 6.5 markedly reduces the fraction of phenylpropionyl units going to acylamino acid (and its ester), whereas a rise in pH to 7.8 or 8.5 increases this fraction only slightly. As was to be expected, a decrease in the initial concentration of Phe-OMe lowers the amount of acylamino acid formed, but

TABLE VII: Rate of Transferase Action of Beef Liver Esterase with Methyl β-Phenylpropionate (Ppr-OMe) as Donor and L-Phenylalanine Methyl Ester as Acceptor.[a]

	Reaction Components Found (μequiv of Ppr or Phe/ml)							
Time (min)	Ppr-OMe	PprOH	Ppr-Phe-OMe	Ppr-Phe	Phe-OMe	Phe	Phe-Phe-OMe	Phe-Phe
1.5	2.3	1.9	0.3	0.1	7.9	1.5	0	0
3.0	0.9	2.8	0.8	0.1	6.5	1.8	1.0	0
6.0	0.3	3.2	0.6	0.4	4.5	2.4	1.3	0.6
12.0	0	3.3	0.5	0.7	2.4	3.3	1.7	1.2
25.0	0	3.1	0.2	1.1	0.8	4.4	0.8	2.3

[a] Concentration of Ppr-OMe, 4.84 μmoles/ml; concentration of Phe-OMe, 10 μmoles/ml; dioxane, 0.25%; enzyme concentration, 0.24 unit/ml of incubation mixture; pH 7.2; 37°.

TABLE VIII: Transferase Action of Beef Liver Esterase with Methyl β-Phenylpropionate (Ppr-OMe) as Donor and L-(or D-)-Phenylalanine Methyl Ester as Acceptor.[a]

		Reaction Components Found (μequiv of Ppr or Phe/ml)							
pH	Initial Phe-OMe[b] (μmoles/ml)	Ppr-OMe	PprOH	Ppr-Phe-OMe	Ppr-Phe	Phe-OMe	Phe	Phe-Phe-OMe	Phe-Phe
6.5	10	0.01	3.5	0.20	0.64	4.1	3.3	1.1	1.1
7.2	10	0	3.1	0.16	1.1	0.8	4.3	0.8	2.3
	10[c]	0	4.4	0	0.04	0.4	9.2	0	0.5
	5	0	3.5	0.01	0.86	0.3	2.6	0.2	0.8
	2.5	0	4.0	0	0.40	0.14	1.4	0	0.32
	1	0.1	4.2	0	0.16	0.08	0.67	0	0.04
7.8	10	0	2.8	0.28	1.2	0.3	4.5	1.0	2.5
8.5	10	0.06	2.3	0.10	1.4	0	4.3	0.6	3.2
	10[c]	0	4.3	0	0.05	0.2	9.1	0	0.4

[a] Concentration of Ppr-OMe, 4.82 μmoles/ml; dioxane, 0.25%; enzyme concentration, 0.24 unit/ml of incubation mixture; 37°; 25 min. [b] Unless otherwise stated, the L isomer was present. [c] D-Phenylalanine methyl ester was used.

it is of interest that even at an initial ratio of fatty acid ester to amino acid ester of 4.8:1, significant amounts of acylamino acid are formed.

In Table VIII are included data for the enzymic reaction in which methyl β-phenylpropionate (4.84 mM) was the acyl donor and D-phenylalanine methyl ester (10 mM) was the potential acceptor amine. The data at pH 7.2 and 8.5 show that the yield of acylamino acid in this case is about 4% of that formed when L-phenylalanine methyl ester is the acceptor. In confirmation of the results of Krenitsky and Fruton (1966), the fraction of D-phenylalanine methyl ester that is converted into Phe-Phe (or its ester) is very small, representing about 5% of the amino acid ester that had undergone reaction.

Examination of the ability of other amino acid esters to act as acceptor amines for the β-phenylpropionyl group showed that whereas Gly-OMe, Ala-OMe, Leu-OMe, and Ile-OMe all were converted into a significant extent to their acyl derivatives, no Ppr-Gly or Ppr-Ile could be detected by gas–liquid partition chromatography (Table IX). On the other hand, Ppr-Leu-OMe had undergone extensive hydrolysis to the corresponding acylamino acid, and Ppr-Ala-OMe had been hydrolyzed slightly. These results are consonant with earlier observations (Krenitsky and Fruton, 1966) that Gly-OMe and Ile-OMe are relatively resistant to the hydrolytic action of beef liver esterase, whereas Ala-OMe is hydrolyzed slowly and Leu-OMe is hydrolyzed rapidly. The data in Table IX also indicate that the effect of a change in pH from 7.2 to 8.5 on the fraction of phenylpropionyl units going to acylamino acid ester is more marked in the case of the aliphatic amino acid esters than with L-phenylalanine methyl ester; this result is consistent with the higher pK_a values (*ca.* 7.5–7.7) for the aliphatic amino acid esters. When L-phenylalaninol (pK_a = 9.5) was tested as an acceptor amine under the conditions given in Table IX at pH 7.2, there was no evidence for the formation of β-phenylpropionyl-L-phenylalaninol by gas–liquid partition

TABLE IX: Transferase Action of Beef Liver Esterase with Methyl β-Phenylpropionate (Ppr-OMe) as Donor and Various Amino Acid Esters as Acceptors.[a]

Amino Acid Ester	pH	Reaction Components Found (μmoles/ml)			
		Ppr-OMe	Ppr-OH	Ppr-X-OMe[b]	Ppr-XOH[b]
Gly-OMe	7.2	0.01	4.8	0.06	0
	8.5	0	4.3	0.24	0
Ala-OMe	8.5	0	4.3	0.21[c]	0.08[c]
Leu-OMe	7.2	0.04	4.6	0.07[c]	0.11[c]
	8.5	0	4.2	0.18[c]	0.39[c]
Ile-OMe	7.2	0.02	4.5	0.24	0
	8.5	0	4.2	0.57	0

[a] Concentration of Ppr-OMe, 4.84 μmoles/ml; concentration of amino acid ester, 10 μmoles/ml; dioxane, 0.25%; enzyme concentration, 0.24 unit/ml of incubation mixture; 37°; 25 min. [b] X denotes the residue of the amino acid in the product. [c] This value was obtained by gas–liquid partition chromatography at 200° (see Table I); retention time for Ppr-Ala-OMe, 7.1 min; for Ppr-Leu-OMe, 14.2 min.

chromatography, and the amount of β-phenylpropionic acid found corresponded closely to the amount of ester (4.8 μmoles/ml) that had disappeared. At pH 8.5, however, per 4.8 μmoles of the ester that had reacted, 0.4 μequiv of β-phenylpropionyl units appeared in the product of the transfer reaction, and only 3.6 μmoles of the acid was formed. Presumably, a significant portion of the acylamino alcohol was left in the aqueous phase during the course of preparation of samples for gas–liquid partition chromatography.

In Table X data are presented for the enzymic transfer of hexanoyl units from methyl hexanoate to the methyl esters of L-phenylalanine, glycine, and L-isoleucine. As was noted in the Experimental Section, methyl hexanoate proved to be volatile during the course of the preparation of samples for gas–liquid partition chromatography, and reliable data for the amounts of unreacted fatty acid ester and of fatty acid formed by enzymic hydrolysis could not be obtained by the method used in this work. It is clear from Table X, however, that the extent of acyl transfer to the three amino acid esters is appreciable, and is similar to that observed with methyl β-phenylpropionate as the acyl donor. This similarity in the extent of transfer is consistent with the relative rates of enzymic hydrolysis of the two fatty acid esters (Table VI). As in the experiments with β-phenylpropionate, the hexanoyl-L-phenylalanine methyl ester was readily hydrolyzed by the enzyme to form the acylamino acid, whereas the acylamino acid esters derived from glycine and L-isoleucine were not hydrolyzed to a detectable extent. It may be added that in the experiments with methyl hexanoate and L-phenylalanine methyl ester, the reaction mixtures were also analyzed for Phe-OMe, Phe, Phe-Phe-OMe, and Phe-Phe by the gel filtration method; the distribution of these products was found to be similar to that observed with methyl β-phenylpropionate as the acyl donor (Table VII).

Discussion

The data presented above provide additional strong evidence for the ability of beef liver esterase to catalyze the transfer of acyl groups from esters of suitable fatty acids and amino acids to the α-amino group of L-amino acid esters. The enzyme preparation employed in this work (specific activity 30 units/mg of protein) appears to be essentially monodisperse upon ultracentrifugation in 0.02 M phosphate buffer (pH 6.85) (*ca.* 8 S) and upon

TABLE X: Transferase Action of Beef Liver Esterase with Methyl Hexanoate (Hex-OMe) as Donor and Various Amino Acid Esters as Acceptors.[a]

Amino Acid Ester	pH	Time (min)	Products Found (μmoles/ml)	
			Hex-X-OMe[b]	Hex-XOH[b]
Phe-OMe	7.2	5	1.0	0.28
		10	1.0	0.48
		25	0.42	1.0
	8.5	25	0.33	1.35
Gly-OMe	8.5	25	0.24[c]	0
Ile-OMe	8.5	25	0.68[c]	0

[a] Concentration of amino acid ester, 10 μmoles/ml; concentration of methyl hexanoate, 4.0 μmoles/ml; dioxane, 0.25%; enzyme concentration, 0.24 unit/ml of incubation mixture; 37°. [b] X denotes the amino acid residue. [c] This value was obtained by gas–liquid partition chromatography at 166° (see Table I); retention time for Hex-Gly-OMe, 6.1 min; for Hex-Ile-OMe, 14.0 min.

starch gel electrophoresis over the pH range 5.5–8 (isoelectric point, *ca.* pH 6.0). The details of these studies will be reported in a future communication; they are mentioned here in support of the view that the transferase activity is an intrinsic property of beef liver esterase.

In confirming the results of Krenitsky and Fruton (1966) on the aminoacyl transferase activity of beef liver esterase, Benöhr and Krisch (1967) reported that a purified preparation of pig liver esterase (Krisch, 1963, 1966; Boguth *et al.*, 1965; Franz and Krisch, 1968) was unable to effect the synthesis of Phe-Phe from L-phenylalanine methyl ester. As noted above, the sample of pig liver esterase (Barker and Jencks, 1967) tested in this work did cause the formation of Phe-Phe-OMe, but with a lower efficiency than that found for the beef liver enzyme. The apparent contradiction between our results and those of Benöhr and Krisch (1967) may be attributed to the fact that these workers used thin-layer chromatography for the detection and identification of the products, whereas we used the quantitative gel filtration method developed in this laboratory.

Krenitsky and Fruton (1966) raised the possibility that the beef liver enzyme reponsible for acyl transfer to amino acid esters is composed of catalytic subunits that cooperate to bring closer together the α-amino group of a potential acceptor (on one subunit) and the acyl group of a potential donor (on another subunit). The identification of the transferase activity with liver esterase makes this possibility even more attractive, as it has been shown that pig liver esterase (Horgan *et al.*, 1966; Heymann and Krisch, 1967; Barker and Jencks, 1967) and beef liver esterase (Benöhr and Krisch, 1967) have a particle weight of *ca.* 170,000 (sedimentation coefficient, *ca.* 8 S) corresponding to a dimeric form of the enzyme, with two active sites as judged by the reaction with *p*-nitrophenyl diethyl phosphate (E600). Dissociation to particles of *ca.* 85,000 (4 S) was found to be effected at acid pH values or by dilution (Barker and Jencks, 1967; Benöhr and Krisch, 1967). It may be added that our purified enzyme preparation is dissociated at pH 3.95 to particles having a sedimentation coefficient of *ca.* 4 S.

It was noted above that the partition of the phenylalanyl units of Phe-OMe in favor of transfer to water is favored by dilution of the enzyme (Table III) or by the addition of 2.5% dioxane (Table V). A possible effect of dioxane is to increase slightly the apparent pK_a of Phe-OMe, thus decreasing the amount of conjugate base for action as an acceptor amine, but it is also known that dioxane promotes the dissociation of the oligomeric enzymes glutamic dehydrogenase (Churchich and Wold, 1963) and sulfatase (Nichol and Roy, 1966). The possibility exists, therefore, that the ability of beef liver esterase to effect peptide synthesis from Phe-OMe is related to subunit interaction, and that the lower efficiency of pig liver esterase in this regard is a reflection of a difference in the nature of the subunit interaction for this enzyme.

The demonstration that the liver esterases are all inhibited by DFP, and that a seryl residue in the sequence Gly-Glu-Ser-Ala of horse liver esterase is phosphorylated by ^{32}P-labeled DFP (Jansz *et al.*, 1959; Blakely *et al.*, 1967), makes it probable that the catalytic action of these enzymes involves the intermediate acylation of a serine residue in the active site, as in the action of chymotrypsin (Hartley, 1960; Bender and Kézdy, 1965). That chymotrypsin can catalyze acyl transfer from suitable substrates to the α-amino group of amino acid residues has long been known (Johnston *et al.*, 1950b; Blau and Waley, 1954). Chymotrypsin, however, is much less effective as a catalyst of such transamidation reactions than papain or ficin (Johnston *et al.*, 1950a; Dowmont and Fruton, 1952; Mycek and Fruton, 1957; Brubacher and Bender, 1966), whose catalytic action involves the intermediate acylation of a cysteine residue (Stockell and Smith, 1957; Lowe and Williams, 1965; Kirsch and Igelström, 1966). Even more effective in this regard is beef spleen dipeptidyl transferase, an oligomeric thiol enzyme (Metrione *et al.*, 1966) which catalyzes the polymerization of dipeptide amides (or esters) with high efficiency (Jones *et al.*, 1952; Nilsson and Fruton, 1964; Heinrich and Fruton, 1968). It has been suggested (Voynick and Fruton, 1968) that this polymerization may involve the cooperative action of catalytic sites on separate subunits, and that dipeptidyl groups in adjacent subunits react during the chain propagation reaction.

The results reported in the present communication, taken together with earlier work on liver esterases, make it probable that beef liver esterase, under suitable conditions of pH and of concentrations of enzyme, acyl donor, and amine acceptor, acts as an efficient transferase because the partition of the acyl-enzyme favors reaction with an amine nucleophile rather than reaction with water. The analytical methods developed in this work, involving the use of gas–liquid partition chromatography and of gel filtration on Sephadex G-10, make possible the quantitative determination of all the reaction components as a function of time. The closer definition of the specificity of beef liver esterase with respect to the acyl donor and the acceptor amine permits a more rational choice of reactants for kinetic studies. Such studies may be expected to provide data to test postulated mechanisms in the action of the enzyme. In particular, the application of theoretical treatments such as those of Durell and Fruton (1954) and of Seydoux and Yon (1967) for the partition of an acyl group of an acyl-enzyme between reaction with an amine acceptor and reaction with water may yield valuable insights into the questions raised in the foregoing discussion regarding the mechanism of the reaction and the role of subunit interaction in the transfer process. Such studies are in progress in this laboratory.

The intracellular function of the liver esterases is unknown. Because of the ability of some of these enzymes to effect peptide synthesis, and their localization in the microsomal fraction of liver homogenates (Krisch, 1963), their possible role in the biosynthesis of peptide bonds does not appear to be excluded. Recent reports on the transfer of acylamino acyl units (formylmethionyl, acetylphenylalanyl, etc.) from tRNA to puromycin, in the presence of a ribosomal enzyme preparation termed "peptidyl synthetase" (Zamir *et al.*, 1966; Monro and

Marcker, 1967; Monro, 1967; Gottesman, 1967), invite further experiments designed to explore this possibility.

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