Application of the Temperature-Jump Technique to the Study of Phospholipid Dispersions[1]

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Abstract: Temperature-jump experiments performed on suspensions of phosphatidylserine vesicles (liposomes) reveal a relaxation process having a phospholipid concentration dependent relaxation time. The addition of calcium ions or cholesterol to the suspensions has little or no effect on the relaxation time. Furthermore, liposomes which have been osmotically shrunk in sucrose give rise to relaxation times which are identical, within experimental error, with those obtained with osmotically swollen liposomes. The relaxation time exhibits a dependence upon the length of the sonication time of the liposome stock solutions, and the rate is considerably reduced upon addition of polylysine to the suspensions. Although a definite mechanism cannot be established, a simple mechanism consistent with all of the data is a conformational change within liposome aggregates, with the rate of the change being dependent on the size of the aggregate.

Many of the major reactions of intermediary metabolism and macromolecular biosynthesis are catalyzed by enzymes which are attached to biological membranes. However, the study of reactions occurring in heterogeneous membrane systems is extremely difficult, both to carry out experimentally and to interpret. Phospholipid spherules (liposomes) provide appealing model membranes for the study of the dynamics of membrane-mediated processes. Liposomes are closed concentric vesicles of phospholipid bilayers possessing membrane-like properties.[3-5] Moreover, various polypeptides and proteins can be bound to the spherules[6,7] to form membrane-like vesicles.[6]

One of the principle factors contributing to the lack of study of heterogeneous membrane systems has been the difficulty of application of suitable physical probes. Ultrasonic attenuation measurements in suspensions of phospholipids have suggested rapid dynamic processes can occur within phospholipid vesicles.[8]

The temperature-jump technique has found wide application for the study of homogeneous enzymic reactions.[9,10] The present investigation was undertaken in an attempt to assess the usefulness of the temperature-jump method for studying the dynamics of processes occurring in suspensions of phospholipids and membranes. The results of this work indicate homogeneous temperature jumps can be applied to suspensions of liposomes. A relaxation process has been observed which is probably related to conformational changes within liposome aggregates.

Experimental Section

Materials. Phosphatidylserine from bovine brain and phosphatidylcholine from egg yolk were obtained and analyzed as previously described.[8] Phenol red indicator from Fisher Scientific Co. was recrystallized from water. Neutral red indicator from Eastman Organic Chemicals was used as obtained. All other reagents used in this study were of commercial analytical grade.

Preparation of Liposome Solutions. An aliquot of a 3:1 chloroform–methanol solution of phospholipid was placed in a round-bottomed flask. The solvent was evaporated on a rotatory evaporator, resulting in the formation of a thin film of phospholipid which covered the bottom of the flask. The film was taken up in an aliquot of 0.1 *M* KCl, the volume of the aliquot usually being such as to yield a solution 0.085% by weight of phospholipid (or 1.0×10^{-3} *M* in phospholipid assuming a molecular weight of 850). The flask was swirled to completely remove the film and the stock solution was sonicated in an ice bath under nitrogen using a 20-kc Heat systems sonifier. Except for the sonication time dependence study, sonication times were generally 15 min. Electron microscopy suggests that the unsonicated liposomes typically have a diameter of 500 Å–1 μ; the average diameter decreases to 300–400 Å and the liposomes become more uniform in size on sonication.[11] Stock solutions were prepared fresh before each experiment.

Mixed phospholipid–cholesterol dispersion were prepared by adding an aliquot of a chloroform–methanol solution of each to a round-bottomed flask and proceeding as described above.

Dispersions of indicator dye- or sucrose-containing liposomes were prepared by taking the thin film of phospholipid up in an aliquot of a 0.1 *M* KCl solution which contained the indicator or sucrose at the desired concentration.

Osmotically swollen liposomes were formed by diluting an aliquot of liposomes containing 0.1 *M* KCl and 0.05 *M* sucrose with 0.1 *M* KCl. Similarly, osmotically shrunken liposomes were formed by diluting an aliquot of liposomes containing only 0.1 *M* KCl with a solution of 0.1 *M* KCl, 0.1 *M* sucrose.

Solutions were prepared for the experimental measurements by pipetting together aliquots of phospholipid stock solution, indicator dye, and concentrated potassium chloride (sufficient to bring the final ionic strength to 0.1 *M*) in a volumetric flask. The pH was adjusted using very small volumes of concentrated HCl and NaOH. Solutions containing calcium were prepared by addition of concentrated $CaCl_2$.

Temperature-Jump Measurements. The temperature-jump relaxation spectrum of each dispersion was measured at 25° using procedures and equipment described previously.[12,13] The photographs of oscilloscope traces were analyzed by means of a curve tracer interfaced with an IBM 1800 digital computer. The relaxation time was calculated from a least-squares analysis of the logarithm of the signal amplitude *vs.* time.

Results

Since phospholipid dispersions do not have appreciable light absorption except that due to light

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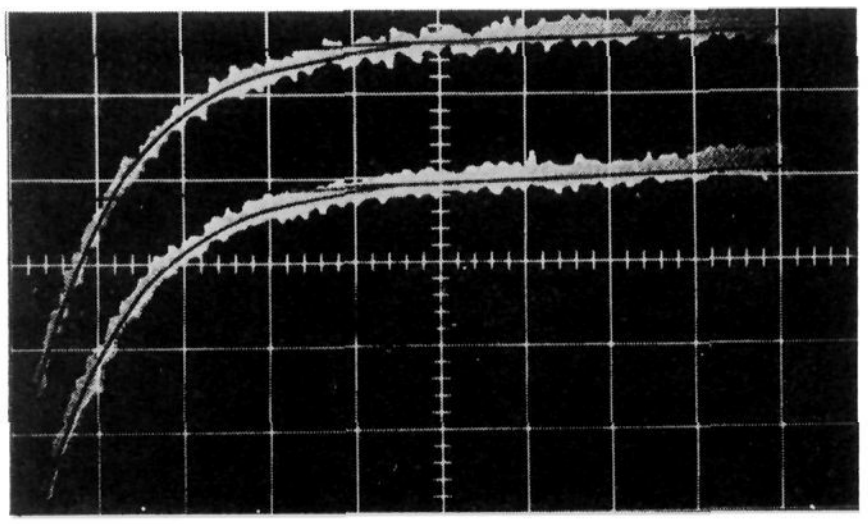


Figure 1. Typical relaxation traces for the PS–phenol red system. The horizontal scale is 1 msec/large division and the vertical scale is in arbitrary units of absorbancy. Experimental conditions: 1.0×10^{-4} *M* PS, 2.0×10^{-5} *M* phenol red, 0.1 *M* KCl, pH 7.5, 25°.

scattering, the pH indicator dye phenol red was used to monitor the relaxation of the dispersions spectrophotometrically. Solutions containing 1×10^{-5} *M* to 9×10^{-4} *M* phosphatidylserine (PS) and 2.0×10^{-5} *M* phenol red at an ionic strength of 0.1 *M* and a pH of 7.5 exhibited one and possibly two relaxation effects. The slower of the two effects was small in amplitude and not very reproducible; it may be an artifact due to convection occurring in the temperature-jump cell at times longer than a few hundred milliseconds. The faster effect, however, was large in amplitude and was relatively reproducible for solutions prepared from the same stock solution of PS (Figure 1). Only the faster effect could be studied in detail.

As expected blank solutions lacking only PS or indicator exhibited no relaxation effects. Furthermore, the effect observed with PS and indicator vanishes in solutions buffered at pH 7.5 with 0.01 *M* Tris buffer.

For large variations in indicator concentration the relaxation time remained essentially constant (Table I).

Table I. Dependence of Relaxation Time on Indicator Dye[a]

10^5 PS, *M*	10^{-5} indicator, *M*	$1/\tau$, sec^{-1}
5	1 (PR)	687 ± 88
5	20 (PR)	821 ± 140
5	2 (PR[b])	841 ± 105
5	2 (PR[c])	653 ± 115
10	2 (PR)	1130 ± 190
10	10 (PR)	1580 ± 130
10	2 (NR)	1750 ± 260

[a] μ = 0.10 *M*, pH 7.5, 25°; PR = phenol red, NR = neutral red. [b] Indicator added to exterior of liposomes. [c] Indicator trapped inside of liposomes.

In addition, PS liposomes containing trapped phenol red exhibited relaxation times identical, within experimental error, with PS liposomes to which phenol red was added externally (Table I). Preparation of PS liposomes containing phenol red followed by isolation of the dye-containing liposomes from the dye in the external media on G-25 Sephadex indicated that the diffusion of the dye across the bilayers of a liposome of PS is relatively slow, requiring a few hours for equilibration.

Experiments performed with neutral red, an indicator whose acid–base color change is the reverse of that for

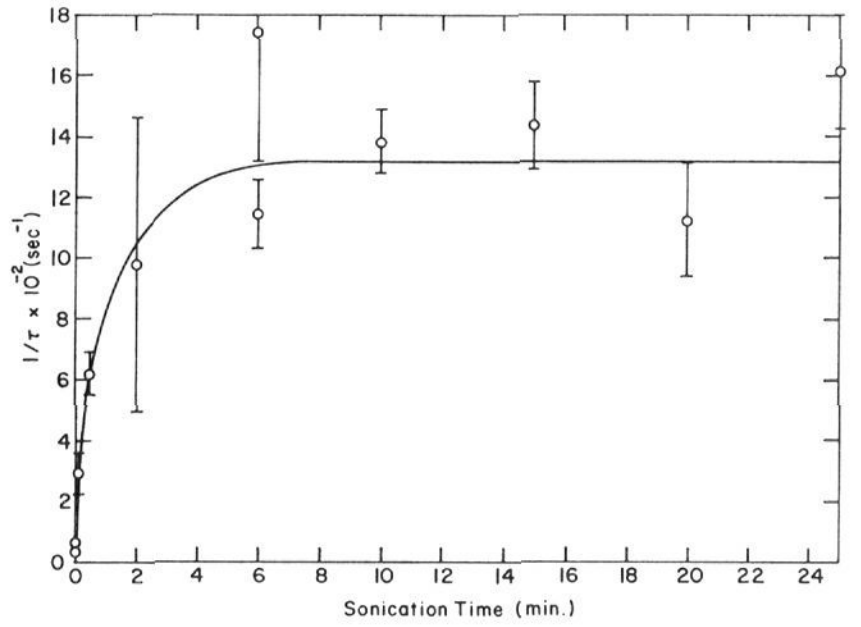


Figure 2. Dependence of the reciprocal relaxation time on sonication time. Each point was prepared from a separate stock solution which had been sonicated the indicated length of time. Experimental conditions: 1.0×10^{-4} *M* PS, 2.0×10^{-5} *M* phenol red, 0.10 *M* KCl, pH 7.5, 25°.

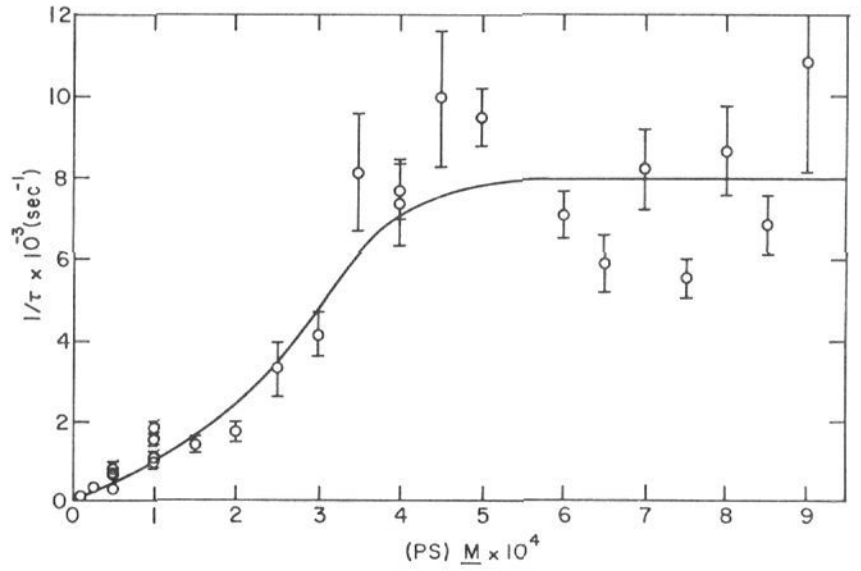


Figure 3. A plot of $1/\tau$ *vs.* concentration of phosphatidyl-L-serine: 2.0×10^{-5} *M* phenol red, 0.10 *M* KCl, pH 7.5, 25°. The solid line drawn through the points has no theoretical significance.

phenol red, revealed a relaxation effect, opposite in amplitude to that of phenol red, with a very similar relaxation time (Table I). A faster effect of low amplitude was also observed with neutral red and is attributed to a dye–liposome interaction. Increasing the concentration of neutral red resulted in the formation of a red precipitate believed to be a dye–liposome complex. Solutions without PS, but otherwise identical in composition, did not yield precipitates. Similarly, no precipitates were observed in solutions of PS and phenol red.

The relaxation time of PS suspensions was observed to depend upon the length of the sonication time of the PS stock solution in the manner shown in Figure 2. The reciprocal relaxation time increases and eventually levels off as the sonication time is increased. Each point of Figure 2 was prepared from a separate stock solution which had been sonicated the indicated length of time.

The dependence of the relaxation time on PS concentration is shown in Figure 3. The solid line drawn through the points has no theoretical significance and is merely used to emphasize the trend in the data. The

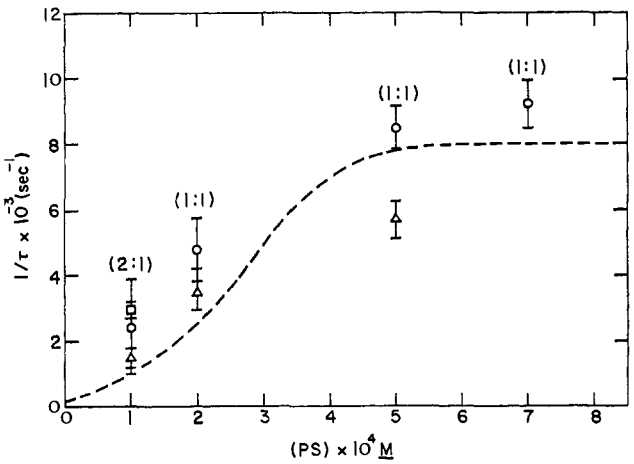


Figure 4. The effect of calcium ion and cholesterol on the reciprocal relaxation time. The dashed line indicates the position of the solid line of Figure 3: ○, cholesterol (ratio in parentheses is molar ratio of PS:cholesterol); △, 2:1 PS:Ca (molar ratio); □, 1:2 PS:Ca (molar ratio).

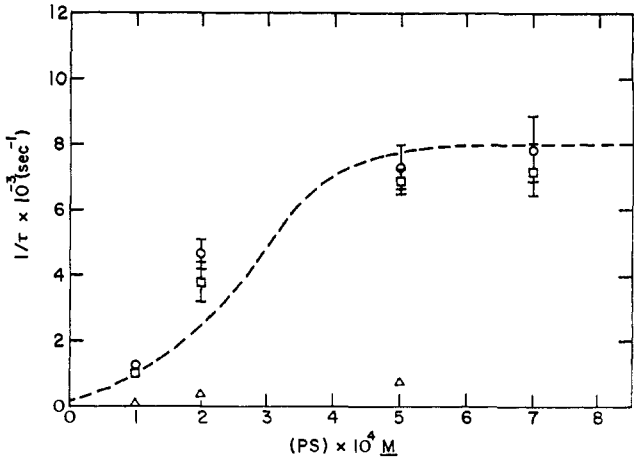


Figure 5. The effect of osmotic swelling and shrinking and the addition of polylysine on the reciprocal relaxation time. The dashed line indicates the position of the solid line of Figure 3: ○, shrunk in sucrose; □, swollen in sucrose; △, PS:polylysine (2.5:1) molar ratio).

scatter in the data appears to arise primarily from inherent irreproducibilities in the preparation of stock solutions. The lipid is not entirely pure and air oxidation undoubtedly occurs. The error bars reflect the uncertainty in the relaxation time from several measurements on a given solution. Maintenance of the pH at 7.5 in unbuffered solution proved to be difficult and frequent readjustments of pH were made between measurements. Qualitatively, the rate increases as the pH increases. Some pH drift in the temperature-jump apparatus was unavoidable; the error bars also reflect the uncertainty introduced by these pH changes. In spite of the scatter, an obvious trend is observed. The reciprocal relaxation time increases with PS concentration and eventually levels off at a value independent of the PS concentration. The suspensions were becoming visibly turbid at concentrations of approximately 9×10^{-4} *M* PS, and data could not be obtained at higher PS concentrations.

The relaxation times of dispersions containing cholesterol and PS were not dramatically different from those of PS alone (Figure 4). Similarly, the addition of calcium to PS dispersions had little apparent effect on the relaxation time (Figure 4).

Relaxation measurements made on suspensions of liposomes which had been osmotically shrunk in sucrose yielded relaxation times which are identical, within experimental error, with those obtained from measurements on suspensions of liposomes which had been osmotically swollen in sucrose (Figure 5).

The addition of polylysine to a dispersion of PS gave rise to considerably slower relaxation times (Figure 5). The data of Figures 4 and 5 display the same behavior as the data of Figure 3: an increase in the reciprocal relaxation time with increasing PS concentration followed by a leveling off at a value independent of the PS concentration.

Discussion

The relaxation processes observed in this study clearly are associated with the phospholipid since control experiments in which phospholipid is omitted do not exhibit these effects. Suspensions of phosphatidylcholine did not exhibit relaxation effects under the conditions of this study. However, this is not totally unexpected since phosphatidylserine has a net negative charge, and in addition has both a carboxyl and an amino group which are undoubtedly serving as indicators of the relaxation process. The lack of an observable relaxation process with phosphatidylcholine may be due to a problem of detection rather than to the absence of chemical relaxation. Although the ionizable group (or groups) involved in the relaxation process cannot be identified at the present time, the rates of the ionization processes are certainly too rapid to account for the observed relaxation process. The mechanistic possibilities can be divided into the following categories: indicator–liposome interactions, liposomal aggregation, transport of diffusion of some species across one or more bilayers of a liposome, intraliposomal conformational changes, and liposome aggregate conformational changes. Each of these possibilities will now be examined in light of the experimental evidence.

The observed effect is probably not due to a dye–liposome interaction for several reasons. Firstly, the observed changes in the absorbance of the solutions result from the dye functioning as a pH indicator as evidenced by the fact that no effect is observed in buffered solutions. Hence, the observed absorbance change is not due to a chromophoric shift in the absorption spectrum of the dye resulting from binding to a liposome. This, of course, does not preclude the possibility that a specific liposome–dye interaction occurs, but it seems unlikely. Secondly, a dye–liposome interaction should exhibit a relaxation time which is dependent upon dye concentration. Experimentally, no such dependence is observed. Thirdly, one would expect the rate of a dye–liposome interaction to be dependent on the structure and charge of the dye involved. However, essentially the same relaxation time is obtained with both neutral red (which is either positively charged or neutral) and phenol red (which is either singly or doubly negatively charged). Finally, within experimental error, identical relaxation times are obtained whether the indicator dye is added to the exterior

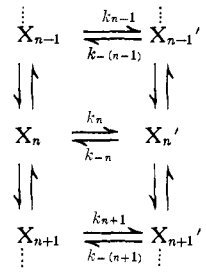
of the liposome, is trapped within the liposome, or is equilibrated between the inside and outside of the liposome. Thus, a dye–lipsome interaction is probably not responsible for the observed relaxation effects.

Some degree of aggregation undoubtedly occurs in suspensions of liposomes. Assuming an average liposome is composed of a thousand phospholipid molecules (certainly a lower limit),[14] the liposome concentrations of the experiments vary an order of magnitude on either side of 10^{-7} M. A relaxation time of 1 msec would then imply a minimum second-order rate constant for aggregation of 10^{10} M^{-1} sec^{-1}. Since the PS liposomes diffuse relatively slowly and are negatively charged, this value is unreasonably large. Moreover, the concentration dependence of the relaxation time is inconsistent with second- or higher order reactions occurring.

Extensive studies of the diffusion of molecules and ions across the bilayers of phospholipid spherules have shown that diffusion rates can be markedly altered by the addition of various agents such as calcium ion or cholesterol.[3,15] In particular, the addition of cholesterol generally tightens the liposome structure by "solidifying" the hydrocarbon tails[16] and/or diluting the surface charge resulting in a decreased permeability of the bilayers.[3,15] Experimentally, the addition of calcium or cholesterol has little or no effect on the observed relaxation rate. Furthermore, osmotically shrunken liposomes in which the phospholipid molecules are more tightly packed might be expected to exhibit permeabilities different from those of osmotically swollen liposomes in which the phospholipid molecules are more loosely packed. However, the relaxation times for osmotically shrunken and swollen liposomes are identical. Therefore, transport or diffusion across bilayers is probably not responsible for the observed relaxation process.

An intraliposomal conformational change is not consistent with the observed dependence of the relaxation rate on PS concentration. Since each solution was prepared by diluting similar stock solutions, the average size of the liposomes in each solution is approximately the same, while the concentration of liposomes varies as a function of the PS concentration. Hence, a conformational change involving individual liposomes should exhibit a concentration-independent rate. Furthermore, one might expect cholesterol addition or osmotic distortion of the liposomes to influence the rate of an intraliposomal conformational change, but no such influence is found.

The observed relaxation effects could arise from changes in conformation of aggregates of liposomes. If the rate of the conformational change depends upon the extent of aggregation, then the observed concentration dependence of the relaxation rate could be explained. Such a reaction scheme is depicted schematically below. X_n represents an aggregate of n liposomes, X_n' represents an aggregate of n liposomes in a conformation different from that of X_n, and the k's are rate constants describing the rates of the conformational changes. As discussed previously, relaxation times for the vertical steps (aggregation) should be considerably slower than the observed relaxation times, which are attributed to the conformational changes (horizontal steps). Indeed, as already stated, slower low-amplitude effects may have been observed, but were not reproducible and were so slow as to be at the limit of reliable data obtainable from the temperature-jump equipment used. Since the horizontal steps of the reaction scheme are rapid compared to the vertical steps, one might expect to observe a spectrum of relaxation times, one relaxation time for each horizontal step, or conformational change occurring in solution. The experimental observation of only one apparent relaxation effect probably reflects a relatively sharp distribution of aggregate sizes. The concentration independence of the relaxation rate at high phospholipid concentrations may be attributed to the existence of an upper limit to the size of liposome aggregates which are stable under the conditions of this study. Alternatively, if the average environment of the liposomes within the aggregate is important in determining the rate of change of conformation of the aggregate, then the average environment of liposones in large aggregates may become independent of the aggregate size.



The effect of sonication of the liposome stock solutions is to reduce the average size of the liposomes and increase the surface area and possibly the degree of aggregation. Thus, the observed increase in reciprocal relaxation time with sonication time is not inconsistent with an aggregate-dependent conformational change. Calcuim, cholesterol, and osmotic shrinking and swelling apparently have little effect on aggregation. Note that although the size of the liposome changes both on sonication and on osmotic shrinking and swelling, in the former case the number of phospholipid molecules per liposome is also affected. Therefore, identical behavior with these two treatments would not be expected.

An interpretation of the observed decrease in relaxation rate upon the addition of polylysine to PS suspensions is not immediately apparent. Polylysine binds to PS liposomes through ionic and hydrophobic interactions and results in the formation of aggregates of liposomes having a decreased number of bilayers.[6] Polylysine is positively charged at the pH of this investigation and considerable neutralization of the negative charge of the PS occurs. Furthermore, very large aggregates are formed.[6]

In conclusion, a mechanism involving an aggregational conformational change appears to be most consistent with the available experimental evidence. While this cannot be regarded as a unique explanation of the data, a number of other mechanisms are clearly

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inconsistent with the experimental results. In any event, this study indicates that temperature-jump relaxation studies on heterogeneous dispersions of phospholipids are possible and is indicative of the feasibility of applying this technique to the study of the dynamics of membrane-medicated processes.

A Search for an Intermediate in Carboxypeptidase A Catalyzed Ester Hydrolyses

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Abstract: The kinetics of the carboxypeptidase A catalyzed hydrolyses of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate (I) and *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate (II) have been studied under substrate in excess and enzyme in excess conditions in an attempt to obtain evidence concerning the possible formation of covalent enzyme–substrate complexes as intermediates in these reactions. Stopped-flow kinetic measurements with I at high enzyme and substrate concentrations failed to reveal the accumulation of any detectable concentration of an intermediate differing significantly in its ultraviolet–visible absorption spectrum from a spectrum of a mixture of the substrates or of the products. Furthermore, a comparison of the k_{cat} values for I and II indicates that the breakdown of an acyl enzyme, *trans*-*p*-nitrocinnamoyl-carboxypeptidase A, cannot be rate limiting in the hydrolysis of II. Also the k_{cat} value is increased and the K_M value is decreased for the reaction of I relative to the corresponding kinetic parameters for the unsubstituted ester, *O*-(*trans*-cinnamoyl)-L-β-phenyllactate. The lack of evidence for the transient formation of covalent enzyme–substrate complexes in the hydrolyses of the *p*-nitrocinnamoyl esters is not surprising when two of the reasonable mechanisms for carboxypeptidase action which involve the catalytic participation of glutamate 270 in the enzyme are considered. The hypothetical intermediate in one mechanism would be a tetrahedral adduct formed by the attack of water on the carbonyl group of the ester assisted by the participation of glutamate 270 as a general base catalyst and the zinc ion at the active site as an electrophilic catalyst. In the other proposed mechanism the hypothetical intermediate might be an acid anhydride formed from the attack of glutamate 270 acting as a nucleophile on the carbonyl group of the ester. In either of the mechanisms considered the intermediates formed would be expected to be very labile indeed.

Bovine pancreatic carboxypeptidase A (CPA) is a zinc-containing metalloenzyme with a molecular weight of approximately 34,000 which catalyzes the hydrolyses of polypeptides at the C-terminal peptide bond, especially if the terminal residue contains an aromatic group. CPA also catalyzes the hydrolysis of the analogous types of ester substrates, *O*-acyl derivatives of α-hydroxy acids.[4-11] This paper is concerned with the esterase action of CPA.

In many cases the "turnover" kinetics observed for the CPA-catalyzed hydrolysis of ester substrates has been complicated by competitive product inhibition,[4,7-9] substrate inhibition,[8] and substrate activation.[6] Much of the previous work with ester substrates has been concerned with describing these effects. In the present work an attempt has been made to probe the mechanism of action of CPA by trying to identify the individual steps of the catalytic reaction.

The existence of acyl-enzyme intermediates has been suggested for CPA-catalyzed reactions, although there is, as yet, no experimental evidence for such species.[12] It might be mentioned that a covalent intermediate has been detected for another zinc metalloenzyme, *viz. E. coli* alkaline phosphatase.[13] These factors led us to conduct ester hydrolysis experiments with CPA under conditions that would favor the detection of any intermediate(s). Accordingly, we employed high enzyme and substrate concentrations in our study. Further, we utilized substrates which had low K_M values and which contained suitable chromophores. Finally, we made use of rapid reaction techniques.

Previous work in this laboratory had made use of cinnamoyl esters which have an absorption maximum in the vicinity of 280 mμ.[10] These esters were unsuitable for studies employing high enzyme concentrations since CPA has a very strong absorption band in this

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