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The Amino Acid Composition of Bovine Pancreatic Carboxypeptidase A*

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The amino acid composition of bovine carboxypeptidase A_α has been determined for the twice-crystallized enzyme prepared from purified procarboxypeptidase A. Internal standards were used to enhance the precision of the estimations of the amino acid composition and to permit the direct determination of the weight concentration of the protein. The weight concentration has been used for calculations of the absorbancy index and of the specific enzymatic activity towards two substrates, carbobenzoxyglycyl-L-phenylalanine and hippuryl-DL-phenyllactate. Carboxypeptidase A_α contains 307 amino acid residues. Its amino acid composition is similar to those of bovine carboxypeptidase A_γ and A_δ, except for five to seven amino acids which are lacking in the latter two enzymes, apparently by the deletion of a fragment from the amino terminal region of these molecules.

Crystalline carboxypeptidase A was isolated by Anson (1937) from an exudate of thawing glands of bovine pancreas, and by Allan *et al.* (1964) from an extract of an acetone powder of the freshly-collected glands. Most of the work reported in the literature on carboxypeptidase A has been carried out with one or the other of these two preparations (Neurath, 1960; Vallee *et al.*, 1963). More recently, Cox *et al.* (1964) developed a method of isolation of the enzyme based on the activation of the partially purified zymogen. This method yields carboxypeptidase A of a high specific activity even after the first crystallization. The present paper describes some of the chemical and enzymatic properties of this enzyme.

A precise amino acid analysis is a fundamental prerequisite for the elucidation of the primary structure of any protein, and such an analysis has been performed in the present work with the necessary accuracy and reliability by means of the technique of ion-exchange chromatography (Spackman *et al.*, 1958) augmented by the use of internal standards, as suggested by Walsh and Brown (1962). These measurements have provided a reliable measure of the concentration of solutions of the protein, which in turn was used as a basis for the calculation of certain chemical, physical, and biological parameters of the enzyme. The amino acid composition of this enzyme (carboxypeptidase A_α) has been compared with those of carboxypeptidase A prepared by the method of Anson (1937) and Allan *et al.* (1964), respectively.[1]

A_α Amino terminal alanine (prepared according to Cox *et al.*, 1964).
A_β Amino terminal serine (occurs in variable yield in all preparations).

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[1] Bovine pancreatic carboxypeptidase A, prepared by the various methods of isolation, comprises several different chemical species of the enzyme. The following differences have been characterized (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1963):

A_γ Amino terminal asparagine (occurs as major component in preparation according to Anson, 1937). In contrast to A_δ, the apoenzyme of this chemical species cannot be fully reactivated by zinc (Vallee *et al.*, 1960).
A_δ Amino terminal asparagine (occurs as major component in preparations according to Allan *et al.*, 1964). The apoenzyme of this chemical species can be fully reactivated by zinc (Vallee *et al.*, 1960).

Experimental Procedure

Materials

Carboxypeptidase A_α was isolated from purified procarboxypeptidase by the method of Cox *et al.* (1964) and crystallized twice. The crystals were stored at 4° as a slurry in 0.001 M potassium phosphate buffer, *p*H 7.5. Before use, the crystals were washed three times with cold distilled water, and dissolved in 1 M NaCl–0.001 M Tris, *p*H 8.0. Carboxypeptidase A_γ prepared according to Anson (1937) as modified by Putnam and Neurath (1946) was obtained from the Worthington Biochemical Corporation. Carboxypeptidase A_δ was prepared by the method of Allan *et al.* (1964).

Chromatographically pure CGP[2] and β-thienyl alanine[3] were obtained from Mann Research Laboratories, Inc. Hippuryl-DL-phenyllactate was synthesized in this laboratory by a modification of the procedure of Snoke *et al.* (1948). α-Amino-β-guanidopropionic acid was a product of the California Corporation for Biochemical Research, and norleucine was obtained from the Nutritional Biochemical Corporation.

For hydrolysis of proteins, concentrated HCl was used from freshly opened bottles of Baker & Adamson reagent grade chemical. Tris was purified according to Fossum *et al.* (1951), and recrystallized from ethanol-water solutions. A 2 M solution at *p*H 8.0 was passed through a column of Chelex-100 (Bio-Rad Laboratories) in order to remove the contaminating heavy metal ions.

[2] The following abbreviations are used: CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactic acid; AGP, α-amino,β-guanido propionic acid.

[3] β-Thienylalanine appears on the chromatogram between norleucine and tyrosine.

Hydrolysis and Amino Acid Analysis

Walsh and Brown (1962) have proposed the use of norleucine as an internal standard for amino acid analysis by the chromatographic technique of Spackman *et al.* (1958). This internal standard, which is added prior to hydrolysis and is completely stable, permits a correction for mechanical errors during the steps leading to analysis of protein hydrolysates. The protein hydrolysates were prepared as follows: to 2.5 ml of norleucine solution (containing 10 μmoles/ml in 0.1 N HCl) was added the required amount of alkali to neutralize the acid, 2.0 ml of 0.2 M NaCl–0.001 M Tris, *p*H 8.0, and 5.0 ml of the protein solution. Suitable aliquots of the protein-norleucine mixture containing about 9.2 mg of the protein were transferred to heavy walled test tubes, and enough water and HCl were added to give a final volume of 4.0 ml in 6 N acid. The tubes were chilled in an acetone–dry ice mixture until the solutions were almost frozen, and the solutions were then evacuated with an oil pump. The tubes were allowed to warm up slowly, sealed, and left at room temperature for 10 minutes before immersion in an *n*-butyl phthalate bath maintained at a constant temperature of 105° ± 0.5°. A total of eight tubes was used, corresponding to duplicates at four different hydrolysis times of 23.5, 47.5, 72.5, and 105.5 hours. After hydrolysis, the contents of the tubes were frozen overnight, then the hydrolysates were evaporated to dryness in a rotary evaporator under reduced pressure at 40–42°. The dried hydrolysate was dissolved in phosphate buffer at *p*H 6.8 and allowed to stand exposed to the air for 4 hours to oxidize cysteine to cystine.[4] The solution was then acidified and a suitable volume of 0.2 N sodium citrate buffer, *p*H 2.2, was added, as well as a standard mixture of both β-thienylalanine and AGP. Aliquots were analyzed in a Spinco Model 120 amino acid analyzer using a 150-cm column for acidic and neutral amino acids and a 15-cm column for the basic amino acids.

The internal standards, β-thienylalanine for the long column and AGP for the short column, were added to provide a measure of the daily reliability of the instrument, and to permit a daily correction for any difference in the analytical sensitivity between the long and short columns resulting from errors in pipetting, pump rates, ninhydrin stability, etc. A control run for the base line stability and another for the calibration with a synthetic amino acid mixture and the internal standards were made immediately before and after the analyses. The aliquots of the test samples were chosen to permit double readings of the "normal" and "suppressed" peaks, within the range of maximum accuracy of the absorbance scale for the standards and reference amino acids.

Calculations

In calculating the absolute amounts of amino acids per ml of stock protein solution, correction was first made on the basis of recovery of the daily standards, β-thienylalanine and AGP. After balancing the long and short columns, the yields of the amino acids were computed on the basis of recovered yields of norleucine. The following example illustrates the calculations. Protein solution 1.0 ml containing exactly 1.0 μmole of norleucine is hydrolyzed, and the dried hydrolysate is dissolved in a final volume of 5.0 ml of buffer containing exactly 1.0 μmole each of β-thienylalanine and AGP. Aliquots (2.0 ml) then contain 0.400 μmole of β-thienylalanine and 0.400 μmole of AGP. In a typical example, the recoveries on the long and short column are 0.395 and 0.410 μmole, respectively. The yields of the other amino acids, including that of norleucine, are then corrected by multiplying all yields of the long column by 400/395 and those of the short column by 400/410. This serves to ensure that the yields of the two columns are strictly comparable. Based on the recovery of norleucine in this aliquot, the amino acid concentrations corresponding to 1.0 ml of the original protein solution are calculated.

Determination of "Half-Cystine"

Vallee *et al.* (1960) have shown that metal-free apocarboxypeptidase A contains one sulfhydryl group, evidently derived from a cysteinyl residue, and Walsh *et al.* (1962) have demonstrated that, following reduction and alkylation, two *S*-carboxymethylcysteine residues can be found in an acid hydrolysate of the protein derivative. The term "half-cystine" is used herein strictly in an operational sense to denote the amino acids determined as cysteic acid in an acid hydrolysate of the performic acid oxidized protein or as *S*-carboxymethylcysteine in an acid hydrolysate of the protein after reduction followed by alkylation. The data thus obtained make no inferences concerning the state in which these amino acids and their derivatives exist in the native protein. The following procedures were used to estimate half-cystine.

Performic Acid Oxidation.—A solution of carboxypeptidase A_α in 0.2 M NaCl–0.005 M Tris, *p*H 8.0, was heated in a boiling water bath for 4 minutes. Vigorous shaking was maintained during heat denaturation to keep the insoluble protein in the form of fine suspension. After cooling, the precipitate was collected by centrifugation, washed twice with 0.1 M ammonium formate buffer, *p*H 3.5, and twice with distilled water, and was then lyophilized. The oxidation of the lyophilized sample was performed at 0° for 14 hours, according to Hirs (1956), and cysteic acid was determined after acid hydrolysis. No internal standards were used in the present case since the oxidized protein was insoluble; however, the quantitation was related to the recoveries of alanine, glycine, and phenylalanine in each analysis.

Reduction and Alkylation (Walsh et al., 1962).—To 140 mg of the enzyme in 30.0 ml of 1 M NaCl–0.1 M Tris, *p*H 8.0, was added 30.0 ml of freshly prepared solution of 8 M urea in the same buffer. Three hundred μl of β-mercaptoethanol was added to the protein solution, and the contents were kept at 0° for 1 hour. Iodoacetate (12.5 mmoles) in 4 M urea in the same buffer was added to the protein solution, and at the end of 2 hours at 0° the carboxymethylated protein was precipitated by the addition of acetic acid and heating in a boiling water bath for 3 minutes. The insoluble protein was washed three times with distilled water, and *S*-carboxymethylcysteine was estimated in an acid hydrolysate. To prevent losses through oxidation, pure nitrogen was bubbled through the protein sample for 10 minutes before the tubes were sealed for hydrolysis. The quantitation of carboxymethylcysteine was computed on the basis of the recovery of alanine and glycine in the hydrolysate.

Determination of Tryptophan

The tryptophan content of the protein was determined by the method of Bencze and Schmid (1957) from the ultraviolet absorption spectrum as a function of *p*H. Tryptophan was also determined according to the method of Patchornik *et al.* (1958) by following spectrophotometrically the oxidation of tryptophan residues after the addition of *N*-bromosuccinimide. The reagent (Arapahoe Chemical) was recrystallized

[4] Personal communication from Dr. S. Moore.

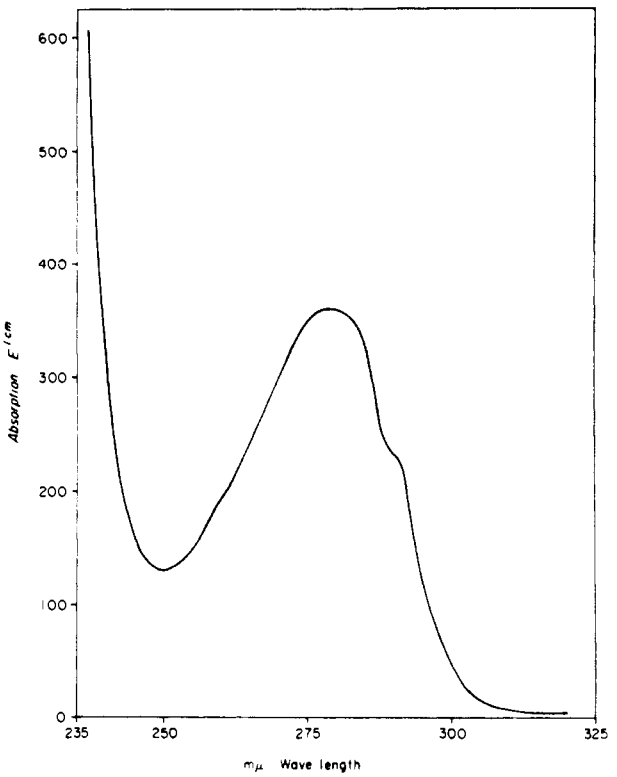

FIG. 1.—Ultraviolet absorption spectrum of carboxypeptidase A_α in 0.5 M Na Cl and 10^{-2} M Tris, *p*H 7.0.

from acetone-water solution before use, and the oxidation was done in 10 M urea adjusted to *p*H 4.0 with acetic acid.

Elemental Sulfur Analysis

Crystals of carboxypeptidase A_α were washed three times with distilled water and twice with acetone, and were finally dried *in vacuo* at 60° over NaOH pellets. Fifty mg of the dry powder, exactly weighed, was used for sulfur analysis according to the method of Alicino (1958), except that barium perchlorate was used instead of barium acetate. In order to apply a correction for the moisture content of the protein, a known amount of the same powder weighed at the same time was hydrolyzed in 6 N HCl with norleucine as internal standard, and amino acids were analyzed as previously described.

Ultraviolet Absorption and Enzyme Activity

A stock solution of carboxypeptidase A_α containing an exact amount of norleucine was diluted with 0.5 M NaCl–0.01 M Tris, *p*H 7.0, and the absorption was measured in a Zeiss PMQ II spectrophotometer within the range of 235–320 mμ against the appropriate blank without the protein (Fig. 1). The curve was drawn manually from duplicate readings 5 mμ apart on the edges of the peak, and each $^1/_2$ mμ for the peak itself. Six independent dilutions, corresponding to absorbancy values of 0.070–0.400 at the maximum, were used for the measurements, and aliquots of the same diluted samples were used for the determinations of enzyme activity. The exact concentration of the protein in the stock solution was determined in terms of the alanine content as described above. The molar concentration of the protein was calculated from the number of residues of alanine in the protein.

Peptidase activity towards CGP was measured by the method of Keller *et al.* (1956). Esterase activity measurements were made by recording the rate of hydrolysis of the substrate HPLA. Difficulties in the crystallization of the L-isomer account for the use of the racemate. The conditions for the assay, as described here, have been established empirically and applied for routine tests in this laboratory for the past three years, with a reproducibility greater than 4% (provided that the temperature of reaction was rigorously controlled [±0.1°]). One assay only requires 5–10 minutes.

The substrate solution is prepared by dissolving the sodium salt of racemic HPLA (or the free acid) in buffer, *p*H 7.5, the final composition being 10^{-2} M HPLA, 5×10^{-3} M sodium Veronal, and 4.5×10^{-2} M NaCl. This solution is stored frozen in aliquots of 3 ml each, the volume required for one assay. A *p*H-stat comprising a TTT-1 Radiometer Autotitrator and a recorder (Ole Dich, Copenhagen) is used. The *p*H is controlled at 7.50 with standard sodium hydroxide solution (0.07–0.1 N), at 25°. The enzyme is added as a small aliquot of 10–150 μl of a solution containing 0.02–0.5 mg/ml of enzyme, in order to minimize dilution. Since this concentration corresponds to that required for absorbancy measurements, specific activities can be determined directly from these solutions.

TABLE I

AMINO ACID COMPOSITION[a] OF HYDROLYSATES OF CARBOXYPEPTIDASE A_α

Amino Acid	Hours of Hydrolysis							
	23.5	23.5	47.5	47.5	72.5	72.5	105.5	105.5
Lysine	14.8	15.1	15.4	15.2	15.0	14.8	15.0	14.7
Histidine	8.0_5	8.0_4	8.3_5	8.2_1	8.1_6	8.0_9	8.3_7	8.1_5
Arginine	11.2	11.1	11.4	11.2	11.0	10.9	11.2	10.9
Aspartic acid	27.8	27.6	27.4	27.7	27.5	27.5	27.2	27.2
Threonine	25.1	25.3	24.0	23.9	23.3	23.1	22.7	22.3
Serine	30.0	29.6	27.7	27.3	25.7	25.6	24.0	23.6
Glutamic acid	24.5	24.6	24.1	24.1	24.1	23.9	23.7	24.0
Proline	9.6_3	9.8_3	9.6_9	9.7_3	9.6_3	9.7_1	9.7_4	9.6_6
Glycine	22.6	22.4	22.2	22.6	22.6	22.3	22.4	22.5
Alanine				(20.0)				
Valine	14.3	14.4	14.9	15.3	15.5	15.8	15.7	15.8
Methionine	2.9_7	2.9_4	3.0_3	3.0_1	3.0_0	3.0_8	2.9_4	2.9_5
Isoleucine[b]	17.7	17.7	19.4	19.6	20.1	20.2	20.4	20.3
Leucine	23.3	23.2	23.2	23.4	23.0	23.2	23.4	23.1
Tyrosine	19.4	19.1	19.2	19.4	19.0	18.9	19.2	19.0
Phenylalanine	16.1	15.9	16.2	16.1	15.9	15.9	16.3	15.9

[a] The data are expressed as amino acid residues per molecule, assuming 20 residues of alanine per molecular weight of 34,600. [b] *allo*-Isoleucine included (peaks measured by paper-weight method, Yamasaki *et al.*, 1963).

TABLE II
AMINO ACID COMPOSITION OF CARBOXYPEPTIDASE A_α

Residues	Mean ($\bar{X}$) Residues/ Molecule	$\frac{100}{\bar{X}}$ ± %	Nearest Integral Number Residues/ Molecule
Lysine	15.00	1.54	15.0
Histidine	8.18	1.59	8.0
Arginine	11.11	1.53	11.0
Aspartic acid[a]	27.9	0.42	28.0
Threonine[a]	27.8	0.68	28.0
Serine[a]	33.0	0.73	33.0
Glutamic acid[a]	25.0	0.56	25.0
Proline	9.70	0.62	10.0
Glycine	22.45	0.85	22.5
Alanine	20.0	0.58	20.0
Valine[b]	15.8	1.08	16.0
Half-cystine[c]			2.0
Methionine	2.99	1.67	3.0
Isoleucine[b]	20.3	0.59	20.0
Leucine	23.22	0.65	23.0
Tyrosine	19.15	0.99	19.0
Phenylalanine	16.04	1.00	16.0
Tryptophan[d]	7.82		8.0
Total:			307–308

[a] Extrapolated to zero time of hydrolysis (Fig. 2). [b] Extrapolated to 110 hours of hydrolysis (Fig. 2). [c] See Table III. [d] Mean of 3 determinations.

Under these experimental conditions, the reaction rate is linear for more than 60% of the hydrolysis of the ester and the data can be treated in terms of zero-order kinetics (Putnam and Neurath, 1946), despite the anomalies described by Snoke *et al.* (1948).

RESULTS

Amino Acid Composition.—Table I lists the recoveries of amino acids from the hydrolysates of carboxypeptidase A_α at various time intervals; Table II summarizes these composition data. In calculating the number of residues, the assumption was made that one molecule of the enzyme contains 20 residues of alanine. The selection of this amino acid as a reference was prompted by practical considerations. Alanine is stable during acid hydrolysis, and its content is such that it can be accurately estimated in all the analyses with a minimum standard deviation. The choice of 20 alanine residues is also substantiated by the following observations: (a) The molecular weight of carboxypeptidase A as determined by sedimentation analysis (Smith *et al.*, 1949; Smith and Stockell, 1954; Cox *et al.*, 1964; Neurath, 1960) is in good agreement with the molecular weight calculated from the composition based on 20 alanine residues per molecule. (b) For each amino acid residue, the difference between the experimental mean and the nearest integral number is at the minimum at this value for alanine residues. (c) Twenty alanine residues provide the best agreement with the molecular weight determined from the residues that occur in small amounts, i.e., three methionine and two half-cystine residues. The chosen value for alanine is consistent with the total sulfur content of the protein.

The scattering of the experimental results was calculated in terms of standard deviation (with $N - 1$ degrees of freedom), and the mean values for amino acids were expressed as number of residues per molecule of the protein (Table II). When the standard deviations were expressed as percentage of the mean of the number of residues, they differed from this mean by less than 1.7% for all the amino acids.

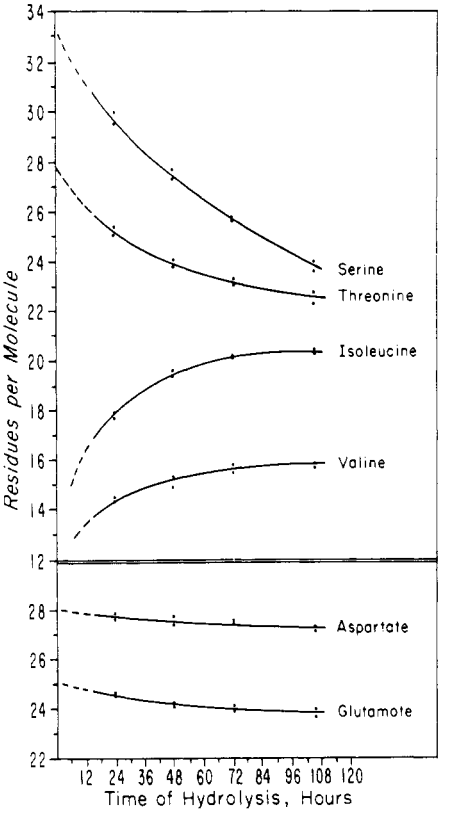

FIG. 2.—Recoveries of unstable amino acids and of valine and isoleucine as a function of time of hydrolysis.

Three groups of amino acids may be distinguished on the basis of their recoveries with respect to time of hydrolysis. (1) Alanine, phenylalanine, glycine, leucine, and tyrosine are stable even up to 105.5 hours of hydrolysis. Carpenter and Chrambach (1962) pointed out recently that tyrosine is the only amino acid which exhibits zero-order rate of decomposition and that this loss might be mediated by oxygen and heavy metal contaminants. In the present analysis, precautions were taken to exclude as much oxygen as possible during hydrolysis, and heavy metal contamination was kept at a minimum by working with metal-free reagents and metal-free surfaces (Thiers, 1957). Under these conditions tyrosine appeared to be stable during acid hydrolysis. (2) Isoleucine and valine show a regular increase approaching a maximum level asymptotically after 50 hours of hydrolysis (Fig. 2). (3) Glutamic and aspartic acids are destroyed very slowly within 105.5 hours, the loss corresponding to 4% of the zero time or approximately one residue per molecule.

As expected, the hydroxyamino acids, particularly serine, are destroyed more rapidly. Within the time limits of the observations, the decomposition of serine averages 1.8 residues per day and that of threonine 0.8 residue per day. The rate of decay is not actually zero order, as is apparent from the curvature of the experimental data (Fig. 2). A plot of $\log_e$ concentration vs. time (Fig. 3) shows that the measurements do not fit straight lines defined by the least squares method, even when the experimental range of scatter of $|2\ \sigma|$ is allowed for, indicating that the decay does not obey first-order kinetics. The obvious curvature in both diagrams demonstrates that the order is higher than one and therefore the extrapolated numbers are too low. Since the average daily destruction (7.5 σ for serine and 4.2 σ for threonine) exceed in both cases

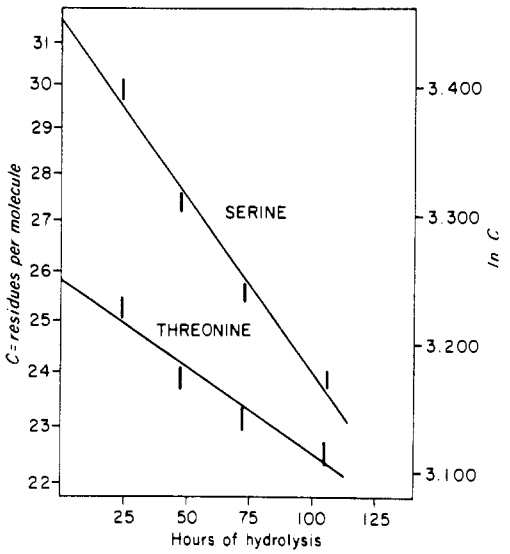

FIG. 3.—Lack of correlation of the rate of destruction of serine and threonine in a first-order plot. The intervals of variation corresponding to duplicate determinations cover a range of $-\sigma$ to $+\sigma$ around the mean of each pair of measurements. The straight lines are those defined by the application of the least squares method.

the experimental range of scatter, a more adequate extrapolation appears to be justified.

The general equation for reactions of nth order,

$$-\frac{d(c)}{dt} = k(c)^n \qquad (1)$$

can be applied to the decay curves of serine and threonine, where c is the concentration (in number of residues per molecule) at time t. If the order of the reaction proves to be constant throughout the time limits of observations, a particular value can be assigned to n for which the coordinates of the experimental points will fall on a straight line (W. O. McClure, unpublished). This allows a linear extrapolation to be applied beyond the first point (c_0 at time t_0 = 23.5 hours). From the integrated form of equation (1), two working expressions have been defined:[5]

for serine: $c^{-2.896} = c_0^{-2.896} + \chi t$

$$(\chi = 5.82 \times 10^{-7}) \qquad (3)$$

for threonine: $c^{-10.65} = c_0^{-10.65} + \chi t$

$$(\chi = 3.395 \times 10^{-17}) \qquad (4)$$

The curves in Figure 2 are the graphical translation of these equations, and their close fit to the experimental points is evident.

Since the chemical processes initiating the partial destruction of the hydroxyaminoacids during the early hours of hydrolysis are not completely understood, some uncertainty remains as to what limit of time the data should be extrapolated (Rees, 1946; Desnuelle and Bonjour, 1951). Conventionally, the recoveries of serine and threonine are extrapolated to zero time and this value is taken as the actual content. According to equation 3, the initial values of serine at time zero, and after 1, 2, and 3 hours of hydrolysis, are 33.1_2, 32.9_6, 32.7_8, and 32.6_2 residues per molecule, respectively. They converge towards 33 residues per molecule, a value which corresponds to that obtained after 1 hour of hydrolysis at 105°. The comparable numbers for threonine obtained from equation 4 are 28.0, 27.8, 27.6, and 27.4 residues per molecule, respectively. They approach 28, a value which is consistent with the normal range of scatter up to 2–3 hours of hydrolysis, and can be taken as an estimate of the initial composition.

All the amino acids gave values very near integral numbers except glycine, which gave consistently 22.5 residues.

The tryptophan content as determined by the method of Bencze and Schmid (1957) amounts to 7.95 residues per molecule. This is in good agreement with the value of 7.72 residues obtained by a modification (Yamasaki *et al.*, 1963) of the method of Goodwin and Morton (1946). A value of 7.78 residues was obtained by the procedure of Patchornik *et al.* (1958). In the latter method, the assumption was made that the absorbancy of free tryptophan and of tryptophan residues in the protein are the same. All three of these methods gave values approximating 8 tryptophan residues.

Distribution of Sulfur.—The results in Table III summarize the distribution of sulfur-containing amino acids in the protein. The analyses by various methods are consistent with 2 residues of half-cystine and 3 residues of methionine; the elemental sulfur content of the protein is entirely accounted for by methionine and half-cystine.

Molecular Weight.—From the data of Table II, a molecular weight for the protein was derived and a maximum error assigned to it. Depending upon the number of residues of glycine (22 or 23), the total number of residues in the protein is either 307 or 308. The corresponding molecular weight of the protein (carboxypeptidase A_α), including 1 gram atom of zinc per molecule (Vallee and Neurath, 1955), would be 34,679 or 34,622. This value does not include the amide groups as these have not been determined in this work. Since each substitution of a carboxyl by an acid amide group decreases the molecular weight by only one, a molecular weight of 34,600 can be assigned to the protein with an error of ±80, or less than 0.3%. This value is in close agreement with a value of 34,300 obtained by sedimentation and diffusion methods (Smith *et al.*, 1949) for carboxypeptidase A_γ.

Ultraviolet Absorption.—The absorption spectrum of carboxypeptidase A prepared from purified zymogen is shown in Figure 1. The maximum of the curve is located at 278 mμ. A plot of protein concentration versus absorbancy at the maximum, at various dilutions of the sample, was made and the slope was determined by a least squares method for a total of twenty-one readings with two instruments used at random. A t test verified that the intercept of this line did not depart from the origin. Assigning 95% confidence limit, and with standard deviations calculated with $(N - 2)$ degrees of freedom, the extinction coefficients of the protein are:

$$E^{278}_{\text{mg}\cdot\text{ml}^{-1}} = 1.88 \pm 0.01 \text{ mg}\cdot\text{ml}^{-1}\cdot\text{cm}^{-1}$$

V (coefficient of variation) = 0.3%

$$E^{278}_{\text{mM}} = 64.9 \pm 0.2 \text{ mmole}\cdot\text{ml}^{-1}\cdot\text{cm}^{-1}$$

V (coefficient of variation) = 0.6%

[5] The values of the exponents have been determined by a generalized method of half-times, applied to the progress curves within the range of the known values. The following expression was derived from equation 1:

$$\log (t_{i+1} - t_i) = (1 - n) \log c_i + \text{constant} \qquad (2)$$

where c_i is the number of residues of serine or threonine at time t_i. A series of at least 5 decrements were taken from the origin, as c_i to $\beta c_i = c_{i+1}$. An arbitrary constant, β, is usually slightly smaller than 1. A plot of log Δt_i versus log c_i verified that in both cases the experimental points were in a linear array.

TABLE III
DETERMINATION OF HALF-CYSTINE

Compound	mg S/100 mg Protein	Number of Measurements	Number of Residues per Molecule: Mean[a] ($\bar{X}$)	Number of Residues per Molecule: Standard[b] Deviation $\pm\sigma$
1. *Sulfur*, elementary analysis on dry weight basis	0.471 0.456	2		
On amino acid analysis basis	0.467 0.452		4.99 4.86	— —
2. *Oxidized* carboxypeptidase A_α (performic oxidation)		4		
Cysteic acid			1.99[c]	0.02
Methionine sulfone			2.64[c]	—
3. *Reduced* + *carboxymethylated* (mercaptoethanol, iodoacetamide)		4		
Carboxymethylcysteine			1.89	0.06
Methionine			2.60	0.23
Homoserine lactone *S*-carboxymethylhomocysteine Homoserine			Traces[d]	

[a] Based on 20 alanine, 16 phenylalanine per molecule of carboxypeptidase A_α (residues per molecule). [b] Standard deviation with $(N - 1)$ degrees of freedom. [c] In control analysis performed after oxidation of a standard mixture of amino acids, the recoveries were 0.96 residues of cysteic acid and 0.79 residues of methionine sulfone per equivalent. [d] Traces were identified in all the chromatograms, but not quantitated; partial alkylation of methionine occurred to various extents, since operational conditions for alkylation were not identical.

TABLE IV
COMPARISON OF AMINO ACID COMPOSITION OF CARBOXYPEPTIDASE A PREPARED BY DIFFERENT METHODS

Amino Acid	A_α[a]	A_γ[b]: Mean Value $\bar{X}$	A_γ[b]: 100 $\sigma/\bar{X}$ (%)	A_γ[b]: Nearest Integers	A_δ[c]: Mean Value $\bar{X}$	A_δ[c]: 100 $\sigma/\bar{X}$ (%)	A_δ[c]: Nearest Integers
Lysine	15	15.01	2.9	15	14.57	4.8	14–15
Histidine	8	7.71	1.9	8	7.69	1.8	8
Arginine	11	9.88	2.3	10	9.63	2.6	10
Aspartic acid	28	26.50	1.8	26–27	26.90	1.9	26–27
Threonine	28	23.40	1.6	23–24	24.40	2.0	24–25
Serine	33	30.00	1.1	30–31	29.80	2.0	29–30
Glutamic acid	25	24.93	1.6	24–25	25.00	0.8	25
Proline	10	9.83	1.3	10	9.61	4.2	10
Glycine	22.5	22.45	2.3	22.5	22.48	1.8	22.5
Alanine	20	19.20	—	19	19.20	—	19
Half-cystine	2			2	1.96	9.3	2
Valine	16	15.60	1.2	16	16.10	1.3	16
Methionine	3	2.67	1.6	3	3.00	3.5	3
Isoleucine	20	19.80	1.0	20	19.90	2.4	20
Leucine	23	22.90	1.0	23	22.40	1.7	22–23
Tyrosine	19	18.80	1.3	19	19.10	1.3	19
Phenylalanine	16	14.56	1.3	15	15.44	1.2	15–16
Tryptophan	8			8	7.90		8

[a] Cox *et al.* (1964). [b] Anson (1937). [c] Allan *et al.* (1964).

The latter value is based on a molecular weight of 34,600 ± 80. It is very close to that obtained recently for carboxypeptidase A_δ by Simpson *et al.* (1963) using a different procedure.

Enzyme Activity.—The rate of hydrolysis of CGP was determined at twelve different concentrations of the enzyme covering the range of 0.6×10^{-4} to 23×10^{-4} mg·ml^{-1} of protein. Progress curves for the release of phenylalanine versus time involved at least four points and the reaction did not exceed 18% of hydrolysis of the substrate. A first-order plot was established for each run, and the first-order rate constant was related to each enzyme concentration as the slope of the least squares line. Expressed as first-order rate constant in decimal logarithms, the proteolytic coefficient for the enzyme is 3.19 ml·mg^{-1}·min^{-1} of carboxypeptidase A_α. The proteolytic coefficient C computed on a molar basis is equal to 110.1 ml·μmole^{-1}·min^{-1} of carboxypeptidase A_α at 25°, *p*H 7.5, and an initial substrate concentration of 0.025 M.

The rate of hydrolysis of HPLA was determined at three dilutions of the sample which were made up for absorbancy measurements. The apparent zero-order rate constant, as the average of six measurements, $k_{0(app)}$ is equal to 0.208 μmole·min^{-1}·μg^{-1} of carboxypeptidase A_α (coefficient of variation, 4%) concentration of the L-substrate of 0.005 M at 25° and *p*H 7.5.

Comparison with Carboxypeptidases Prepared by Other Methods.—Table IV presents a comparison of the amino acid composition of carboxypeptidase A_α with those of carboxypeptidases A_δ and A_γ. The experimental data for the latter two preparations show a slightly larger scatter than for carboxypeptidase A_α. For both carboxypeptidase A_δ and carboxypeptidase A_γ the protein concentration was first established on the arbitrary basis of 20 residues of alanine per mole-

cule, and the mean values of the different analyses were compared with the nearest integral numbers in each case, taking the standard deviation as an estimate of the variation. On this basis, the composition pattern of both carboxypeptidase A_δ and carboxypeptidase A_γ revealed that most of the data deviated quite markedly from the results in Table II (carboxypeptidase A_α) and that, for 6 or 7 residues on both preparations, the results could no longer be defined by a unique integer. Moreover, the deviations appeared to have the same direction in all cases. Calculations showed that a basis of 19.2 (Bargetzi *et al.*, 1964) residues of alanine in both carboxypeptidase A_δ and carboxypeptidase A_γ would give the most favorable conditions whereby all the experimental means would best approach the nearest integral numbers. Because of an increased scatter, only linear extrapolation was made to correct for the destruction of serine and threonine in the case of carboxypeptidase A_δ and A_γ, and the intercept at zero time was calculated by the method of least squares. As a result of this approximation, the values for these amino acids in both A_δ and A_γ may be somewhat low. The destruction of aspartic and glutamic acids is not apparent, except by larger standard deviations.

Discussion

Smith and Stockell (1954) reported the amino acid composition of carboxypeptidase A prepared by the method of Anson (1937) (A_γ). Considering that automation and refinement of the techniques of amino acid analysis have been introduced since that time, the agreement between their values and the present data is remarkably good.

A cursory comparison of the data presented in Table IV indicates that the three preparations are very similar. Indeed, this was to be expected since the enzyme was isolated from the same source, though by different procedures. However, a careful examination of the data revealed some significant differences in the composition of the enzyme prepared by the method of Cox *et al.* (1964) (A_α) and those prepared by the methods of Anson (A_γ) and of Allan *et al.* (1964) (A_δ), respectively. These differences are: alanine (1 residue), arginine (1 residue), aspartic (1 residue), serine (1–2 residues), and threonine (1–2 residues). It is probable that carboxypeptidase A_α may contain these residues in an additional sequence at either end of the molecule. This conclusion presumes that the activation of crude extracts containing procarboxypeptidase A (yielding A_δ and A_γ) and of the purified zymogen (yielding A_α) each proceeds by slightly different pathways. Recent data from this laboratory (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1963) strongly support this possibility as they indicate that the enzyme (A_α) prepared from the purified zymogen contains an N-terminal alanine in contrast to asparagine found for A_γ (Thompson, 1953) and for carboxypeptidase A_δ (Coombs and Omote, 1962). The significance of this difference at the amino terminus, particularly as it relates to its involvement in the binding of zinc, remains to be elucidated. The high precision and reliability of the amino acid analysis, as described in the present paper, have enabled the identification of some subtle but significant differences between seemingly identical proteins derived from the same source by different methods of preparation.

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