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).

References

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663, 777.

Bethune, J. L., and Ulmer, D. D. (1963), *Federation Proc. 22*, 594.

Blout, E. R. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., ed., New York, McGraw-Hill, 238.

Boyer, J. H. (1952), *J. Am. Chem. Soc. 74*, 6274.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coleman, J. E., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2244.

Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4*, 247.

Koshland, D. E., Jr. (1960), *Advan. Enzymol. 22*, 45.

Li, T. K., Ulmer, D. D., and Vallee, B. L. (1962), *Biochemistry 1*, 114.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Rudolph, H. (1955), *J. Opt. Soc. Am. 45*, 50.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Slobodian, E., Mechanic, G., and Levy, M. (1962), *Science 135*, 441.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Vallee, B. L. (1964), *Federation Proc. 23*, 8.

Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 109.

Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.

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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[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).

activities. However, these acylated enzymes undergo a time-dependent restoration of peptidase activity and return to almost normal esterase activity owing to spontaneous deacylation of the active center, accounting for the results previously reported (Vallee *et al.*, 1963; Riordan and Vallee, 1963). Since these observations are analogous to those on model systems employing monophenyl esters of dicarboxylic acids (Bruice and Pandit, 1960), carboxypeptidase was modified with the anhydrides of the same dicarboxylic acids. Comparison of the rates of spontaneous hydrolysis of these esters with those observed for the recovery of peptidase activity of the respective acyl enzymes provides additional evidence for the participation of tyrosyl residues in the activity of carboxypeptidase. They demonstrate further that the restoration of activity is indeed caused by spontaneous deacylation of the active-center tyrosyl residues.

Experimental

Twice-recrystallized bovine pancreatic carboxypeptidase prepared by the method of Anson (1937) was obtained from the Worthington Biochemical Corp., Freehold, N. J. The zinc-protein ratio was between 0.97 and 1.02 g-atoms/mole based on a molecular weight of 34,600 (Bargetzi *et al.*, 1963). Succinic and glutaric anhydrides were obtained from Eastman Organic Chemicals, Inc., and were used without further purification. Methylsuccinic, α,α-dimethylglutaric, and β,β-dimethylglutaric anhydrides were obtained from K and K Laboratories and were recrystallized from glacial acetic acid before use. Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957).

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as previously described (Coleman and Vallee, 1960). Activity is expressed as an apparent proteolytic coefficient, C, defined as $\log a_0/a$ per min per μmole enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively.

Esterase activity was determined by recording the rate of hydrolysis of hippuryl-DL-β-phenyllactate using a *p*H-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen) (Snoke *et al.*, 1948). Activities are expressed as zero-order velocity constants, k, with units of μeq H^+ liberated per min per μmole enzyme. *Protein concentrations* were measured by absorbance at 278 mμ with a Zeiss PMQ II spectrophotometer, using a molar absorptivity for native carboxypeptidase of 6.42×10^4 M^{-1} cm^{-1}. Spectra were obtained with a Cary Model 15 automatic recording spectrophotometer. The *p*H of buffers was determined with a Radiometer Model PHM4 equipped with a Radiometer GK 2021 electrode. *Acylations* were routinely carried out at 0–4° by adding a 48-fold molar excess of solid anhydride to a well-stirred solution of carboxypeptidase (4–10 mg/ml) in 0.02 M sodium Veronal–2 M NaCl, at *p*H 7.5, on the *p*H-stat (Riordan and Vallee, 1963). The reaction was considered complete after 30 minutes when base uptake had ceased. Excess reagent was then removed by dialysis for 36–72 hours against the same buffer to maintain constant *p*H and ionic strength and to avoid spurious reactions. Alternatively, to follow the time course of the reaction immediately subsequent to the addition of anhydride, the reaction was stopped by 100-fold dilution. Control experiments demonstrated that the amount of anhydride remaining under these conditions did not interfere with the assay. In some experiments the competitive inhibitors β-phenylpropionate, indole-3-acetate, or phenylacetate were used as protective agents during succinylation (Riordan and Vallee, 1963). In such instances, aliquots were removed from the succinylation-reaction mixture, diluted 1000-fold, and assayed at 25° rather than at 0°, thereby circumventing the inhibitory effects of these agents.

The degree of modification of the free amino groups of carboxypeptidase was determined by means of the ninhydrin reaction (Moore and Stein, 1948) using phenylalanine as a standard.

The time course of the effect of acylation on the activity was followed by removing aliquots from the acylation-reaction mixture and, after dilution, assaying both peptidase and esterase activities. After 30 minutes excess anhydride was removed by gel filtration at 4° and aliquots of the enzyme-containing effluent fraction were diluted and assayed at various times.

For deacylation the acylenzymes were exposed to 1.0 M hydroxylamine for 10 minutes in 0.02 M sodium Veronal–2.0 M NaCl, *p*H 7.5, 20°, followed by dialysis. Spectral changes during deacylation with 0.04 M hydroxylamine were followed in 0.01 M Tris–2 M NaCl at 20°.

Amidination was performed for 2 hours in 0.02 M sodium Veronal–2.0 M NaCl, *p*H 8.5, with a 100-fold molar excess of methylacetimidate prepared according to the procedure of Hunter and Ludwig (1962).

Results

Enzymatic Consequences of Succinylation.—Succinylation of carboxypeptidase with succinic anhydride at *p*H 7.5, 4°, for 30 minutes followed by 36–72 hours' dialysis against cold 0.02 M sodium Veronal–2 M NaCl buffer, *p*H 7.5, results in an increase both of peptidase and of esterase activities when measured at the end of dialysis. The increase varies from 20 to 40% of the control as the molar excess of anhydride employed is increased from 24- to 96-fold (Table I). These changes in activity after succinylation are accompanied by a decrease in free amino groups from 15 to about 4 equivalents of phenylalanine per mole of succinyl enzyme. The results measured after 36 hours of dialysis are virtually identical when the acylation reaction is carried out both in the presence and absence of 0.1 M β-phenylpropionate (Table I).

Table I

Succinylation of Carboxypeptidase with Succinic Anhydride: Enzymatic Activities and Free Amino Groups[a]

Molar Excess Anhydride	Esterase ($k \times 10^{-3}$)	Peptidase (C)	Equivalents Phenylalanine/Mole Enzyme
0	6.8	30.8	15.1
24	8.6	36.3	5.4
48	8.8	38.3	4.8
48[b]	9.5	41.9	4.7
96	9.4	40.2	3.7

[a] Succinylation at *p*H 7.5, 0.02 M Veronal–2 M NaCl, 0°, 30 minutes, followed by 36-hour dialysis. Esterase activity measured at 25°, peptidase measured at 0°. [b] Succinylation in the presence of 0.1 M β-phenylpropionate.

Thus the effects of succinylation on enzymic activity appear to be strikingly different from those of acetylation with acetic anhydride or acetylimidazole when both are measured under similar conditions after 36–72 hours

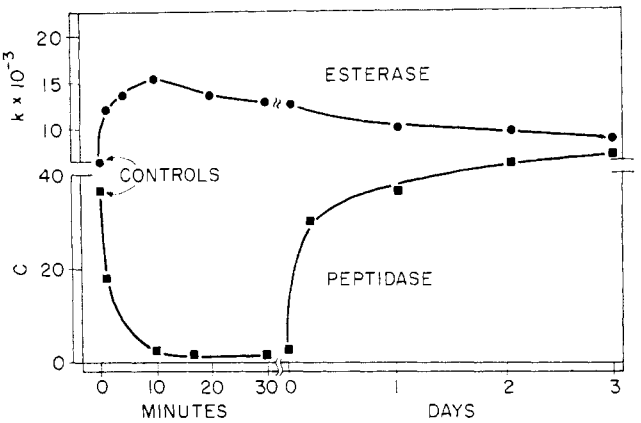


FIG. 1.—Progression of changes in esterase (●) and peptidase (■) activities during succinylation of carboxypeptidase (6 mg/ml) with succinic anhydride (48-fold M excess). Reaction was performed in 0.02 M sodium Veronal–2 M NaCl, *p*H 7.5, 0–4°, on the *p*H-stat. Activities were determined immediately following dilution of an aliquot of the reaction mixture into the same buffer. After 30 minutes the reaction mixture was passed over a 1 × 30-cm column of Sephadex G-25 equilibrated with the same buffer, and the protein-containing effluent fraction was kept at 4° and assayed at the times indicated.

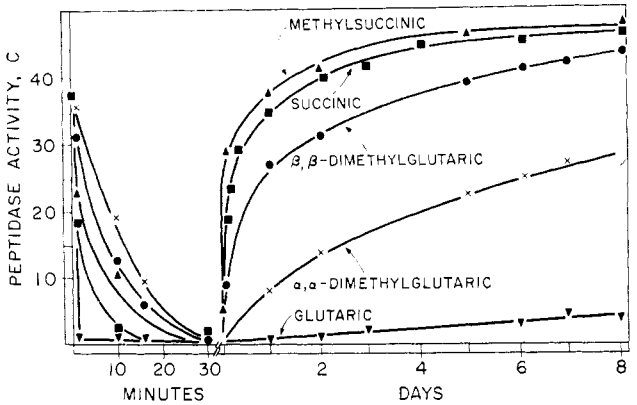


FIG. 2.—Progression of changes in peptidase activities during acylation of carboxypeptidase (4–10 mg/ml) with methylsuccinic (▲), succinic (■), β,β-dimethylglutaric (●), α,α-dimethylglutaric (×), and glutaric (▼) anhydrides (48-fold M excess). Reactions were performed as in Fig. 1.

of dialysis in order to remove the excess anhydride (Riordan and Vallee, 1963).

However, succinylcarboxypeptidase prepared in this manner is still susceptible to acetylation which induces the same functional consequences as obtained on acetylation of the native enzyme: peptidase activity is abolished and esterase activity increases to more than 600% of the unmodified control. Iodination of succinylcarboxypeptidase yields similar results (Table II).

TABLE II

ACETYLATION AND IODINATION OF SUCCINYLCARBOXYPEPTIDASE: ENZYMATIC ACTIVITIES[a]

Carboxypeptidase	Esterase ($k \times 10^{-3}$)	Peptidase (C)
Succinyl	8.8	38.3
Acetylsuccinyl	42.7	0.3
Iodosuccinyl	25.5	3.6

[a] Succinylation with a 48-fold molar excess of anhydride at *p*H 7.5, 0.02 M Veronal–2 M NaCl, 0°, 30 minutes followed by 48-hour dialysis. Acetylation of succinylcarboxypeptidase with 60-fold molar excess of acetylimidazole, 30 minutes; iodination with 35-fold molar excess of I_2 in 0.5 M KI, 20°, 10 minutes.

Apparently at the termination of dialysis the two tyrosyl residues susceptible to *O*-acetylation (Simpson *et al.*, 1963; Riordan and Vallee, 1963) or to iodination (Vallee *et al.*, 1963) are not succinylated. The residues may be refractory to reaction with succinic anhydride, or *O*-succinyltyrosyl groups may be unstable once formed.

Since it could be shown that excess anhydride does not interfere with the assays, the latter alternative was susceptible to examination by measuring esterase and peptidase activities throughout the course of the succinylation reaction (Fig. 1). Immediately following the addition of the anhydride, peptidase activity decreases rapidly and is abolished within 30 minutes. During the first 10 minutes of the reaction, esterase activity increases to about 300% of the control then decreases slightly to about 250% after 30 minutes.

A succinylenzyme with very low peptidase activity and increased esterase activity can be obtained similarly if the reaction mixture is passed over Sephadex equilibrated with 0.02 M sodium Veronal–2 M NaCl, *p*H 7.5, in lieu of dialysis. Incubation at 4° of the enzyme obtained in this manner restores peptidase activity to about 50% within 4 hours and to about 125% of the control after 72 hours. At the same time esterase activity decreases, also reaching about 125% of the control after 72 hours. The final values are the same as those obtained after dialysis of succinylcarboxypeptidase at 4° for 72 hours.

Succinylation of carboxypeptidase in the presence of 10^{-1} M β-phenylpropionate, indole-3-acetate, or phenylacetate prevents the changes in both esterase and peptidase activities which occur in the first half hour of reaction (Table III). This protective effect,

TABLE III

PROTECTION OF THE ACTIVE CENTER OF CARBOXYPEPTIDASE AGAINST SUCCINYLATION BY INHIBITORS: ENZYMATIC ACTIVITIES AND FREE AMINO GROUPS[a]

Inhibitor	Esterase ($k \times 10^{-3}$)	Peptidase (C)	Equivalents Phenylalanine/Mole Enzyme
None	13.7	2	4.6
β-Phenylpropionate	9.8	129	4.1
Indole-3-acetate	9.7	125	4.9
Phenylacetate	10.4	124	5.8

[a] Succinylation in the presence or absence of 0.1 M inhibitor at *p*H 7.5, 0.02 M Veronal–2 M NaCl, 0°, 30 minutes. Aliquots were removed, diluted 1:1000, and assayed without prior dialysis. Esterase and peptidase activities measured at 25°.

analogous to that observed on acetylation, is due apparently to blocking of the active center tyrosyl residues of the enzyme by the competitive inhibitors.

Mechanism of Deacylation of Succinylcarboxypeptidase.—The chemical events leading to the spontaneously reversible alterations of peptidase and esterase activities on succinylation of carboxypeptidase were extended by means of analogous studies with other dicarboxylic acid anhydrides. The time courses for the loss of peptidase activity following addition of methylsuccinic, α,α-dimethylglutaric, β,β-dimethylglutaric, or glutaric anhydrides were compared. The results closely resemble those obtained by means of

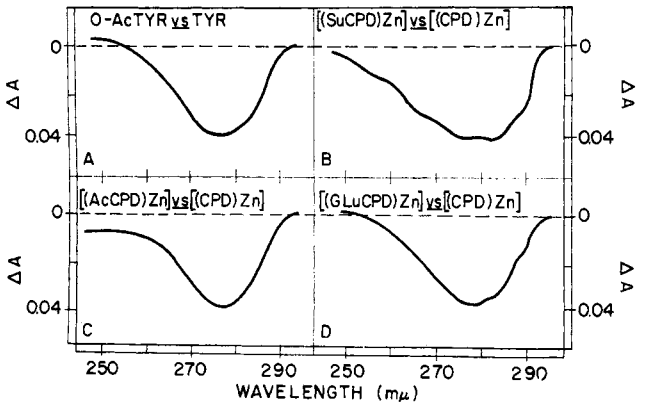


FIG. 3.—Difference spectra of *N,O*-diacetyltyrosine (*O*-AcTyr) versus tyrosine (Tyr) (A), and native carboxypeptidase versus succinylcarboxypeptidase (B), acetylcarboxypeptidase (C), and glutarylcarboxypeptidase (D). The concentration in (A) was 3.2×10^{-5} M and in (B), (C), and (D) the concentration was 7.5×10^{-6} M. All difference spectra were obtained in 0.01 M Tris–1 M NaCl (*p*H 7.5) buffer at 20° using a Cary Model 15 recording spectrophotometer with 1-cm cells. Abbreviations: [(CPD)Zn], native zinc carboxypeptidase; [(SuCPD)Zn], succinylcarboxypeptidase; [(GluCPD)Zn], glutarylcarboxypeptidase; [(AcCPD)Zn], acetylcarboxypeptidase.

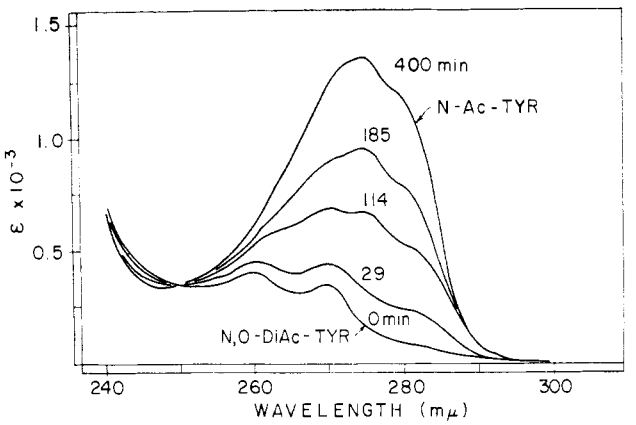


FIG. 4.—Progression of changes in the absorption spectrum of *N,O*-diacetyltyrosine (*N,O*-DiAc-Tyr) on deacetylation with 0.01 M hydroxylamine in 0.01 M Tris–1 M NaC (*p*H 7.5) buffer at 20°. The zero-time spectrum corresponds to an identical sample but is diluted with buffer not containing hydroxylamine. Intermediate spectral scans were run starting from 300 mμ at 29, 114, and 185 minutes. Scanning time was 75 seconds. The spectrum after 400 minutes was identical with that of a sample of *N*-acetyltyrosine (*N*-Ac-Tyr).

succinylation. In each instance peptidase activity falls virtually to zero within 30 minutes after the addition of anhydride (Fig. 2), and esterase activities increase to between 250 and 300% of the control (Fig. 1). However, there are marked differences in the rates at which the peptidase activities are restored to the different acylenzymes. While succinylcarboxypeptidase exhibits 50% peptidase activity after 4 hours and becomes maximally active only after 24–36 hours (*vide supra*), methylsuccinylcarboxypeptidase recovers 125% of the control peptidase activity within 6 hours. The time span required for reactivation of the substituted glutarylenzymes depends on the nature and position of the substitution. Thus, after 24 hours, β,β-dimethylglutarylcarboxypeptidase is about 50% active while the α,α-dimethylglutarylenzyme has recovered only 17% of its activity. Glutarylcarboxypeptidase has only 10% of the peptidase activity of the control as long as 192 hours after the end of the glutarylation reaction.

Rate constants for the recovery of peptidase activity for the various acylated carboxypeptidases were calculated from the half-times of the reaction (Table IV, column 3) and are compared with the pertinent rate constants for the spontaneous hydrolysis of the corresponding mono-*p*-bromophenyl esters of the same dicarboxylic acids (Bruice and Pandit, 1960) (Table IV, column 2). The relative order of the rate constants obtained with these model compounds and those found here for the acylcarboxypeptidases are in remarkably good agreement; this correlation provides strong support for the inference that spontaneous deacylation of the active-center tyrosyl residues is the chemical event which is responsible for the restoration of peptidase activity.

TABLE IV
RATES OF DEACYLATION OF *p*-BROMOPHENYL ESTERS AND ACYLCARBOXYPEPTIDASES[a]

Ester or Acylenzyme	Ester (k'_1 min^{-1})	Acylenzyme (k'_1 hr^{-1})
Glutaryl	0.0038	0.001
α,α-Dimethylglutaryl	0.0138	0.010
β,β-Dimethylglutaryl	0.073	0.042
Succinyl	0.94	0.175
(di)[b]Methylsuccinyl	2.61	2.31[b]

[a] Rate data for esters obtained spectrophotometrically (Bruice and Pandit, 1960) in 0.05 M acetate, μ = 0.65 M, with KCl; solvent 50% (v/v) dioxane-water; Na 0.05 M, 30°, at an apparent *p*H 7.00 (except dimethylsuccinyl ester, which was at *p*H 6.64). Data for acylenzymes obtained from half-times of recovery of peptidase activity in 0.02 M Veronal–2 M NaCl, *p*H 7.5, 4°. [b] Methylsuccinic anhydride was employed in lieu of the dimethylsuccinyl ester as in Bruice and Pandit (1960).

Spectral evidence for the functional role of tyrosyl residues in carboxypeptidase has already been reported (Simpson *et al.*, 1963; Riordan and Vallee, 1963). Acetylation of the tyrosyl residues of carboxypeptidase and of other proteins (Wacker *et al.*, 1964) with acetic anhydride and acetylimidazole causes a marked decrease in absorbance between 250 and 300 mμ and results in a characteristic difference spectrum with a maximum at 278 mμ. Similarly, difference spectra for succinyl-, acetyl-, and glutarylcarboxypeptidases versus native carboxypeptidase (Fig. 3B,C,D, respectively) were obtained immediately after their preparation and when these derivatives were still virtually devoid of peptidase activity. These difference spectra, virtually identical with that of *N*-acetyltyrosine versus *N,O*-diacetyltyrosine (Fig. 3A), demonstrate that *initially* succinylation and glutarylation indeed modify tyrosyl residues of the enzyme.

Deacetylation of *N,O*-diacetyltyrosine with 0.01 M hydroxylamine at *p*H 7.5 restores the characteristic spectrum of *N*-acetyltyrosine (Fig. 4). The spontaneous recovery of peptidase activity by succinylcarboxypeptidase is accompanied by an analogous increase in absorbance at 278 mμ (Figs. 1 and 2). The increase in peptidase activity and absorbance at 278 mμ are very closely correlated (Fig. 5). Further, the spontaneous recoveries both of activity and absorbance at 278 mμ can be accelerated by hydroxylamine. Deacylation of succinylcarboxypeptidase with 1.0 M hydroxylamine at *p*H 7.5, 20°, restores the spectrum characteristic of the native enzyme within 10 minutes (Fig. 6). Under similar conditions spontaneous deacylation restores the spectrum in 24–36 hours.

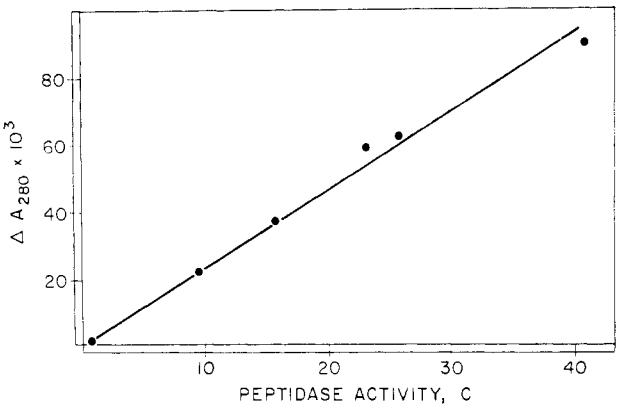


FIG. 5.—Correlation of spontaneous recovery of peptidase activity of succinylcarboxypeptidase expressed as the proteolytic coefficient, *C*, with the increase in absorbance at 280 mμ. Native enzyme was succinylated in 0.01 M Tris–2 M NaCl but otherwise as under Experimental. The sample isolated after gel-filtration was maintained at 20° and activity and absorbance were determined at 0, 1, 3, 5, 7, and 24 hours.

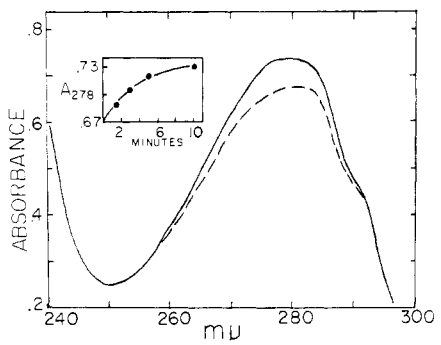


FIG. 6.—Succinylcarboxypeptidase (- - -) without peptidase activity was deacylated with 1.0 M hydroxylamine–0.01 M Tris–2 M NaCl, *p*H 7.5, 20°. After 10 minutes the spectrum of native carboxypeptidase (———) was restored. The time course for the restoration of absorbance at 278 mμ is indicated in the inset.

With glutarylcarboxypeptidase, spontaneous changes in either absorbance or activity occur at a much slower rate. However, in this instance, too, the rates of recovery both of absorbance at 278 mμ and of peptidase activity can be accelerated markedly by hydroxylamine (Fig. 7). These data demonstrate that the functional tyrosyl residues acylated by dicarboxylic acid anhydrides are operationally identical with those modified by the monocarboxylic acid anhydrides.

DISCUSSION

Two tyrosyl residues have been shown to play an essential role in the function of carboxypeptidase. Their *O*-acetylation with acetic anhydride or acetylimidazole causes significant spectral changes which are accompanied by a marked increase in esterase and abolition of peptidase activities (Simpson *et al.*, 1963; Riordan and Vallee, 1963). The enzymatic consequences of acetylation can be prevented both by peptide substrates and by inhibitors of the enzyme. Thus the inhibitor, β-phenylpropionate, has been shown to block selectively the acetylation of 2 tyrosyl residues in the active center. These tyrosyl residues are also susceptible to acylation with a series of monocarboxylic acid anhydrides, resulting in losses of peptidase activity which are commensurate with corresponding increases in esterase activity and, in turn, characteristic of each one

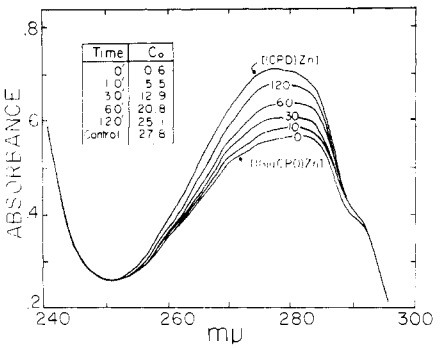


FIG. 7.—Progression of changes in absorption spectrum of glutarylcarboxypeptidase on deacylation with 0.04 M hydroxylamine–0.01 M Tris–2 M NaCl, *p*H 7.5, 20°. This concentration of hydroxylamine, 1/50 of that in Figure 6, was employed here to obtain rates slow enough to document the detailed spectral changes with time. The numbers on the curves correspond to minutes of incubation of the enzyme with hydroxylamine. The uppermost spectrum is that of native carboxypeptidase. Inset: Changes in peptidase activity (C_0) accompanying deacylation of glutarylcarboxypeptidase with hydroxylamine. Aliquots were removed directly from the cuvet employed for spectrophotometry, diluted, and assayed. Abbreviations as in Fig. 3.

of the reagents in this series. Increase in the chain length or in the bulk of their side chains renders the anhydrides progressively less effective (Riordan and Vallee, 1963).

These data are in accord with similar observations on the reactivities of these anhydrides in other systems (Dixon and Neurath, 1957). Although the magnitude of the simultaneous increase of esterase and decrease of peptidase activities subsequent to acylation with monocarboxylic acid anhydrides varies quantitatively, qualitatively the changes are similar for all.

The consequences of acylation with dicarboxylic acid anhydrides, however, differ from the results obtained with monocarboxylic acid anhydrides in kind, not in degree. Thus, under the same conditions of chemical modification and removal of excess anhydride by dialysis, i.e., 48-fold M excess followed by dialysis for 36–72 hours, succinylation *increases both* esterase and peptidase activities to about 125% of the control. Since these enzymatic changes do not coincide with the pattern of those observed on the derivatives prepared with monocarboxylic acid anhydrides, the hypothesis that succinic anhydride might not be able to react with the critical tyrosyl residues of carboxypeptidases was entertained. Alternatively, it was presumed that the succinyl-*O*-tyrosine linkage is unstable and on hydrolysis yields a product whose enzymatic characteristics differ minimally from those of the native enzyme.

Previous studies with carboxypeptidase have demonstrated that only 6–7 of the 19 tyrosyl residues dissociate "normally" with an apparent *pK* of 10.5 and are therefore considered to be "free" (Simpson *et al.*, 1963). In accord with these data, the residues were found to be readily accessible to reaction with acetic anhydride and acetylimidazole. The two "functional" tyrosyls are among the reactive residues (Riordan and Vallee, 1963). The apparent lack of susceptibility of tyrosyl residues to succinylation could imply that the two essential tyrosyl residues, accessible to hydronium ions and monocarboxylic acid anhydrides, might be sterically inaccessible to succinylation.

The features of carboxypeptidase which might account for such a high degree of reagent specificity were therefore examined further. When assays are per-

formed immediately after the addition of succinic anhydride *but prior to the removal of the excess anhydride by dialysis*, peptidase activity is decreased to zero and esterase activity increases 3-fold (Fig. 1)—changes similar to those observed on acylation with monocarboxylic acid anhydrides; dissimilarities become apparent only with time. The activities of the succinylenzyme undergo rapid alterations in the first 24 hours after reaction. Peptidase activity increases progressively and reaches 125% of the control after 36–48 hours, while esterase activity falls from 300 to 125% during the same interval. In contrast, the acetylated enzyme is quite stable; its esterase activity remains at about 600–700% of the native enzyme for up to 30 days at 4°, and at this time peptidase activity is still less than 10%—evidence of the stability of the tyrosyl ester bond (J. F. Riordan and B. L. Vallee, unpublished experiments). Apparently, solvent hydrolysis cannot account for the restoration of peptidase activity to the succinylenzyme.

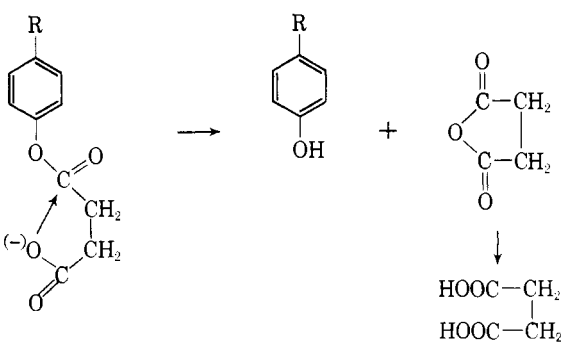
However, the existence of a free carboxyl group in *O*-succinyl-tyrosyl residues presents a potentially important modus for a hydrolytic mechanism. Intramolecular nucleophilic catalysis of ester hydrolysis has been studied extensively in several model systems (Morawetz and Zimmering, 1954; Morawetz and Westhead, 1955; Bruice and Sturtevant, 1959). The initial observations by Edwards (1950) of the abnormal spontaneous hydrolysis of aspirin led to a variety of mechanistic suggestions based on cyclic intermediates formed by attack of the carboxylate anion on the carbonyl carbon of the ester group (Chanley *et al.*, 1952; Davidson and Auerbach, 1953; Garrett, 1957). Acetate-ion catalysis of the hydrolysis of several phenylacetates (Bender and Turnquest, 1957; Bender and Neveu, 1958; Bruice and Lapinski, 1958) was thought to support such a mechanism (Bender *et al.*, 1958), although recent data indicate that these bimolecular hydrolytic reactions may proceed via general base catalysis (Butler and Gold, 1962). In the case of aspirin hydrolysis (Bender *et al.*, 1958) indirect evidence for the intermediate formation of an anhydride has been obtained from isotopic labeling experiments and direct evidence based on studies of the solvolysis of the *p*-methyoxyphenyl ester of exo-3,6-endoxo-Δ^4-tetrahydrophthalic acid has been recorded (Bruice and Pandit, 1960).

Such observations suggest that the time-dependent recovery of peptidase activity subsequent to inactivation by succinylation might be the result of *deacylation* proceeding via intramolecular catalysis by the carboxylate group.

Intramolecular nucleophilic catalytic hydrolysis of esters of aliphatic alcohols by carboxylate ion has only been reported for methyl hydrogen phthalate (Bender *et al.*, 1958), while bimolecular nucleophilic catalysis of esters of aliphatic alcohols occurs only with difficulty, if at all (Bender and Neveu, 1958; Kirsch and Jencks, 1964). In this regard, it has been reported that the alkaline hydrolysis of the dimethyl ester of succinic acid proceeds more rapidly than that of the monomethyl ester (Halonen, 1955). In contrast, phenolic esters are hydrolyzed readily. It is therefore significant that acetylcarboxypeptidase undergoes ready bimolecular nucleophilic catalysis. Hydroxylamine rapidly deacetylates this derivative, thereby fully reversing both the spectral and enzymatic effects either of acetic anhydride or acetylimidazole providing the first indication of the involvement of tyrosyl residues in the mechanism of carboxypeptidase (Vallee *et al.*, 1963; Simpson *et al.*, 1963; Riordan and Vallee, 1963).

Bruice and Pandit (1960) have examined the effects of ring size, steric compression, and steric hindrance on the rates of formation of cyclic intermediates during the hydrolysis of monophenyl esters of a series of dibasic acids. Methyl or dimethyl substitution of the α-carbon atom increased the rate of solvolysis apparently by hindering the rotation of the free carboxylate group away from the ester carbonyl-carbon atom, thereby favoring the formation of the transition state. The ready hydrolysis of succinyl esters as compared with glutaryl esters was thought to result from fewer bonds having to be "frozen into position" upon entering the transition state.

Therefore a series of acylcarboxypeptidases with dicarboxylic acid anhydrides analogous to the esters studied by Bruice and Pandit were prepared to compare relative rates of deacylation. Table III lists the rates of spontaneous deacylation of the modified carboxypeptidases calculated from the data on recovery of peptidase activity in Figure 2, and also the pertinent ones of those reported for the model compounds by Bruice and Pandit (1960). The order of the rates of deacylation of the model compounds and those of the acylcarboxypeptidases are in surprisingly good argeement considering that the data for carboxypeptidase had to be obtained at 4° in 2.0 M NaCl–0.02 M sodium Veronal buffer, while those for the model compounds were measured at 30°, in 50% dioxane acetate buffer, μ = 0.65. Such correlation strongly suggests that hydrolysis of *O*-acyl-tyrosyl bonds in these acyl enzymes proceeds via a mechanism involving intramolecular nucleophilic catalysis by a carboxylate group of analogous phenolic esters:



In the model system this mechanism has been substantiated by obtaining direct evidence for the formation of the anhydride intermediate (Bruice and Pandit, 1960).

The acetylation of tyrosyl residues results in a marked spectral decrease between 260 and 290 mμ, which is a measure of the degree of acetylation of tyrosyl residues of proteins. The qualitative similarity of the difference spectra for succinyl- or glutarylcarboxypeptidase versus native carboxypeptidase with those for acetylcarboxypeptidase versus native carboxypeptidase and for *N,O*-diacetyltyrosine versus *N*-acetyltyrosine demonstrates the *O*-acetylation of tyrosyl residues in both succinyl- and glutarylcarboxypeptidase (Fig. 5). Moreover, the one-to-one correlation of the spontaneous recovery of peptidase activity with the spontaneous restoration of absorbance at 278 mμ (Fig. 5) is further experimental proof of the functional role of these tyrosyl residues in the active center of the enzyme. The rates of recovery both of activity and absorbance can be accelerated markedly by hydroxylamine as expected under these circumstances. At a concentration of 1.0 M, hydroxylamine completely reverses the effects of succinylation within 10 minutes (Fig. 6) compared with 36 hours when the spontaneous process is allowed to go to completion. Even glutarylcarboxypeptidase, which is

stable for days, can be deacylated by 0.04 M hydroxylamine in 2 hours with concomitant restoration of activity and increase in absorption at 278 mμ (Fig. 7).

The increase in both esterase and peptidase activities to 125% of the control at a time when the spontaneous deacylation of the tyrosyls is completed merits additional discussion. The increase in activity is not prevented by acylation in the presence of the inhibitor, β-phenylpropionate, but does seem to be related to the molar excess of anhydride employed (Table I). It is approximately the same for methylsuccinyl-, succinyl-, and glutarylcarboxypeptidases. In fact, a similar small increase in activity is observed after acetylation with acetic anhydride in the presence of β-phenylpropionate—conditions which prevent the specific effects on activity owing to *O*-acetyltyrosine formation (Table V). Both succinylation and acetylation also block

TABLE V

ESTERASE AND PEPTIDASE ACTIVITIES AND FREE AMINO GROUPS OF NATIVE AND MODIFIED CARBOXYPEPTIDASES[a]

Carboxypeptidase	Esterase ($k \times 10^{-3}$)	Peptidase (C)	Equivalents Phenylalanine/Mole Enzyme
Native	6.8	30.8	15.1
Acetyl[b]	7.2	36.8	4.8
Succinyl[c]	8.8	38.3	4.8
Amidinated[d]	5.5	26.2	4.7

[a] Esterase at 25° and peptidase activity at 0°. [b] A 48-fold molar excess of acetic anhydride in 0.1 M β-phenylpropionate–0.02 M Veronal–2 M NaCl, *p*H 7.5, 0°, 30 minutes, followed by 48-hour dialysis. [c] Same, except 48-fold molar excess of succinic anhydride and without β-phenylpropionate. [d] A 100-fold molar excess of methylacetimidate in 0.02 M Veronal–2 M NaCl, *p*H 8.5, 0°, 2 hours, followed by 48-hour dialysis against the same buffers at *p*H 7.5.

amino groups, of course, and thereby alter the net charge on the protein molecule. Acetylation converts the positively charged ammonium ion to a neutral amido group while succinylation produces a negatively charged amido group. The hypothesis that the increase in net negative charge on the protein molecule is responsible for the resultant changes in activities is supported by the results of amidination (Table V), which blocks the amino groups of carboxypeptidase to the same extent as acetylation. However, in this instance, the positively charged amino group is replaced by the even more basic, positively charged amidino group, though tyrosyl groups are not modified. Peptidase and esterase activities are not increased following amidination. In fact, there is even a slight decrease in both activities. Electrophoretic mobility is the only pertinent physical property of carboxypeptidase found altered so far by succinylation (Vallee, 1964; J. L. Bethune, D. D. Ulmer, and B. L. Vallee, to be published). In regard to the final activities of succinylcarboxyl peptidase, it does not appear likely that modification of a particular amino acid residue in the enzyme is important. Rather it would seem that electrostatic factors operating to increase either the attraction between substrate and the active center of the enzyme or the elimination of product constitute the basis of the activity changes. The relationship between net protein charge and the kinetic parameters reflecting enzymatic activities is under continuing investigation.

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REFERENCES

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663, 777.
Bargetzi, J.-P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.
Bender, M. L., Chloupek, F., and Neveu, M. C. (1958), *J. Am. Chem. Soc. 80*, 5384.
Bender, M. L., and Neveu, M. C. (1958), *J. Am. Chem. Soc. 80*, 5388.
Bender, M. L., and Turnquest, B. W. (1957), *J. Am. Chem. Soc. 79*, 1656.
Bruice, T. C., and Lapinski, R. (1958), *J. Am. Chem. Soc. 80*, 2265.
Bruice, T. C., and Pandit, U. K. (1960), *J. Am. Chem. Soc. 82*, 5858.
Bruice, T. C., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc. 81*, 2860.
Butler, A. R., and Gold, V. (1962), *J. Chem. Soc.*, 1334.
Chanley, J. D., Gindler, E. M., and Sobotka, H. (1952), *J. Am. Chem. Soc. 74*, 4347.
Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.
Davidson, D., and Auerbach, L. (1953), *J. Am. Chem. Soc. 75*, 5984.
Dixon, G. H., and Neurath, H. (1957), *J. Biol. Chem. 225*, 1049.
Edwards, L. J. (1950), *Trans. Faraday Soc. 46*, 723.
Garrett, E. R. (1957), *J. Am. Chem. Soc. 79*, 5206.
Halonen, E. A. (1955), *Acta Chem. Scand. 9*, 1492.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Am. Chem. Soc. 84*, 3491.
Kirsch, J. F., and Jencks, W. P. (1964), *J. Am. Chem. Soc. 86*, 837.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Morawetz, H., and Westhead, E. W. (1955), *J. Polymer Sci. 16*, 273.
Morawetz, H., and Zimmering, P. E. (1954), *J. Phys. Chem. 58*, 753.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.
Thiers, R. E. (1957), *Methods Biochem. Analy. 5*, 273.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.
Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 109.
Wacker, W. E. C., Riordan, J. F., and Vallee, B. L. (1964), Abstr. 1st European Fed. Soc., London, March 23–25, p. 10.