Histidine at the Active Site of Phospholipase A_2†

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ABSTRACT: Porcine pancreatic phospholipase A_2 and its zymogen prophospholipase A_2 exhibited complete loss of (potential) catalytic activity upon treatment with *p*-bromophenacyl bromide. The reaction followed first-order kinetics and identical rates of inactivation were observed for both proteins. Studies of the pH dependence of the inactivation reaction indicated that a group with a p*K* around 6.1 is involved. Incorporation studies using ^{14}C-labeled *p*-bromophenacyl bromide showed that loss of enzymatic activity was accompanied by the incorporation of 1.1 [^{14}C]-*p*-bromophenacyl residues and by the loss of approximately 1 histidyl residue per mole of protein. The histidine involved in the inactivation was identified as histidine-53 (numbering of residues as in the zymogen). In both proteins about 10% of the incorporated radioactivity appeared to be associated with histidine-121. The active enzyme, as well as the zymogen, were effectively protected against the inactivation by divalent metal ions (Ca^{2+} and Ba^{2+}) or substrate analogs in the monomeric state. The efficiency of the protection of these specific ligands was determined by quantitative treatment of the protection data according to the procedure of M. C. Scrutton and M. F. Utter [(1965), *J. Biol. Chem. 240*, 3714]. In this manner the dissociation constants of the various phospholipase A_2–ligand complexes could also be determined, and these were shown to be in excellent agreement with those obtained by other direct binding methods. Comparison of the dissociation constants determined for a series of lysolecithins revealed that hydrophobic bonding makes a major contribution to the interaction of phospholipase A_2 with monomeric substrate analogs. The inactivation rate of the active enzyme was enhanced in the presence of substrate analogs in a concentration range above the critical micellar concentration, whereas the rate of inactivation of the zymogen was not influenced by the presence of a phospholipid–water interface. The rate enhancement was shown to be caused by a solubilization of *p*-bromophenacyl bromide in the phospholipid micelles, combined with the formation of an enzyme–interface complex. The inability of the zymogen to form this complex thus explains the difference in the effects of micellar substrate analogs. From these results it is concluded that histidine-53 is involved in the active site of phospholipase A_2 and that the active site must preexist for a main part already in the zymogen.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 1,2-diacyl *sn*-phosphoglycerides (van Deenen and de Haas, 1964). Together with other lipolytic enzymes it shares the property that the rate of hydrolysis increases several orders in magnitude when the substrate passes from a monomeric to an aggregated state (Desnuelle, 1971; Wells, 1972; de Haas *et al.*, 1971). The increased catalytic efficiency toward substrates present as lipid–water interfaces is the main characteristic differentiating lipolytic enzymes from enzymes catalyzing similar reactions on nonlipid substrates. One of the most important questions concerning the study of phospholipase A_2 and other members of the class of lipolytic enzymes is therefore: To what extent is this special requirement of substrate organization reflected in the structure and properties of the active site?

In recent years a number of pure phospholipases A_2 have been investigated for substrate requirements, metal ion activation, and kinetic parameters (see preceding paper, Pieterson *et al.* (1974a) and references therein). The primary structure has been determined for the porcine pancreatic (de Haas *et al.*, 1970a,b) and the bee venom enzymes (Shipolini *et al.*, 1971). Thus far, however, little information has been obtained concerning the amino acid residues involved in substrate binding and catalysis.

In contrast to the "serine"-esterases like acetylcholine esterase and proteolytic enzymes like trypsin and chymotrypsin, phospholipases A_2 obtained from snake venom (Saito and Hanahan, 1962; Salach *et al.*, 1971) or mammalian tissue (Postema, 1968) are not inhibited by organophosphorus compounds such as diisopropyl fluorophosphate.

Among the phospholipase A_2 studied so far, only the *Naja naja* venom enzyme has been reported to contain one or more essential histidines (Salach *et al.*, 1971), a conclusion which was based on photooxidation experiments. However, a correlation between the loss of activity and the oxidation of histidines was not shown. Therefore, taking into account the lack of specificity of dye-sensitized photooxidation, this conclusion must be considered as tentative.

The results of chemical modifications of the *Crotalus adamanteus* phospholipase A_2 have been described recently by Wells (1973). Two tryptophans as well as a single abnormally ionizing lysine appear to be essential for the catalytic activity of this dimeric enzyme. Chemical modification of tyrosyl or histidyl residues had no effect on enzymatic activity.

In the present paper it will be shown that porcine pancreatic phospholipase A_2 as well as its zymogen are completely inactivated upon reaction with *p*-bromophenacyl bromide. Results will be discussed in terms of a probable involvement of histidine-53 in the active site of the enzyme. Some preliminary results of this study have been published elsewhere (Bonsen *et al.* 1972a).

Experimental Section

Materials and Methods. Pure porcine pancreatic prophospholipase A_2 was obtained as described recently (Nieuwen-

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huizen *et al.*, 1974). Phospholipase A_2 was prepared by activation of the pure zymogen with trypsin as is described in the preceding paper. 1-*sn*-Phosphatidylcholines (D-lecithins) with two identical short-chain fatty acids were prepared as described previously (Bonsen *et al.*, 1972b). 1-Acyl-*sn*-3-phosphatidylcholines (lysolecithins) and 1-decanoyl-*sn*-glycero-2-phosphorylcholine were prepared from the corresponding diacyl compounds by phospholipase A_2 breakdown. 2-Decanoyl-*sn*-glycero-1-phosphorylcholine was prepared from the diacyl compound by degradation with pancreatic lipase (Slotboom *et al.*, 1970). [U-^{14}C]acetic anhydride was obtained from the Radiochemical Centre, Amersham, England. *p*-Bromophenacyl bromide was obtained from Fluka A.G. All other reagents and materials were of the purest grade available and used without further purification.

Synthesis of [^{14}C]-*p-Bromophenacyl Bromide.* A two-step procedure described in the textbook of Vogel (1948) was used for the synthesis of ^{14}C-labeled *p*-bromophenacyl bromide. The procedure was carried out on a 75-fold reduced scale in special microscale glassware. [U-^{14}C]acetic anhydride (1 mCi) was diluted with redistilled acetic anhydride to a specific activity of 431,000 dpm/μmol before use. All other reagents and solvents were used in an amount proportional to the starting amount of [^{14}C]acetic anhydride. The final product was treated with Norit in hot ethanol to remove traces of colored impurities and finally recrystallized from hot ethanol. A main fraction (373 mg) was obtained in a 21% yield. The product (mp 109°) had a specific radioactivity of 215,000 dpm/μmol (99% of the calculated value) and cochromatographed on thin-layer chromatography (tlc) in different solvent systems with commercially available *p*-bromophenacyl bromide. Moreover, this preparation inactivated phospholipase A_2 with a rate identical with the one observed for the commercial preparation. The product was stored dissolved in benzene (373 mg/25 ml) in the dark at room temperature and appeared to be stable over a period of more than 18 months.

Inactivation Procedures. Experiments to measure the rate of inactivation of phospholipase A_2 and prophospholipase A_2 by *p*-bromophenacyl bromide under different conditions were performed using a 0.1 M sodium cacodylate–HCl buffer containing 0.1 M NaCl at 30°. The same buffer was used throughout the whole pH range studied (5.0–7.5). Tlc indicated that *p*-bromophenacyl bromide is completely stable under the conditions used for inactivation of phospholipase A_2. Control experiments showed that no spontaneous activation of the zymogen occurred during the inactivation and subsequent procedures.

Treatment of Protection Data. As will be shown below the inactivation of phospholipase A_2 and prophospholipase A_2 by *p*-bromophenacyl bromide follows essentially first-order kinetics. This means that inactivation rates can be measured conveniently using a semilogarithmic plot of residual activity *vs.* time and can be expressed as the half-time ($t_{1/2}$). The same was observed in the presence of protecting ligands like divalent metal ions (see preceding paper) or substrate analogs (see below). Therefore, the method developed by Scrutton and Utter (1965) could be adopted to study the effect of protecting ligands in a quantitative manner. For the case of a single protecting ligand L eq 1 holds, where $t_{1/2}^0$ and $t_{1/2}$ are

$$\frac{t_{1/2}^0}{t_{1/2}} = \frac{k_2}{k_1} + K_L\left[\frac{1 - (t_{1/2}^0/t_{1/2})}{[L]}\right] \quad (1)$$

observed half-times for the inactivation in the absence and presence of L, k_1 and k_2 are the rate constants for the inactivation reaction of the free enzyme and of the enzyme–ligand complex, respectively, K_L is the dissociation constant of the enzyme–ligand complex, and [L] is the equilibrium concentration of the protecting ligand which equals $[L]_{total}$ provided $[L]_{total} \gg$ [protein]. A plot of $t_{1/2}^0/t_{1/2}$ against the bracketed term of eq 1 thus yields a straight line from which the slope equals K_L and the ordinate intercept equals k_2/k_1. In this way values are obtained for two parameters determining the protective effect of a given ligand at a certain concentration. The values for K_L can be compared with those obtained by direct binding methods which gives a criterion for the reliability of the method. Moreover, the value found for the ordinate intercept gives a direct measure of the efficiency of the protection: in other words, to what extent is there a direct competition of the protecting ligand and the irreversible inhibitor for the same site. In the optimal case occupation of this site should be mutually exclusive and k_2 should equal zero.

Assay. Phospholipase A_2 activities were routinely determined using the egg-yolk lipoprotein assay procedure as described previously (de Haas *et al.*, 1968). Identical rates of inactivation were obtained when the phospholipase A_2 activity was assayed with L-dioctanoyllecithin as substrate (de Haas *et al.*, 1971).

Radioactivity Measurements. The radioactivity of proteins or peptides was determined in a Packard Tri-Carb liquid scintillation counter using a dioxane based scintilland (7.0 g of diphenyloxazole, 0.3 g of 2,2-*p*-phenylenebis(4-methyl-5-phenyloxazole) and 100 g of naphthalene in 1 l. of dioxane). Efficiencies were determined using the external standard. Samples were kept in the dark for at least 30 min prior to counting. Radioactive peptides were located on paper with a tlc scanner (Berthold LB 2721).

Protein Determination. Concentrations of *p*-bromophenacyl bromide treated phospholipase A_2 or prophospholipase A_2 were determined from amino acid analyses averaging the values for lysine and arginine.

Amino acid analyses were performed by the method of Spackman *et al.* (1958) on a Beckman Unichrom amino acid analyzer. Samples were hydrolyzed for 22–24 hr at 110° in evacuated sealed tubes with 6 N HCl.

Cleavage of Disulfide Bonds. Sulfitolysis of [^{14}C]-*p*-bromophenacyl bromide treated phospholipase A_2 and prophospholipase A_2 was performed as described by Pechère *et al.* (1958).

Cyanogen Bromide Fragmentation. Cyanogen bromide cleavage of [^{14}C]-*p*-bromophenacyl bromide treated *S*-sulfophospholipase A_2 was performed in 70% trifluoroacetic acid as described by Bargetzi *et al.* (1964).

Enzymatic Digestion. Tryptic hydrolyses were performed at pH 8 and 37° with 10% trypsin (w/w) at substrate concentrations of 5 mg/ml.

Peptide Separations. Peptide mixtures obtained by CNBr cleavage or proteolytic digestion of [^{14}C]-*p*-bromophenacyl bromide treated and oxidized proteins were separated on columns of Sephadex G-50 fine or G-25 fine (2 m × 2.5 cm ϕ) in 1% NH_4HCO_3 solution (pH 8). The eluent was analyzed by ultraviolet absorption at 280 and 230 nm. Suitable aliquots were removed from each fraction for radioactivity determination. Further separation of radioactive peptides was done on Whatman paper No. 1 or 3 MM. High-voltage paper electrophoresis was carried out in a buffer of pH 3.5 (pyridine–acetic acid–water (1:10:289 v/v)) at approximately 60 V/cm on a Gilson high-voltage electrophorator (Model D). Paper chromatography was performed in the solvent system 1-butanol–acetic acid–pyridine–water (15:3:10:12 v/v). Detection of peptides on paper was usually achieved with the nin-

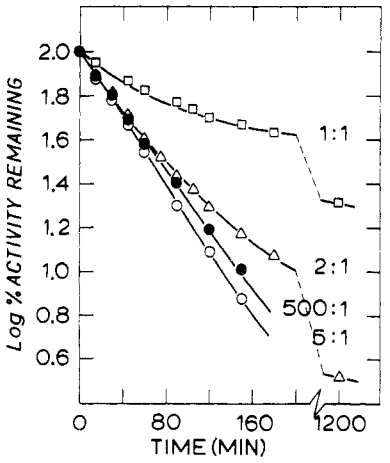

FIGURE 1: Loss of phospholipase A_2 activity as a function of time with different molar excesses of *p*-bromophenacyl bromide. Conditions: 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M. The numbers in the figure represent the molar ratio *p*-bromophenacyl bromide–phospholipase A_2.

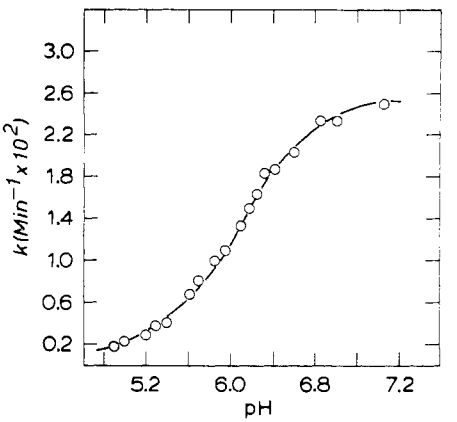

FIGURE 2: The pseudo-first-order rate constant for the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide as a function of pH. Conditions: 0.1 M sodium cacodylate–HCl containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M; 50-fold molar excess of *p*-bromophenacyl bromide.

hydrin reagent (0.1% ninhydrin dissolved in *s*-collidine–acetic acid–1-butanol (2:15:33 v/v)). Histidine or tyrosine containing peptides were made visible with the Pauly reagent (Sanger and Tuppy, 1951).

Results

Kinetics of Inactivation. Figure 1 shows the loss of phospholipase A_2 activity as a function of time upon incubation of the enzyme with different molar excesses of *p*-bromophenacyl bromide. It can be seen that for molar excesses of *p*-bromophenacyl bromide greater than five straight lines are obtained when the residual activity is plotted against time in a semilogarithmic plot. Inactivation under these conditions was complete in about 6 hr and straight lines were observed through 95% inactivation. This indicates that the reaction follows first-order kinetics and that inactivation does not involve sequential modification of groups with different reactivities. No loss of activity was observed after 24 hr when *p*-bromophenacyl bromide was omitted from the incubation mixture. Similar rates of inactivation were obtained when the amount of *p*-bromophenacyl bromide added was varied a factor of 100. The reason for this is the fact that *p*-bromophenacyl bromide has only a very limited solubility in aqueous media. The rate of inactivation appears to be only dependent on the concentration of dissolved *p*-bromophenacyl bromide. Below the solubility limit of *p*-bromophenacyl bromide (Figure 1, twofold excess and 1:1 mixture) straight lines were no longer observed in the semilogarithmic plot of residual activity *vs.* time. From these results one might conclude that the solubility of *p*-bromophenacyl bromide is somewhere in the order of the enzyme concentration used (7×10^{-5} M). This was confirmed by measuring the optical density at 265 nm of a saturated solution of *p*-bromophenacyl bromide in water at 30° using the molar extinction coefficient determined in methanol; this gave a value of about 10^{-4} M.

As is indicated in Figure 1 the inactivation proceeds to completion even when very low excesses of *p*-bromophenacyl bromide are used. This indicates that the inactivation is caused by the modification of a single essential residue.

Similar kinetics was observed when prophospholipase A_2 was used instead of the active enzyme. Half-times of 55 min were observed for both proteins at pH 6.0 and 30° using a 50-fold molar excess of *p*-bromophenacyl bromide (BPB). Using this value a pseudo-first-order and a second-order rate constant can be calculated from the following relation: $k = 0.69/t_{1/2} = k_1[\text{BPB}]^n$, where $t_{1/2}$ is the half-time for the inactivation obtained from a semilogarithmic plot as in Figure 1, k is the pseudo-first-order rate constant, k_1 is the second-order rate constant if we assume n, the number of *p*-bromophenacyl bromide moles involved in the inactivation, to be 1, and [BPB] is the concentration of soluble *p*-bromophenacyl bromide in the reaction mixture. For k and k_1 we find 1.25×10^{-2} min^{-1} and 125 M^{-1} min^{-1}, respectively, taking [BPB] = 10^{-4} M.

Dependence on pH. Figure 2 shows the effect of pH on the rate of inactivation of phospholipase A_2 expressed as the pseudo-first-order rate constant k. The pH dependence follows a sigmoidal curve with an inflection point around pH 6.1, indicating that the rate of inactivation is controlled by a group with an apparent pK of 6.1. Control experiments showed that there was no effect of pH on the solubility of *p*-bromophenacyl bromide.

Protection by Divalent Metal Ions. As was shown in the preceding paper phospholipase A_2 and prophospholipase A_2 are protected against inactivation by the presence of the metal activator Ca^{2+} and the related Ba^{2+} ion. The latter competes with Ca^{2+} for the same site but does not give rise to a ternary Michaelis complex leading to products. Mg^{2+}, which is not a competitive inhibitor, does not exhibit any protective effect. Furthermore it was shown that values obtained for the dissociation constants of the enzyme–Ca^{2+} complex (K_{Ca}) by means of a plot according to Scrutton and Utter (1965) are in close agreement with the values obtained by direct binding methods. The ordinate intercept of this plot yields for both Ca^{2+} and Ba^{2+} a value of 0.07, suggesting that the protection of these ions on the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide is most efficient.

Protection by Substrate Analogs and Products. Kinetic evidence has been obtained which supports the existence of separate binding sites for the substrate (short-chain lecithins) and the metal activator Ca^{2+} (de Haas *et al.*, 1971). Therefore, it was of interest to examine the effect of substrate analogs on the rates of inactivation of phospholipase A_2 and prophospholipase A_2. Figure 3 demonstrates the protective nature of D-lecithins previously shown to be competitive inhibitors (Bonsen *et al.*, 1972c), and the products of the phospholipase A_2 hydrolysis, lysolecithin and fatty acid. Short-chain D-

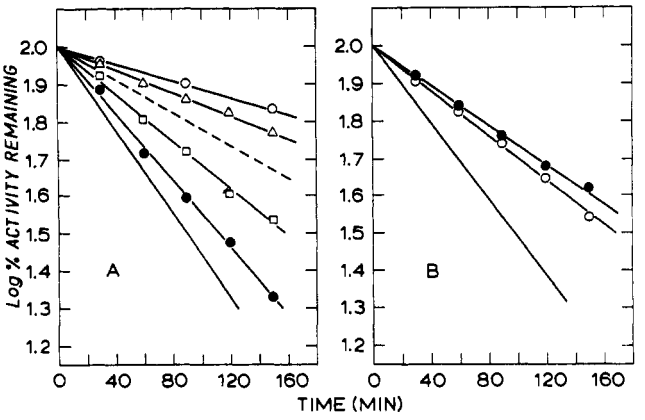

FIGURE 3: A. Effect of substrate analogs on the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide. Conditions: 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M; 50-fold molar excess of *p*-bromophenacyl bromide; (—) no ligand added; (- - - - - -) 5 mM $CaCl_2$; (●—●) 4.4 mM D-dihexanoyllecithin; (○—○) 4.4 mM D-dihexanoyllecithin + 5 mM $CaCl_2$; (□—□) 7.4 mM sodium heptanoate; (△—△) 65.3 mM 1-octanoyllysolecithin. B. Effect of substrate analogs on the inactivation of prophospholipase A_2 by *p*-bromophenacyl bromide. Conditions: see legend of A except prophospholipase A_2 concentration 14×10^{-5} M; (—) no ligand added; (○—○) 1.6 mM D-diheptanoyllecithin; (●—●) 5.0 mM 1-nonanoyllysolecithin.

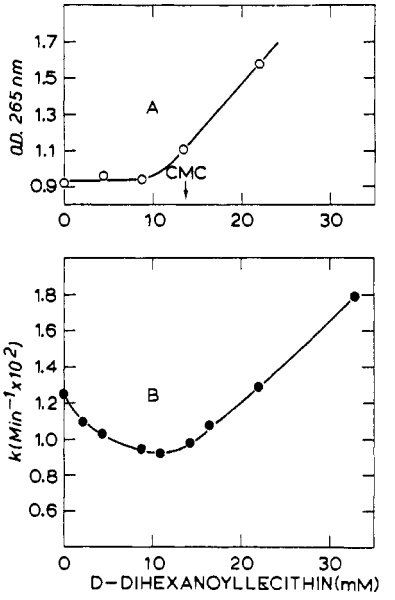

FIGURE 4: A. Solubility of *p*-bromophenacyl bromide in aqueous solution measured by the OD at 265 nm as function of the D-dihexanoyllecithin concentration. The procedure is described in the text. B. The pseudo-first-order rate constant for the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide as function of the D-dihexanoyllecithin concentration. Conditions: 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M; 50-fold molar excess of *p*-bromophenacyl bromide.

lecithin, short-chain lysolecithin, and short-chain fatty acid all protect the enzyme and the zymogen against inactivation by *p*-bromophenacyl bromide. The most effective protection is obtained when both the metal ion (Ca^{2+}) and a D-lecithin (D-dihexanoyllecithin) are present.

With respect to the data presented in Figure 3 it should be emphasized that these results were obtained with the lipids present in a concentration sufficiently below the critical micellar concentration (cmc) to exclude the presence of micellar aggregates. Apparently the protection is the result of the formation of protein monomer complexes.

Rather striking results were observed when the rate of inactivation of phospholipase A_2 was studied over a wide range of D-dihexanoyllecithin concentrations. As is indicated in Figure 4B we observe an increasing protection, expressed as a decrease in the pseudo-first-order rate constant k, of this competitive inhibitor for increasing lecithin concentrations. However, close to the cmc (13 mM) the curve passes through a minimum and at concentrations exceeding the cmc there is no longer protection but rather stimulation of the inactivation reaction. A similar effect was observed using D-diheptanoyllecithin and in this case the minimum appeared at a much lower concentration in agreement with the known cmc value for this compound (2 mM). A possible explanation of this phenomenon involved the effect of increasing concentrations of D-dihexanoyllecithin on the solubility of *p*-bromophenacyl bromide in aqueous solvent. This was investigated in the following experiment: 1 mg of solid *p*-bromophenacyl bromide was added to a series of test tubes containing an aqueous solution of D-dihexanoyllecithin in varying concentration. After incubation at 30° under vigorous shaking during 30 min the undissolved *p*-bromophenacyl bromide was removed by centrifugation and the optical density at 265 nm of the supernatant was measured against a blank containing the same amount of lipid. The results are presented in Figure 4A. As can be seen there is no influence of D-dihexanoyllecithin on the solubility of *p*-bromophenacyl bromide for concentrations below the cmc; however, when the cmc is passed, there is a marked increase in the optical density. We interpret this behavior as the solubilization and probable inclusion of *p*-bromophenacyl bromide in the apolar core of the micelles.

No enhanced inactivation rates were observed when the inactivation was carried out with prophospholipase A_2 instead of the active enzyme. As presented in Figure 5, the expected increasing protection is found for increasing concentrations of D-dihexanoyllecithin below the cmc similar to the effects observed for phospholipase A_2. However, no stimulation of the inactivation is observed for concentrations above the cmc. Apparently both phospholipase A_2 and prophospholipase A_2 are protected against the inactivation by substrate analogs in a monomeric form, while reacting quite differently to the presence of micelles containing solubilized *p*-bromophenacyl bromide.

Attempts to determine the dissociation constants between monomers of D-dihexanoyl- and D-diheptanoyllecithin and

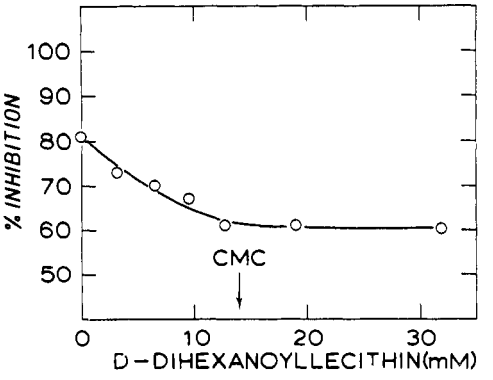

FIGURE 5: Effect of D-dihexanoyllecithin on the inactivation of prophospholipase A_2 by *p*-bromophenacyl bromide. Prophospholipase A_2 (14×10^{-5} M) was incubated with a 50-fold molar excess of *p*-bromophenacyl bromide in 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl and various concentrations of D-dihexanoyllecithin. After 2 hr incubation the residual potential activity was determined after tryptic activation.

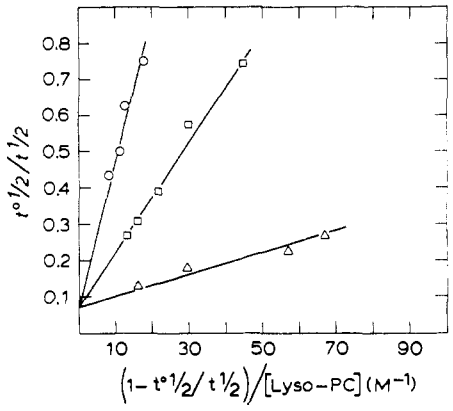

FIGURE 6: Plot according to Scrutton and Utter for the protection of lysolecithin against the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide. Conditions: 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M; 50-fold molar excess of *p*-bromophenacyl bromide; (○—○) 1-heptanoyllysolecithin; (□—□) 1-octanoyllysolecithin; (△—△) 1-nonanoyllysolecithin. $t_{1/2}$ values were obtained from semilogarithmic plots as in Figure 3.

phospholipase A_2 or prophospholipase A_2 by the method of Scrutton and Utter as described above were unsuccesful. The combined effects of a relatively low affinity and a low cmc are responsible for this failure. However, better results were obtained with a series of lysolecithins with varying chain length. Figure 6 shows the plots according to Scrutton and Utter obtained with three different lysolecithins at pH 6.0. Straight lines are obtained with a common intersection on the ordinate. From the value of the ordinate intercept, which equals in this case the ratio of the rate constants for the enzyme–lysolecithin complex and the free enzyme, we can conclude that these compounds give very efficient protection. Moreover, the dissociation constants of the various lysolecithin–phospholipase A_2 complexes, which are given by the slopes, appear to be very much dependent on the length of the fatty acyl chains. This is also illustrated in Table I where the dissociation constants,

TABLE I: Dissociation Constants of Various Lysolecithin–Phospholipase A_2 Complexes at pH 6.0.

Lysolecithin	K^a (M) I	K^a (M) II
1-Heptanoyl-*sn*-glycero-3-phosphorylcholine	4.1×10^{-2}	4.3×10^{-2}
1-Octanoyl-*sn*-glycero-3-phosphorylcholine	1.5×10^{-2}	– –
1-Nonanoyl-*sn*-glycero-3-phosphorylcholine	3.1×10^{-3}	3.2×10^{-3}
1-Decanoyl-*sn*-glycero-3-phosphorylcholine	1.4×10^{-3}	1.2×10^{-3}
1-Decanoyl-*sn*-glycero-2-phosphorylcholine	1.4×10^{-3}	– –
2-Decanoyl-*sn*-glycero-1-phosphorylcholine	0.8×10^{-3}	1.2×10^{-3}
1-Dodecanoyl-*sn*-glycero-3-phosphorylcholine	2.2×10^{-4}	2.0×10^{-4}

[a] I, *K* values derived from plots similar to Figure 6; II, *K* values derived from uv difference spectroscopy (Pieterson, 1973); – –, not determined.

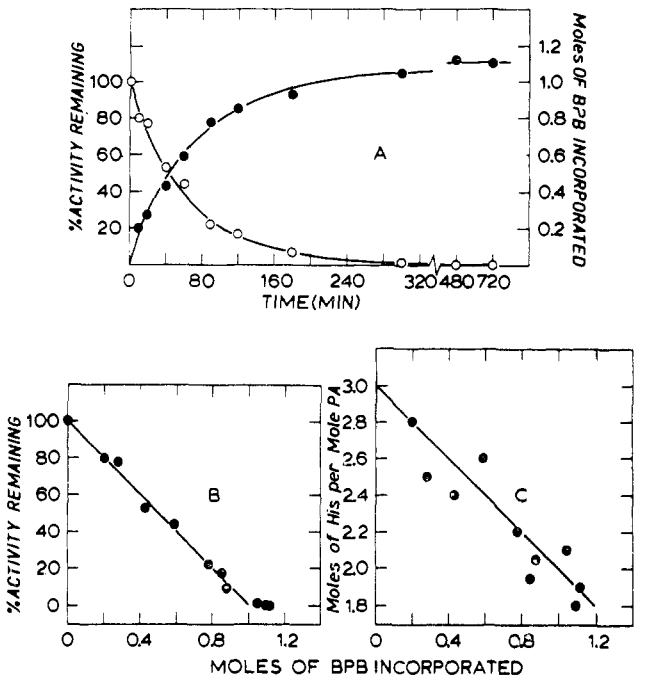

FIGURE 7: A. Loss of phospholipase A_2 activity and incorporation of *p*-bromophenacyl residues as a function of time. Conditions: 0.1 M sodium cacodylate–HCl (pH 6.0) containing 0.1 M NaCl; phospholipase A_2 concentration, 7×10^{-5} M; 50-fold molar excess of [^{14}C]-*p*-bromophenacyl bromide (specific activity 215,000 dpm/μmol). B. Correlation between the loss of phospholipase A_2 activity and the incorporation of *p*-bromophenacyl bromide residues. Replot of the data from Figure 7A. The solid line is the theoretical line for a stoichiometrical inactivation. C. Correlation between the histidine content according to the amino acid analyses and the incorporation of *p*-bromophenacyl bromide residues. The points give the histidine content of phospholipase A_2 samples obtained with the experiment represented in Figure 7A.

determined by protection experiments, for a number of lysolecithins are presented and compared to values obtained from uv difference spectroscopy (Pieterson, 1973). We see that elongation of the fatty acyl chain from 7 to 12 carbon atoms gives a 200-fold decrease in the dissociation constant, whereas variations in the relative positions of the fatty acyl chain and the phosphorylcholine moiety have only a minor influence. Thus it can be concluded that for these monomeric species hydrophobic interaction between the apolar part of the molecule and an apolar site on the enzyme plays a very important role. Furthermore it appears that occupation of this site gives a very effective protection against inactivation of phospholipase A_2 by *p*-bromophenacyl bromide. This indicates that the location of the modified residue is in or very close to the monomer binding site.

Incorporation Studies. [^{14}C]-*p*-Bromophenacyl bromide (specific radioactivity 215,000 dpm/μmol) was used to measure the precise stoichiometry of the inactivation reaction and to correlate the loss of activity with the incorporation of *p*-bromophenacyl residues.

Figure 7A shows the incorporation of [^{14}C]-*p*-bromophenacyl residues and the loss of phospholipase A_2 activity as a function of time. As was already indicated by kinetic evidence mentioned above the stoichiometry of the reaction appears to be close to 1:1. At the moment that complete loss of activity was reached (5 hr reaction) the incorporation was on the average 1.1–1.2 residues/mol. This indicates that besides the reaction which causes the inactivation a slower side reaction takes place leading to incorporation levels somewhat

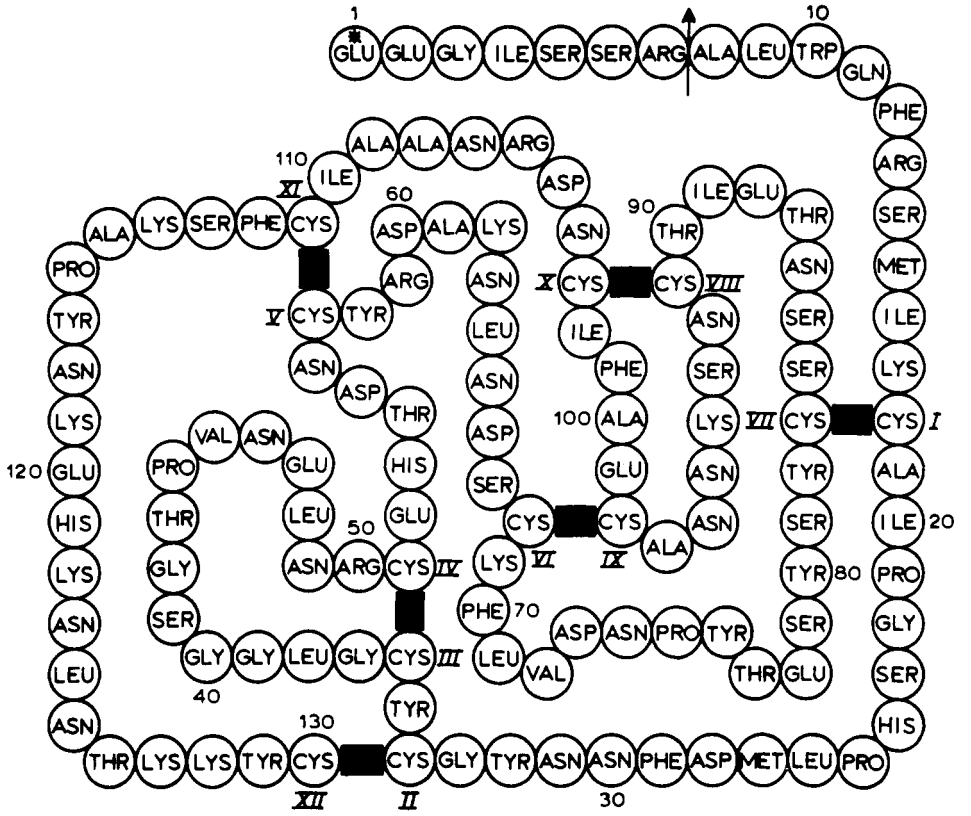

FIGURE 8: Primary structure of porcine pancreatic prophospholipase A_2. * Stands for pyroglutamic acid.

higher than 1:1. Similar results were obtained for prophospholipase A_2 when treated with [^{14}C]-*p*-bromophenacyl bromide under the same conditions.

Figure 7B shows a replot of the data from Figure 7A. The solid line is the one expected for a stoichiometric reaction leading to complete inactivation. From this result it is clear that the complete loss of activity upon incubation with *p*-bromophenacyl bromide is caused by chemical modification of a single essential residue.

A first indication of the nature of the modified residue was obtained from amino acid analyses of partially or completely inactivated phospholipase A_2 and prophospholipase A_2 samples. Figure 7C shows the histidine content of the modified phospholipase A_2 in the relation to the number of [^{14}C]-*p*-bromophenacyl residues incorporated in the protein. Although there is some scattering in the points it seems clear that incorporation of 1 mol of *p*-bromophenacyl bromide is accompanied by the loss of approximately 1 histidyl residue. Such a result can be expected if an N-alkylated derivate of histidyl imidazole is formed during the reaction. Derivatives of this type are known to be stable during the acid hydrolysis (Shaw, 1967). The only significant deviation in the amino acid analysis of *p*-bromophenacyl bromide inactivated phospholipase A_2 is observed in the histidine content. The methionine content appeared to be unchanged. This is of special interest because methionine is a possible site for alkylation by phenacyl bromides (Sigman *et al.*, 1969).

Identification of the Modified Histidines. Phospholipase A_2 and prophospholipase A_2 both contain three histidines in positions 24, 53, and 121, respectively (Figure 8, numbering of residues as in prophospholipase A_2). To determine which of these histidines is the site of modification by *p*-bromophenacyl bromide correlated with the loss of enzymatic activity, the following strategy was applied.

[^{14}C]-*p*-Bromophenacyl bromide treated phospholipase A_2 (200 mg, containing 1.1 *p*-bromophenacyl residues/mol) was converted to the *S*-sulfo derivative and subsequently treated with CNBr. Sephadex filtration on a column of G-50 fine yielded three principal peptides.

Table II shows the results of the amino acid analyses and the assigned sequences of these peptides. The incorporated radioactivity remains associated with the large CNBr fragment Asp(28)–Cys(130) (peptide I) containing His-53 and His-121.

In order to isolate peptides containing His-53 and His-121, the large peptide fragment I was subjected to extensive tryptic hydrolysis. Incubation was performed for 36 hr in the presence of 10^{-2} M $CaCl_2$ to prevent autolysis of trypsin. Sephadex filtration of the obtained peptide mixture on a column of G-25 fine yielded three radioactive fractions marked IA, IB, and IC containing 14, 63, and 8% of the radioactivity applied to the column, respectively. Fraction IA consisted of undigested material and was not further examined. Paper electrophoresis of fraction IB showed a complex mixture of peptides; however, one very acidic radioactive peptide was present. This peptide was further purified by paper electrophoresis and chromatography. Fraction IC contained a single radioactive peptide.

Table III shows the amino acid analyses, the final recoveries, and the assigned sequences of the peptides isolated from fractions IB and IC. These results indicate that His-53 is the primary site of modification and that modification of this residue leads to an inactive phospholipase A_2. Furthermore, approximately 10% of the incorporated radioactivity appears to be associated with His-121. Apparently His-121 becomes partially labeled during the inactivation reaction leading to incorporation levels somewhat higher than exactly 1 mol per mole of protein. However, it is clear that modification of this

TABLE II: Amino Acid Analyses and Assigned Sequences of the Peptides Obtained after Cyanogen Bromide Fragmentation of [^{14}C]-*p*-Bromophenacyl Bromide Treated Phospholipase A_2.

	Peptide					
	I		II		III	
	Amino Acid Analyses					
	Expected	Found	Expected	Found	Expected	Found
Lys	8	7.7	1	1.2		
His[a]	2	1.1	1	1.0		
Arg	3	3.0			1	0.9
Asp	23	20				
Thr	7	5.0				
Ser	8	6.6	1	1.0	1	0.9
Glu	6	6.2			1	1.0
Pro	4	3.6	2	2.0		
Gly	5	4.4	1	1.0		
Ala	6	6.0	1	1.1	1	1.0
$^1/_2$-Cys	11	10.5	1	0.7		
Val	2	1.8				
Met						
Ile	3	2.7	2	1.8		
Leu	5	4.8	1	1.1	1	1.0
Tyr	8	6.9				
Phe	4	3.6			1	0.9
Trp					1	Nd
Homoser + homoser lacton			1	1.1	1	+[b]
Yield[c] (%)	74		60		42	
Recovery of radioactivity (%)	77					
Sequence	Asp(28)–Cys(130)		Ile(16)–Met(27)		Ala(8)–Met(15)	

[a] No histidine derivative was detectable. [b] Quantitative determination impeded by poor resolution. [c] Calculated from the amino acid analyses. Nd, Not determined.

TABLE III: Amino Acid Analyses and Assigned Sequences of the Radioactive Peptides Obtained after Tryptic Digestion of Peptide I.

	Peptide			
	IB		IC	
	Amino Acid Analyses			
	Expected	Found	Expected	Found
Lys			1	0.8
His[a]	1		1	
Arg	1	0.9		
Asp	2	2.0		
Thr	1	0.8		
Glu	1	1.0	1	1.0
$^1/_2$-Cys	2	2.0		
Tyr	1	0.5		
N-terminal[b]	Nd		Glu	
Recovery of radioactivity[c] (%)	27		8	
Sequence	Cys(51)–Arg(59)		Glu(120)–Lys(122)	

[a] No histidine derivative was detectable. [b] Determined with the dansylation method according to Gross and Labouesse (1969). [c] Percentage of the amount of radioactivity present in peptide I after final purification on paper.

residue is not responsible for this loss of enzymatic activity. Similar results were obtained for [^{14}C]-*p*-bromophenacyl bromide treated prophospholipase A_2. Also in this case 10% of the incorporated radioactivity appeared to be associated with His-121.

Discussion

In the present study evidence is presented that porcine pancreatic phospholipase A_2 and its zymogen loose completely their (potential) catalytic activity when the imidazole side chain of histidine-53 is alkylated through reaction with *p*-bromophenacyl bromide. The type of chemical inactivation encountered here is one of the examples where a reagent possessing neither a well-defined group specificity nor a clear structural resemblance with the normal substrate is, nevertheless, able to produce a selective modification of an essential residue in an enzyme. This discussion will be centered around the following questions. (1) Is histidine-53 involved in the active site of the enzyme? (2) What is the meaning of the remarkable similarities with respect to the inactivation process of the active enzyme and the zymogen and how can we understand the difference in the effect of micellar substrate analogs on the inactivation of both proteins? (3) What is the reason for the selectivity of the reaction of *p*-bromophenacyl bromide with phospholipase A_2 and prophospholipase A_2?

The data presented above reveal that two criteria, which in general can be applied to determine whether a specifically

modified residue is in the active site, appear to be met for the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide. The loss of phospholipase A_2 activity shows a 1:1 correlation with the incorporation of *p*-bromophenacyl residues and with the loss of a histidyl residue in the amino acid analyses, the latter being identified as histidine-53.

The protection studies clearly show that both the phospholipase A_2 and the zymogen are efficiently protected against the inactivation by *p*-bromophenacyl bromide by divalent metal ions (Ca^{2+} and Ba^{2+}, see preceding paper) and by substrate analogs present in the monomeric state, the efficiency of the protection being determined by the value of the ordinate intercept in the plots according to Scrutton and Utter. The assumption that lysolecithins are true substrate analogs is supported by the observation that these compounds induce a protein uv difference spectrum essentially identical with the one observed for the corresponding diacyl D compounds. Thus it appears that two conditions for active site modification are fulfilled, and we can conclude that histidine-53 is, in fact, involved in the active site of phospholipase A_2.

Concerning the second question, the experimental data reveal that the active phospholipase A_2 and its zymogen show identical rates of inactivation and very similar labeling patterns by *p*-bromophenacyl bromide. Moreover both proteins are protected in a similar manner by divalent metal ions and substrate analogs in the monomeric form. The most important conclusion that can be drawn from these observations is that, whatever may be the reason for the site specificity of the inactivation reaction, the active site residue histidine-53 is present in the same form in both proteins. This suggests that in the case of phospholipase A_2 the active site at least partially preexists in the zymogen.

One of the most interesting observations made during the protection studies with substrate analogs is the effect of short chain D-lecithins in concentrations above the cmc on the inactivation of both proteins (Figures 4 and 5). The stimulated inactivation of phospholipase A_2 parallels the solubilization of *p*-bromophenacyl bromide by the D-diacyllecithin micelles. The inclusion of *p*-bromophenacyl bromide molecules into the lipid–water interface and the interaction of phospholipase A_2 with this interface would increase the concentration of *p*-bromophenacyl bromide close to the reaction site so that enhanced rates of inactivation are to be expected depending on the total amount of interface available. However, at least two additional factors may contribute to the observed rate enhancement. (1) The interface may induce *via* a local conformational change an increase in the reactivity of the susceptible histidine. (2) The interface may offer a microenvironment which is more favorable for the covalent bond formation to occur. At the moment it is not clear whether any of these possibilities play a significant role. In any case the physicochemical state of the interface, *i.e.*, the mixed aggregate of diacyllecithin and *p*-bromophenacyl bromide, must be such that phospholipase A_2 is able to interact. This is evident from the finding that aggregates of D-didecanoyllecithin which are of the lamellar type and for which phospholipase A_2 has no affinity (compare subsequent paper) are therefore not able to produce the stimulated inactivation, although uv difference spectroscopy indicated that *p*-bromophenacyl bromide is incorporated as well into these structures.

In summary we may say that the characteristics of the inactivation of phospholipase A_2 by *p*-bromophenacyl bromide parallel those of the phospholipase A_2 catalyzed hydrolyses of short-chain lecithins. Monomeric substrates are hydrolyzed very slowly compared to the rate of hydrolysis of the same substrate in an aggregated form provided the enzyme is able to interact with this liquid–water interface. In a similar manner monomers of *p*-bromophenacyl bromide are able to react with the active site residue histidine-53 but the reaction rate is enhanced when *p*-bromophenacyl bromide is present in a lipid–water interface suitable for phospholipase A_2 interaction.

The reasoning outlined above is in agreement with the apparent inability of D-diacyllecithin micelles to stimulate the inactivation of prophospholipase A_2. As is shown in the accompanying paper (Pieterson *et al.*, 1974b) the zymogen has no affinity for lipid–water interfaces of any type and therefore no enhancement of the inactivation rate would be expected.

A more general conclusion to be drawn from the aforementioned data is that the results of chemical modifications of lipolytic enzymes might be very much dependent on the physicochemical state of the employed inhibitor. This is nicely illustrated in a recent paper of Maylié *et al.* (1972) who found that porcine pancreatic lipase is only inhibited by the organophosphorus inhibitor diethyl *p*-nitrophenyl phosphate (E 600) in the presence of micellar bile salts.

With respect to the third question it is apparent that the reaction of *p*-bromophenacyl bromide with phospholipase A_2 and prophospholipase A_2 is site specific in that mainly one of the many possible reaction sites in the protein actually undergoes modification. In addition it should be emphasized that *p*-bromophenacyl bromide is not simply a histidine reagent. Under conditions where a rapid inactivation of phospholipase A_2 took place no reaction was observed between *p*-bromophenacyl bromide and free histidine. This observation together with the inability of alkylating reagents like iodoacetic acid and iodoacetic acid methyl ester to inhibit phospholipase A_2 (experiments not described) suggests that a special complementarity exists between the modification site in the protein and *p*-bromophenacyl bromide which favors covalent bond formation. The fact that efficient protection is obtained by monomeric substrate analogs and that the affinity of phospholipase A_2 for these compounds is dependent on the length of the fatty acyl chains makes it likely that the reactive histidine is close to an apolar site on the protein. This site might help to orient the apolar *p*-bromophenacyl bromide molecule in such a way that covalent bond formation is favored. This would mean that the reaction between *p*-bromophenacyl bromide and histidine-53 is in fact catalyzed by the enzyme and that *p*-bromophenacyl bromide is an inhibitor of the "active site directed" type.

From the observed efficient protection by divalent metal ions and monomeric substrate analogs we can conclude that histidine-53 must be located very close to both the metal ion binding site and the monomer binding site. This suggests that the amphiphatic nature of the substrate molecules might be reflected in the geometry of the active site, which would then contain a polar region where the metal activator is located in the Michaelis complex and an apolar region where the substrate molecule (to be hydrolyzed) is bound *via* hydrophobic interaction. At the moment work is in progress to study the effect of modifications in the *p*-bromophenacyl bromide structure on the inhibitory properties of the halo ketone and to design an active site directed inhibitor more closely approximating the minimal substrate requirements of phospholipase A_2.

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References

Bargetzi, J. P., Thompson, E. D. P., Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964), *J. Biol. Chem. 239*, 3767.

Bonsen, P. P. M., Burbach-Westerhuis, G. J., Haas, G. H. de, and Deenen, L. L. M. van (1972b), *Chem. Phys. Lipids 8*, 199.

Bonsen, P. P. M., Haas, G. H. de, Pieterson, W. A., and Deenen, L. L. M. van (1972c), *Biochim. Biophys. Acta 270*, 364.

Bonsen, P. P. M., Pieterson, W. A., Volwerk, J. J., and Haas, G. H. de (1972a), *in* Current Trends in the Biochemistry of Lipids, Ganguly, J., and Smellie, R. M. S., Ed., London, Academic Press, p 189.

Deenen, L. L. M. van, and Haas, G. H. de (1964), *Advan. Lipid Res. 2*, 167.

Desnuelle, P. (1971), *Biochimie 53*, 57.

Gross, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.

Haas, G. H. de, Bonsen, P. P. M., Pieterson, W. A., and Deenen, L. L. M. van (1971), *Biochim. Biophys. Acta 239*, 252.

Haas, G. H. de, Postema, N. M., Nieuwenhuizen, W., and Deenen, L. L. M. van (1968), *Biochim. Biophys. Acta 159*, 103.

Haas, G. H. de, Slotboom, A. J., Bonsen, P. P. M., Deenen, L. L. M. van, Maroux, S., Puigserver, A., and Desnuelle, P. (1970a), *Biochim. Biophys. Acta 221*, 31.

Haas, G. H. de, Slotboom, A. J., Bonsen, P. P. M., Nieuwenhuizen, W., Deenen, L. L. M. van, Maroux, S., Dlouha, V., and Desnuelle, P. (1970b), *Biochim. Biophys. Acta 221*, 54.

Maylié, M. F., Charles, M., and Desnuelle, P. (1972), *Biochim. Biophys. Acta 276*, 162.

Nieuwenhuizen, W., Kunze, H., and Haas, G. H. de (1974), *Methods Enzymol.* (in press).

Pechère, J. F., Dixon, G. H., Maybury, R. H., and Neurath, H. (1958), *J. Biol. Chem. 233*, 1364.

Pieterson, W. A. (1973), Thesis, State University, Utrecht.

Pieterson, W. A., Vidal, J. C., Volwerk, J. J., and Haas, G. H. de (1974b), *Biochemistry 13*, 1455.

Pieterson, W. A., Volwerk, J. J., and Haas, G. H. de (1974a), *Biochemistry 13*, 1439.

Postema, N. M. (1968), Thesis, State University, Utrecht.

Saito, K., and Hanahan, D. J. (1962), *Biochemistry 1*, 521.

Salach, J. I., Seng, R., Tisdale, H., and Singer, T. P. (1971), *J. Biol. Chem. 246*, 340.

Sanger, F., and Tuppy, H. (1951), *Biochem. J. 49*, 463.

Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 3714.

Shaw, E. (1967), *Methods Enzymol. 11*, 677.

Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., and Vernon, C. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 39.

Sigman, D. S., Torchia, D. A., and Blout, E. R. (1969), *Biochemistry 8*, 4560.

Slotboom, A. J., Haas, G. H. de, Burbach-Westerhuis, G. J., and Deenen, L. L. M. van (1970), *Chem. Phys. Lipids 4*, 30.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Vogel, A. I. (1948), A Textbook of Practical Organic Chemistry, London, Longmans, Green and Co., pp 696, 835.

Wells, M. A. (1972), *Biochemistry 11*, 1030.

Wells, M. A. (1973), *Biochemistry 12*, 1086.