Physicochemical Studies of Acetylcarboxypeptidases. I. *p*H Dependence of Structural Integrity*

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Acetylation of carboxypeptidase with either acetic anhydride or acetylimidazole increases esterase activity and abolishes peptidase activity (J. F. Riordan and B. L. Vallee, *Biochemistry 2*, 1460 [1963]; R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry 2*, 616 [1963]). The structural stability of these enzymes at different *p*H values was examined by optical rotatory dispersion, free-boundary electrophoresis, and esterase activity. The increase in esterase and decrease in peptidase activities of carboxypeptidase acetylated either with acetic anhydride or acetylimidazole are not accompanied by changes in conformation of the enzyme detectable by techniques presently available for this purpose. Such changes as could be detected in the enzyme modified with acetic anhydride could be shown to be owing to denaturation of a fraction of the total population of modified enzyme molecules which were virtually inactive enzymatically and which could be separated from the active fraction by electrophoresis. This effect was barely detectable with acetylimidazole. If conformational changes do occur as a result of acetylation the alterations of enzymatic activity are at present their only detectable manifestation.

The chemical modification of enzymes by site-specific selective reagents is employed widely to identify catalytically active side chains of proteins; it has become an increasingly powerful procedure in studies of the chemical basis of biological specificity. Frequently the involvement of a particular amino acid residue in catalysis has been inferred from losses in enzymatic activity as measured subsequent to modification. In such instances, however, it is not always clear whether loss of function is the *direct* consequence of specific modification of the primary structure at the active center[1] of the enzyme or the *indirect* result of nonspecific changes in secondary or tertiary structure. Acetylcarboxypeptidases, which exhibit striking *increases* in enzymatic activity as a consequence of acetylation of two tyrosine residues (Vallee *et al.*, 1963; Simpson *et al.*, 1963; Riordan and Vallee, 1963), provide unusual opportunities to differentiate between these alternatives. Such a differentiation is possible in this case through the simultaneous application of standard enzymological and physicochemical techniques. The data here presented show that the primary structure of the protein can be modified to yield increased catalytic activity without there being demonstrable changes in secondary or tertiary structure. A preliminary report has been rendered (Bethune and Ulmer, 1963) and an account of the sedimentation properties is in preparation.

Materials and Methods

Beef-pancreas carboxypeptidase A, prepared by the method of Anson (1937) (Worthington Biochemical Corp., Freehold, N. J.) was washed three times with water and dissolved in 2 M NaCl–0.02 M Veronal, *p*H 7.5, prior to use. The chemicals employed in these experiments were of reagent grade and were used without further purification with the exception of acetic anhydride, which was redistilled prior to use. *N*-Acetylimidazole was prepared according to Boyer (1952).

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as described previously (Coleman and Vallee, 1960), and is expressed as an apparent proteolytic coefficient, *C*, defined as log a_0/a per minute per μmole of enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively (Simpson *et al.*, 1963). The assays were carried out at 0° in 0.02 M sodium Veronal, 1 M NaCl buffer, *p*H 7.5; *C* was calculated from the linear portion of the first-order plots observed when hydrolysis did not exceed 15%.

Esterase activity was determined with hippuryl-DL-β-phenyllactate as the substrate, as described previously (Coleman and Vallee, 1961). Assays were performed at 25° with 5 ml of 0.01 M substrate in 0.2 M NaCl–0.005 M Tris-HCl, *p*H 7.5. Activity, expressed as a zero-order velocity constant, *k*, with units of moles of H^+ per minute per mole of enzyme, was measured by titration of the hydronium ions released on hydrolysis of the substrate with 0.1 M NaOH using a *p*H-stat titrator (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). *Free amino groups* were determined by reaction of the protein with ninhydrin (Moore and Stein, 1948; Slobodian *et al.*, 1962), using phenylalanine as a standard.

The stability of the enzyme protein was examined, using esterase activity as a criterion, in the following manner. The native or modified enzymes were dialyzed at discrete *p*H values for 18 hours against buffers adjusted to the desired *p*H. Changes in conformation or structure owing to alterations in the stability of the protein, as a function of *p*H, should become apparent over that period of time. Subsequent to the dialysis the enzyme was assayed immediately for activity under the standard conditions at *p*H 7.5. This assay would be expected to reflect the effect of *p*H on protein stability as opposed to its effect upon the hydrolytic

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[1] The designation "active center" refers to all those features of primary, secondary, and tertiary structure of the enzyme which are required for substrate binding, specificity, and hydrolysis of the substrate. In carboxypeptidase one component of the active center, the essential zinc atom together with its ligands, is referred to as the "active site."

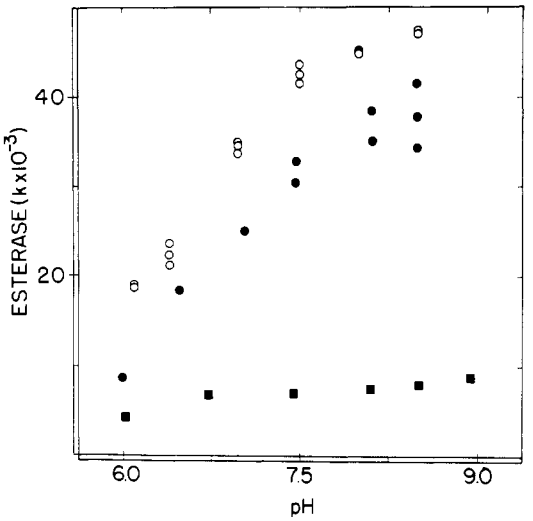

FIG. 1.—Esterase *p*H-rate profiles for native (■), Ac_A-carboxypeptidase (●), and Ac_I-carboxypeptidase, (O). Activities were measured at the *p*H indicated on the abscissa using the substrate hippuryl-DL-β-phenyllactic acid as described under Materials and Methods. At *p*H 8.5, for Ac_I-carboxypeptidase, there are three determinations, two of which coincide.

process. We shall refer to the resultant graphic presentation of these phenomena as *p*H-*stability profiles.*

*p*H-*Rate profiles* of esterase activity were determined for the enzymes dissolved in 0.005 M citrate–0.005 M Veronal–1 M sodium chloride, *p*H 8.0. The substrate was adjusted to the *p*H desired with 1 M sodium hydroxide or 1 M hydrochloric acid just prior to addition of the enzyme. The activity was determined as described above.

Protein concentrations were determined from the absorbance at 278 mμ, using the known molar absorptivity for Ac_A-carboxypeptidase[2] of 5.78×10^4 mole^{-1} cm^{-1} and for Ac_I-carboxypeptidase of 5.9×10^4 mole^{-1} cm^{-1} (Riordan and Vallee, 1963; Simpson *et al.*, 1963). A Beckman Model DU spectrophotometer was used throughout.

Modification of the enzyme was carried out by addition of acetic anhydride at a ratio of 48 moles/mole of enzyme or with acetylimidazole at a 60 moles/mole ratio, in 2 M sodium chloride–0.02 M Veronal, *p*H 7.5 (Riordan and Vallee, 1963; Simpson *et al.*, 1963). The *p*H was maintained at 7.5 in a *p*H-stat by addition of 1 N sodium hydroxide. Acetylation with the anhydride was carried out at 0°, and with acetylimidazole at 20°, for 30 minutes, after which no further base uptake occurred. The modified enzymes were then dialyzed, with three changes of buffer, to the *p*H and buffer composition desired.

Electrophoresis in a Spinco Model H electrophoresis and diffusion apparatus was carried out at 1°, at protein concentrations of from 0.2 to 1 g/dl, and in a field of 1–3 v/cm.

Optical rotatory dispersion was measured by means of a Model 200S–80Q photoelectric spectropolarimeter with an oscillating polarizer prism (O. C. Rudolph and Sons). Either a high-intensity, high-pressure mercury lamp (A–H6, General Electric Co.) or a 450 watt Osram xenon lamp (O. C. Rudolph and Sons) served as light source. Stability of the mercury lamp was improved by circulating cooled, deionized water through the quartz-jacketed lamp housing.

[2] The prefixes Ac_A- and Ac_I- are used to designate carboxypeptidase acetylated with acetic anhydride and acetylimidazole, respectively.

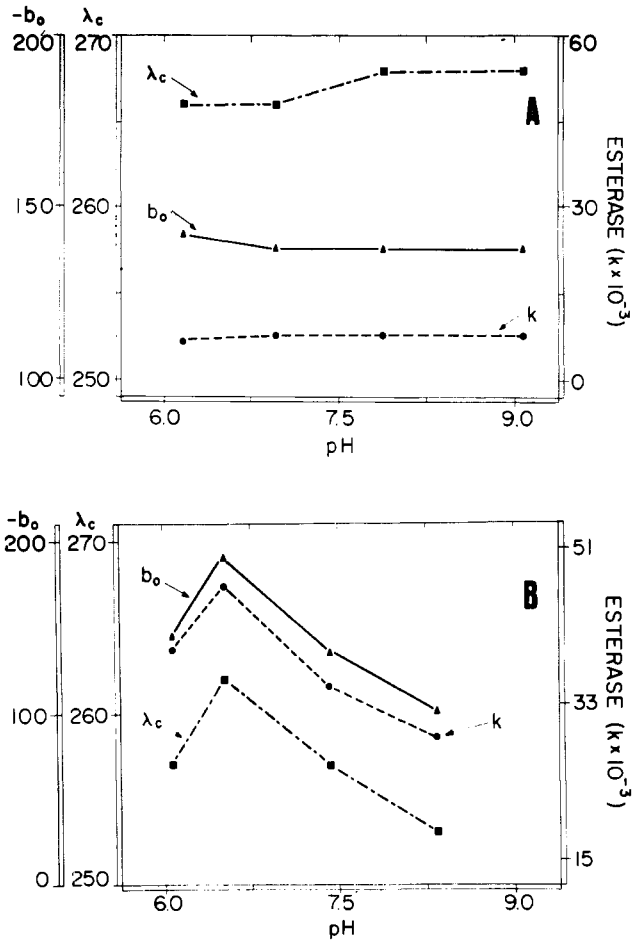

FIG. 2.—Effect of *p*H of incubation on properties of native and Ac_A-carboxypeptidase. (A) Effect on esterase activity (●), b_0 (▲), and λ_c (■) of native carboxypeptidase. The following buffers were employed: *p*H 6.0, 0.1 M acetate–0.5 M NaCl; *p*H 6.5, 0.1 M cacodylate–0.5 M NaCl; *p*H 7–9, 0.02 M Veronal–0.5 M NaCl. (B) Effect of *p*H of incubation on esterase activity (●), b_0 (▲), and λ_c (■) of Ac_A-carboxypeptidase. The buffers used were as in (A), with 0.1 M NaCl, which could be used to reduce convective disturbances in electrophoresis since the acetylated enzyme is more soluble than the native enzyme.

Maximal intensity of illumination was achieved by adjusting the lamp position until the highest photometer response was reached in the wavelength region under study. Most measurements of rotation were performed in 5-cm semimicro polarimeter cells with fused-quartz end plates (O. C. Rudolph and Sons). The temperature of the protein solution was maintained at 10 ± 1° by circulating cooled water through the polarimeter housing from an external bath. Rotational angles were measured by the method of symmetrical angles (Rudolph, 1955) with the instrument adjustment maintained at 5° throughout, while the monochromator slit-width and photometer sensitivity gain were varied to control light intensity. At each wavelength nearly identical slit-widths were employed for the sample and its blank, and it was possible to restrict them to less than 0.15 mm in all instances. Specific rotations were calculated on the basis of protein concentration and are precise to about ±1.0. The dispersion constant, λ_c, was calculated as suggested by Yang and Doty (1957) and b_0 from the Moffitt equation, assuming a λ_c value of 212 mμ (Blout, 1960). Absolute values for specific rotation in the regions of high absorbance of radiation were confirmed at two or more protein concentrations (Urnes and Doty, 1961).

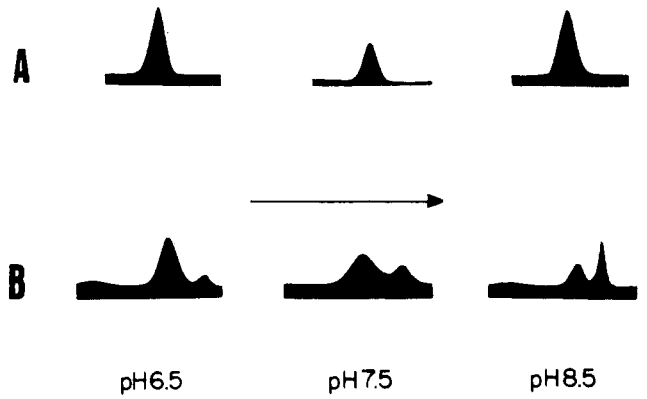

FIG. 3.—Electrophoretic patterns of native and Ac_A-carboxypeptidase at different *p*H values. (A) Native carboxypeptidase after 20 hours at 1.1 v/cm in buffers given in Fig. 2A. The anode is on the right and the direction of motion is as indicated. (B) Ac_A-carboxypeptidase after 15 hours at 2–3 v/cm in buffers as given in Fig. 2B. The area distribution, at pH 7.5, is the same if 0.5 M NaCl is used.

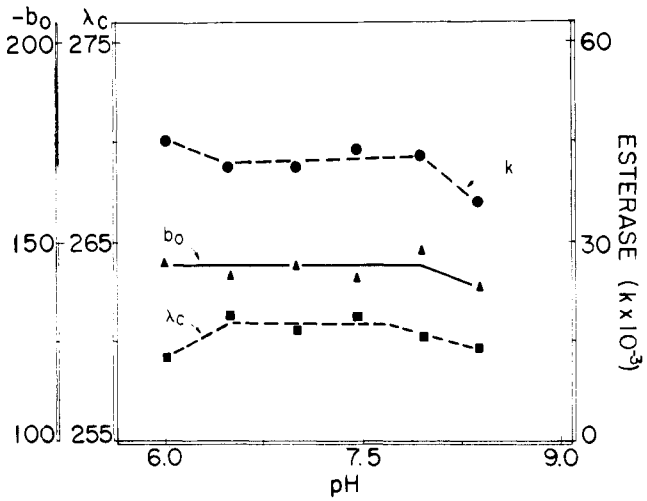

FIG. 4.—Effect of *p*H of incubation on esterase activity (●), b_0 (▲), and λ_c (■) for Ac_I-carboxypeptidase. The buffers used were as in Fig. 2A. The dispersion constants for different enzyme preparations range from 261 to 268 mμ.

RESULTS

The changes in *p*H dependence of the hydrolytic process subsequent to acetylation have been discussed (Riordan and Vallee, 1963), and we have previously reported the *p*H-*rate profiles* of the esterase activities of native and Ac_A-carboxypeptidase (Vallee *et al.*, 1963). In conjunction with other data these kinetic results bear upon the mechanism of action of these enzymes (Vallee *et al.*, 1963). The *p*H-rate profiles are here shown to allow for direct comparison with the *p*H *stability profiles* (Fig. 1). The *p*H-rate profile of native carboxypeptidase is highly reproducible, but that of Ac_A-carboxypeptidase is not, as indicated by the scatter of the pertinent experimental points above *p*H 8. Acetylation with acetic anhydride renders the protein sensitive to a *p*H-dependent alteration which is not encountered in the native enzyme. The stability of both enzymes was therefore investigated as a function of *p*H.

The dispersion constants, λ_c, the b_0 values, and the *p*H-stability profiles of the esterase activities of the two enzymes are compared in Figure 2. Prolonged dialysis against buffers adjusted to *p*H values between 6 and 9 does not affect the optical-rotatory or catalytic properties of the native enzyme (Fig. 2A). Further, only one boundary is seen when electrophoresis of the native enzyme is carried out at *p*H 6.5, 7.5, or 8.5 (Fig. 3A).

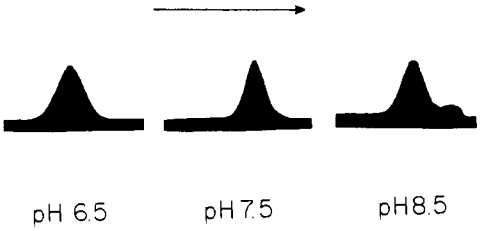

FIG. 5.—Electrophoretic patterns of Ac_I-carboxypeptidase after 20 hours at 1–2 v/cm in the buffers of Fig. 2A. The anode is on the right and the direction of motion is as indicated.

In marked contrast, the b_0 and λ_c values, as well as the esterase activity, of Ac_A-carboxypeptidase vary with the *p*H at which incubation is performed (Fig. 2B). The values of all these parameters rise from *p*H 6 to reach a maximum at *p*H 6.5 and then fall linearly to *p*H 9. Electrophoresis performed at the same *p*H values as for the native enzyme now results in two boundaries (Fig. 3B). The relative area under the faster-moving boundary increases with the *p*H of incubation.

At the conclusion of each run, both boundaries were sampled. The two species differed markedly in esterase activity. After electrophoresis at *p*H 8.5, for example, the faster boundary gave an esterase activity of 3×10^3 moles/mole $min.^{-1}$, much lower even than that for the native enzyme. The slower boundary, on the other hand, contained material with an activity of 42×10^3 moles/mole/min^{-1}, which was much higher than that of the sample after acetylation but before electrophoresis, 24×10^3 moles/mole/min^{-1}. It will be recalled that the esterase activity of native carboxypeptidase is 6–7 $\times 10^3$ moles/mole/min^{-1}.

The lack of reproducibility of the esterase rate profile for Ac_A-carboxypeptidase at high *p*H, the lowered catalytic activity and stability of this modified enzyme under these conditions, and the alteration of the dispersion values in the direction encountered on denaturation all correlated with the appearance of an enzymatically inactive species of Ac_A-carboxypeptidase. Moreover, as the *p*H of incubation increases, an increasing proportion of the chemically modified enzyme is transformed into inactive material, demonstrating the hydrogen-ion dependence of this destabilization.

It is of interest to know whether the induced *p*H instability is a result of modification of tyrosine residues or of modification of other residues which are attacked by acetic anhydride. Acetylimidazole, a milder and more selective acetylating agent than acetic anhydride when employed under the conditions of Simpson *et al.* (1963), was used to investigate this question. At all *p*H values, the esterase activities of Ac_I-carboxypeptidase are higher than the comparable ones of Ac_A-carboxypeptidase.

Prolonged dialysis against buffers adjusted to *p*H values between 6 and 8 affects neither the optical rotatory dispersion nor the catalytic properties of Ac_I-carboxypeptidase (Fig. 4). All the parameters decrease very slightly at *p*H 8.5, and in accord with this, upon electrophoresis a very small, fast boundary composed of inactive material appears at this, but *not* at lower *p*H (Fig. 5). According to these criteria, Ac_I-carboxypeptidase is much more stable than Ac_A-carboxypeptidase. This circumstance is also reflected in the

TABLE I
OPTICAL ROTATORY DISPERSION PARAMETERS FOR NATIVE, Ac_I-, and Ac_A-CARBOXYPEPTIDASES AT *p*H 7.5.

	Native	Ac_I	Ac_A
$[\alpha]_{546}^{10}$	−26	−26	−32
λ_c[a]	266	266	257
b_0	−144	−144	−135

[a] Values for the dispersion constant at *p*H 7.5 are the average of separate determinations on three or more enzyme samples. The range for λ_c for different enzyme preparations varies from 261 to 268 mμ.

reproducibility of the *p*H-rate profile (Fig. 1). The average values of the relevant optical-rotatory parameters for these enzymes, at *p*H 7.5, are given in Table I.

DISCUSSION

It is now generally agreed that the catalytic activity of enzymes resides in an area of the protein which is small when compared to its total surface. Side chains of certain amino acids participate both in catalytic activity and substrate binding (Koshland, 1960). The whole area in contact with the substrates has been designated the "active center." The designation "active site" in carboxypeptidase has been reserved for those residues known to be involved directly in the catalytic process (Vallee *et al.*, 1963). In most enzymes studied so far these amino acid residues are widely separated in the sequence of the primary structure of the protein. They are apparently brought into the biologically active, three-dimensional configuration through specific folding of the primary chain which is stabilized by forces other than peptide bonds.

Chemical modifications of functional residues, critical to substrate binding, catalytic activity, or both, generally completely abolish enzymatic activity (Koshland, 1960). Many of these reagents, or the conditions under which they must be employed, may alter secondary or tertiary protein structure nonspecifically in addition to their site-specific and selective action (Fraenkel-Conrat, 1957). Since both specific and nonspecific interactions cause the same functional consequences, i.e., the abolition of activity, their individual contributions are not easily assessed (Li *et al.*, 1962). Modification of a specific functional residue, however, should change a critical step in the mechanism of the catalytic process, which in some instances may, in fact, then manifest itself as *increased* catalytic activity. This accelerated catalytic rate may be distinguished, of course, from the functionally destructive alterations of secondary and tertiary structure, since under such circumstances the critical, three-dimensional constellation of functional groups comprising the active center may be presumed not to have been destroyed. The joint study of the kinetics and physicochemical parameters of such a system should permit the resolution of the effects on primary from those on secondary or tertiary structure.

The acetylcarboxypeptidases appeared to exhibit the appropriate characteristics for the experimental examination of these propositions. Acetylation of two tyrosyl residues of the active center constituted the requisite modification of primary structure which increases esterase activity 7- to 8-fold. Optical rotatory dispersion served as the criterion of conformational changes, and electrophoresis served to separate material with increased biological activity which had not undergone conformational alteration from that which lost activity on the basis of structural changes.

The prolonged incubation of the enzyme at a given *p*H, followed by determination of activity under standard conditions at *p*H 7.5, separates the effects of structural changes from the specific dependence of the hydrolytic process on hydrogen-ion concentration in all instances.

The *p*H stability of Ac_A-carboxypeptidase differs from that of the native enzyme (Fig. 2). Stability is maximal at *p*H 6.5 where the esterase activity is 48×10^3 moles/mole/min^{-1} (Fig. 2B). The slightly decreased catalytic activity of the enzyme prepared at *p*H 6.0 may be owing to loss of zinc during prolonged dialysis of the acetyl protein at this *p*H. Activity falls to 28×10^3 moles/mole/min^{-1} at *p*H 8. The optical rotatory parameter b_0 reflects these changes; it changes from −198 at *p*H 6.5 to −105 at *p*H 8, while λ_0 decreases from 262 to 253°. On the basis of currently accepted views these changes, though minor, might imply denaturation. As to their origin, the spectropolarimetric data cannot distinguish between a small conformational change in *all* the molecules or, alternatively, a large change in but a small fraction of the total population. The intrinsic features of electrophoresis, however, are well suited for this discrimination if alterations in the charge of the protein accompany the *p*H-dependent instability of activity.

The appearance of a second boundary on electrophoresis, indeed, provided the means to distinguish between the two alternatives suggested by the optical-rotatory-dispersion studies (Fig. 3B). The relative area under the more rapidly moving electrophoretic boundary increased with the *p*H of incubation and the protein in this boundary was virtually inactive. Such activity as was found might, indeed, represent an artifact of the sampling procedure.

Thus all the changes observed are apparently due to denaturation of a fraction of the molecules, and a transport method, like electrophoresis, is required to reveal the existence of this moiety.

Denaturation might be attributed to the fact that acetic anhydride, in contrast to acetylimidazole, acetylates the amino groups of carboxypeptidase in addition to certain tyrosyl residues (Simpson *et al.*, 1963). Hence acetylation of the active center with a reagent devoid of such undesirable side effects might increase activity even further.

This expectation is borne out by acetylation of carboxypeptidase with acetylimidazole. The second, inactive population which lowers the specific activity is eliminated between *p*H 6 and 8. The fast, inactive component, seen only at *p*H 8.5 (Fig. 5), is such a small fraction of the total that its effects upon activity are minimal. None of the changes in λ_c and b_0 are now observed (Fig. 4), and increases in activity, indeed, exceed those brought about by acetic anhydride. Since no absolute criteria for the attainable maximum activity of Ac_A-carboxypeptidase were available, it was not possible to predict that the activity observed at any given *p*H was actually *less* than could be expected from modification of the active-center tyrosyl residues alone.

Though all the findings are in accord with the proposition stated, it might be reasoned that the production of an inactive component in Ac_A-carboxypeptidase could be due to partial acetylation of the tyrosyl groups rather than to the concomitant acetylation of nonfunctional side chains of other residues. It will be recalled, however, that acetylation of carboxypeptidase, employing even suboptimal concentrations of acetic anhydride or acetylimidazole, always results in esterase activities much greater (not less) than those of the native enzyme (Riordan and Vallee, 1963). The mobility of the inactive fraction, obviously higher than that of the active one, indeed suggests *more extensive* acetylation of the protein. The initial population, however,

seems almost homogeneous at *p*H 6.5, and the appearance of the second component depends upon the *p*H of incubation. It is more probable, therefore, that the appearance of a second, inactive species is an indirect consequence of the acetylation of side chains causing *p*H-dependent changes in secondary or tertiary structure. As a consequence, either additional negatively charged groups of the molecule are exposed to the environment, or positively charged groups are hidden from it; in either case the result would be the appearance of two species in solution.

Thus the *increase* in esterase activity induced by acetylation is not accompanied by detectable changes in conformation (Table I).[3] The observed loss in activity and alterations in rotational characteristics are owing directly to alterations in secondary and tertiary structure of a fraction of the total population of molecules and this modification appears to be *independent* of that which occurs at the active center and induces increased activity.

Hence, it must be concluded that the increase in activity is the most sensitive index of changes in primary structure. If these are accompanied by changes in conformation, such alterations cannot be detected by presently available conventional means. It should be emphasized that the investigations here reported bear upon the general problem of assessing the basis of alterations in enzyme activities. Measurement of physicochemical parameters is required to rule out structural changes as the basis of changes in catalytic activity.

[3] In addition, studies of the melting curves and sedimentation properties revealed no conformational changes (Vallee, 1964).

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Succinylcarboxypeptidase*

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Acylation of carboxypeptidase with dicarboxylic acid anhydrides abolishes peptidase activity and increases esterase activity due to modification of tyrosyl residues in the active center of the enzymes, in agreement with previous results obtained by means of acetic and other monocarboxylic acid anhydrides. In contrast to acetylcarboxypeptidase, the enzymes modified by means of dicarboxylic acid anhydrides, owing to spontaneous deacylation, undergo a time-dependent recovery of esterase and peptidase activity, eventually reaching 125% of the control. Correlation of the rates of deacylation of methlysuccinyl-, succinyl-, α,α-dimethylglutaryl-, β,β-dimethylglutaryl-, and glutarylcarboxypeptidases with those for the corresponding esters of *p*-bromophenol demonstrates that the mechanism of deacylation proceeds via intramolecular nucleophilic catalysis by the free acylcarboxylate group. The increase in both activities over those of the native enzyme subsequent to deacylation is postulated to be related to the increase in the net negative charge on the protein.

Acetylation of carboxypeptidase[1] with acetylimidazole or acetic anhydride completely abolishes peptidase activity and concomitantly increases esterase activity 6- to 7-fold (Simpson *et al.*, 1963; Riordan and Vallee, 1963). The changes in enzymatic activity can be prevented by β-phenylpropionate and other competitive inhibitors, as well as by substrates, and can be reversed by deacetylation with hydroxylamine. Spectral changes demonstrate that the alterations in activity are caused by acetylation of two tyrosyl residues at the active center of the enzyme.

The present data show that succinic and other dicarboxylic acid anhydrides also react with the active-center tyrosyl residues of carboxypeptidase resulting in analogous increases in esterase and decreases in peptidase

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† Postdoctoral Fellow of the National Institutes of Health, U. S. Department of Health, Education and Welfare.

[1] "Carboxypeptidase" refers to carboxypeptidase A only. The enzyme used in these studies was prepared from autolyzing bovine pancreas (Anson, 1937) and is predominantly carboxypeptidase Aγ (Bargetzi *et al.*, 1963).