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Action of Carboxypeptidase-A on Bovine Insulin: Preparation of Desalanine-Desasparagine-Insulin*

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In order to make use of carboxypeptidase-A as a tool for determining the differences between various insulins (see following paper), it was necessary to develop conditions which would cleave completely the carboxyl-terminal amino acid residues from insulin. With this view in mind, bovine insulin has been subjected to the action of carboxypeptidase-A under a variety of conditions. The rates of release of the carboxyl-terminal amino acids (1) are much greater for zinc-free insulin than for zinc-insulin and (2) decrease with increasing salt concentration at *p*H 7.4. For carboxyl-terminal alanine and asparagine the rate of release increases from *p*H 7.4 to 9.4, while for carboxyl-terminal aspartic acid the rate of release decreases dramatically over this *p*H range. The action of the enzyme on insulin ceases after the removal of one mole of alanine from the carboxyl-terminal position of the B-chain and one mole of asparagine (or aspartic acid) from the carboxyl-terminal position of the A-chain. Conditions were developed for the complete removal of the carboxyl-terminal amino acids to give desalanine-desasparagine-insulin. The latter has been characterized by countercurrent distribution, amino acid composition, and biological activity; it possesses at best less than 5% of the activity of native insulin in the mouse convulsion test.

Lens (1949) was the first to use carboxypeptidase to determine the carboxyl-terminal group of a protein and reported the release of alanine by the action of the enzyme on insulin. Later Harris (1952) reported the release of asparagine as well as alanine and assigned these amino acids to the carboxyl-terminal positions of the A- and B-chains (Sanger, 1949; Ryle *et al.*, 1955), respectively, of insulin. Although an amount of alanine almost equimolar to the insulin was produced by the action of the enzyme, the asparagine was released at a much slower rate and in a total amount equal to only about 20% of the theoretical yield (Harris and Li, 1952). Nicol and Smith (1956) and Nicol (1960) encountered similar difficulties in securing a complete hydrolysis of zinc-insulin but reported nearly complete liberation of alanine and asparagine, as measured by the spot dilution method (Polson *et al.*, 1947), when acetyl-insulin (Fraenkel-Conrat and Fraenkel-Conrat, 1950) was used as a substrate for the carboxypeptidase.

The present studies were initiated in order to find conditions for the complete release of the carboxyl-terminal amino acids from insulin. As it turned out, most samples of crystalline zinc-insulin of bovine origin gave aspartic acid as well as asparagine and alanine upon treatment with carboxypeptidase-A. This fact had been noted previously (Harris, 1952) but had been attributed to partial hydrolysis of the asparagine during the performance of the procedures used to detect the amino acids released by the action of the enzyme. The present investigations were facilitated by the availability of a sample of insulin which, upon digestion with carboxypeptidase, gave about an equal amount of aspartic acid and asparagine. The sum of the amounts of aspartic acid and asparagine was equal to the amount of alanine released, which in turn amounted to one mole per mole of insulin (m.w. 6000). As shown in a subsequent paper, this particular sample of insulin was made up of about equal amounts of insulin-A and desamido-insulin (Harfenist and Craig, 1952; Harfenist, 1953). By the use of this sample, the effect of several variables on the rate of release of each one of the carboxyl-terminal amino acids was followed in the same experiments.

Of particular importance to the performance of the present experiments was an observation first made by Hill and Smith (1957), who had reported that zinc-insulin was particularly resistant to the action of leucine aminopeptidase, while zinc-free insulin was attacked at a moderate rate. We have made a similar observation in our studies on the action of trypsin on insulin (Young and Carpenter, 1961). Preliminary experiments indicated that zinc-free insulin, prepared by precipitation from acid solution as its hydrochloride, was much more susceptible to attack by carboxypeptidase than zinc-insulin. Consequently, insulin hydrochloride rather than zinc-insulin was used as starting material in all of these studies.

Although Harris and Li (1952) had not been able to secure complete cleavage of asparagine from insulin,

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they had noted a decrease in biological activity (by the mouse convulsion test) accompanying the release of asparagine. Nicol (1960) had reported that the material obtained by the action of carboxypeptidase on acetyl-insulin possessed less than 10% of the biological activity of the original acetyl-insulin, as judged by its ability to lower the blood sugar level of rabbits. Since in these previous studies no products were isolated and characterized, the results on biological activity were subject to several interpretations. One of the goals of this study was to prepare desalanine-desasparagine-insulin in a homogeneous state for chemical and biological characterization.

Experimental

Materials.—Bovine carboxypeptidase which had been treated with diisopropylfluorophosphate was obtained as a suspension of crystals in water from Worthington Biochemical Corporation (Lots No. 629–30 and 6110). Enzyme solutions were prepared by diluting one volume of stock suspension with ten volumes of 10% LiCl and stirring for several hours at 4°. The concentration of enzyme was obtained from optical density measurements at 278 mμ using the value of 1.94 for a 1-cm thick solution of 1 mg/ml (Vallee *et al.*, 1960). Bovine insulin was supplied by Eli Lilly and Company (Lots No. PJ 3371, PJ 3026 and PJ 4812). Samples were converted to their hydrochlorides by precipitation from 0.25 N HCl solution with acetone (Carpenter, 1958).

Amino Acid Analysis.—Amino acid analyses of digests were determined by the method of Spackman, Moore, and Stein (Spackman *et al.*, 1958; Moore *et al.*, 1958) with a Beckman/Spinco Amino Acid Analyzer, Model 120. Routine analyses of aspartic acid, asparagine, and alanine in the enzyme digests were performed on the 50-cm column. The amino acid compositions of insulin and desalanine-desasparagine-insulin were determined on samples which had been hydrolyzed for 6 hours at 120° (Carpenter and Chrambach, 1962) in 0.1% solution in constant boiling HCl. The acid was removed on a rotary evaporator and the residue dissolved in *p*H 2.2 buffer (Moore *et al.*, 1958) for introduction on the column.

Digestion of Insulin by Carboxypeptidase.—Digestions were performed either in buffered solutions or in a *p*H-Stat (Difunctional Recording Titrator, International Instrument Company, Canyon, Calif.). Buffered digestions were performed with a mixed buffer of Tris (Sigma 121) and 2-amino-2-methyl-1,3-propanediol (Eastman Kodak). The buffer was prepared by mixing 125 ml of 0.2 M solutions of each constituent, adjusting to the desired *p*H with 1.0 N HCl, and diluting to 1 liter with distilled water. The temperature of the digestion was maintained at 25° or 37°. The digestion mixtures were stirred magnetically. Insulin concentrations varied from 0.25 to 2 mM and protein weight ratios of insulin to carboxypeptidase were approximately 25:1. Digestions were stopped by the addition of 0.1 N HCl until the *p*H was lowered to between 1 and 2 and the resulting solutions were made up to known volumes (5 to 10 ml). The acidified digests were kept at −10° until amino acid analyses were performed. A volume was taken for amino acid analysis which was estimated to contain 0.2 to 0.5 μmoles of alanine. In the digestions performed in the *p*H-Stat, a solution of insulin-HCl was prepared in distilled water and the *p*H adjusted to the desired value by the addition of 0.1 N NaOH. Nitrogen was passed over the surface of the solution, and the digestion mixture was stirred magnetically. After the *p*H had been maintained for approximately 15 minutes, sufficient carboxypeptidase solution was added to give a weight ratio of insulin to carboxypeptidase of about 25:1. Toluene (50 μl) was added routinely as a preservative to digestions at *p*H 7.4 that proceeded for more than 4 hours. At higher *p*H values, toluene addition was not necessary. Digestions were performed at *p*H 7.4, 8.4, and 9.4. Aliquots of the digestion mixture were removed at various time intervals, acidified with 0.1 N HCl, and stored at −10° until amino acid analyses were performed.

Preparation of Desalanine-Desasparagine-Insulin.—Insulin-HCl (300 mg prepared from PJ 4812) was dissolved in 35 ml of buffer at *p*H 7.4 and the *p*H was adjusted to 7.4 with 0.1 N NaOH. Carboxypeptidase (15 mg in 1.5 ml) and toluene (0.1 ml) were added and the resulting solution was incubated at 37° for 4 hours, at which time an additional 7.5 mg of carboxypeptidase was added. After an additional 4 hours of digestion, an aliquot was removed for amino acid analysis and the remainder of the digest was acidified by the addition of 1.0 N HCl (1 ml). The resulting mixture was centrifuged and the clear supernatant was applied to a dextran gel column (Sephadex G-25, coarse), which was 3 cm in diameter and 32 cm in height. Elution was performed with 0.01 N HCl. The eluate was collected in 7-ml fractions and the location of the desalanine-desasparagine-insulin was determined by optical density at 277 mμ. The proper tubes were pooled and the volume was concentrated to 13 ml on a rotary evaporator with a bath temperature of 25° and with the receiver cooled in a dry ice–isopropanol bath. The sample was isolated as the hydrochloride by precipitation with 18 volumes of acetone. The precipitate was washed twice with ether (U.S.P.) and dried over P_2O_5 to give 200 mg of product.

Countercurrent Distribution.—The solvent system employed was very similar to one reported by Harfenist and Craig (1952). 2-Butanol was purified by refluxing over and distilling from calcium hydride (Carpenter, 1958). Only that portion of the distillate was used which had an optical density of 0.05 or less when read against distilled water at 277 mμ in a 1-cm cuvet. Dichloroacetic acid (DCA) was purified by distillation. A solution of dichloroacetic acid (1.56% w/v) was equilibrated with an equal volume of 2-butanol, and equal volumes (10 ml) of upper and lower layer were used. Desalanine-desasparagine-insulin (735 mg) was dissolved in 90 ml of equilibrated lower layer and introduced into the first nine tubes of a 500-tube instrument. After the performance of 2350 transfers, the desalanine-desasparagine-insulin fraction was located by measurement of optical density at 277 mμ of the lower layers. The upper and lower layers of the appropriate tubes (see Fig. 5) were pooled and enough 6 N HCl was added to make the aqueous layer 0.1 N with respect to HCl. Ether (U.S.P.) from a newly opened can was added in a volume equal to three times that of the upper layer. After the phases had separated, the aqueous phase was extracted twice more with an equal volume of ether. The aqueous layer was brought to *p*H 3.5 by the slow addition of a weak base ion-exchange resin (Amberlite-IR-45) in the carbonate form. The resin was removed by filtration and the filtrate concentrated to one-fiftieth of its volume on a rotary evaporator with a bath temperature of 25° and with the receiver cooled in a mixture of dry ice and isopropanol. The material was precipitated as the hydrochloride by the addition of 18 volumes of acetone. The yield of the material in Fraction A was 280 mg. Nitrogen analysis gave a value of 14.5% after correction for 4.6% moisture. The theoretical value is 15.0% nitrogen, assuming the

molecular weight of the dry hydrochloride to be 5767.

Oxidation of Desalanine-Desasparagine-Insulin and Determination of Carboxyl-Terminal Cysteic Acid.—Desalanine-desasparagine-insulin was oxidized according to the method of Hirs (1956). Performic acid was prepared by allowing a solution of hydrogen peroxide (0.5 ml 30% H_2O_2, Merck Superoxol) and formic acid (9.5 ml, 97%, Eastman Kodak) to stand at room temperature in a stoppered flask for 2 hours. Desalanine-desasparagine-insulin (40 mg) was dissolved in 1.0 ml of 97% formic acid and anhydrous methanol (0.2 ml) was added with stirring. Performic acid solution (2 ml) was added to the insulin solution and the mixture was allowed to react for 2.5 hours at −7 to −10°. The tube was then rinsed with 5 ml of ice water into a flask containing 70 ml water at 0°. The diluted solution was frozen and the solvent removed by lyophilization. A portion of the oxidized material was taken for amino acid analysis. Another portion was treated with carboxypeptidase at *p*H 7.4 and 37° under conditions similar to those described above and then subjected to amino acid analysis.

Assay of Carboxypeptidase.—Carboxypeptidase was assayed by the method of Young (1959). The decrease in the optical density at 230 mμ of a solution of *N*-acetyl-L-phenylalanyl-L-phenylalanine (0.001 M in 0.02 M Tris buffer at *p*H 7.8) was used to follow the action of the enzyme. The rate of change of absorption per minute was a linear function of the enzyme concentration for amounts between 0.2 and 1.0 μg per 1.5 ml of assay solution.

Paper Chromatography.—Paper chromatography was performed by the descending technique with Whatman 3HR paper and the following solvent systems: (A) *n*-butanol–95% ethanol–NH_4OH–H_2O (4:4:1:1) and (B) *n*-propanol–0.05 M sodium pyrophosphate in 0.1 M NaCl at *p*H 7.3 (70:30) (Pechère *et al.*, 1958).

Amino-Terminal Analysis.—Insulin and desalanine-desasparagine-insulin were degraded by a modified phenylisothiocyanate procedure (Edman, 1950). A solution of the derivative (10 mg in 4 ml of 50% aqueous pyridine) was stirred vigorously at 40° during treatment with 25 μl of phenylisothiocyanate for 1.5 hours, while the *p*H was maintained at 9 by the addition of 0.1 N NaOH from a *p*H-Stat. The derivatized protein was precipitated by adjustment of the *p*H to 5, collected by centrifugation, and washed twice with ether (U.S.P.). The dry residue was dissolved in 2 ml of anhydrous trifluoroacetic acid (Eastman Kodak, redistilled) and allowed to stand at room temperature for 3 hours (Guidotti *et al.*, 1962). The trifluoroacetic acid was removed on a rotary evaporator and the residue taken up in 1 ml of water. Acetone (20 ml) was added and the white precipitate that formed was allowed to stand overnight at 4°. The mixture was centrifuged and the precipitate washed twice with 10 ml of ethyl acetate and dried over P_2O_5. The residue was hydrolyzed and the hydrolysate subjected to amino acid analysis (Table I).

The ethyl acetate layers were combined with the acetone mother liquor and extracted with an equal volume of 0.1 N HCl. The organic layer was taken to dryness on the flash evaporator. The residue was hydrolyzed in constant boiling HCl at 150° for 24 hours and the hydrolysate was analyzed for amino acids.

Results

Identification of Products.—The identity of the products produced by the digestion of insulin with carboxypeptidase was tentatively established by the location of the ninhydrin peaks on the trace from the Amino Acid Analyzer and was confirmed by paper chromatography of the digestion mixture in several solvent systems. The paper chromatograms eliminated the possibility of the release of glutamine and serine, both of which emerge with asparagine in the elution sequence used on the Amino Acid Analyzer (Moore *et al.*, 1958). The only amino acids liberated in significant amounts were alanine, asparagine, and aspartic acid. Neither lysine nor cystine, the penultimate amino acids at the carboxyl end, was detected in the enzyme digest. However, the extreme sensitivity of the Amino Acid Analyzer disclosed the presence of trace amounts of free amino acids as contaminants in the carboxypeptidase. Some of these increased in amount during the digestion. Expressed as moles of amino acid per mole of carboxypeptidase (m.w. 34,400), the amounts present before and after 8 hours of self-digestion of carboxypeptidase at *p*H 7.4 and 37° were aspartic acid (0.07, 0.42), threonine (0.45, 1.04), asparagine (0.55, 1.15), glutamic acid (0.28, 0.38), glycine (0.45, 0.55), alanine (0.31, 0.45), and trace amounts of most of the other amino acids present in carboxypeptidase. During a digestion, the molar ratio of insulin to carboxypeptidase was always greater than 50:1. Consequently, at the most, not more than 5% of the alanine, aspartic acid, or asparagine present in a hydrolysate could have come from the enzyme rather than the insulin.

TABLE I
AMINO ACID COMPOSITION OF INSULIN AND DEGRADATION PRODUCTS

	Residues per Mole[a]				
				After Edman Degradation	
	Insulin		Des-alanine-Desas-paragine-Insulin		Des-alanine Desas paragine-Insulin
Amino Acid	Theory	Found		Insulin	
Lysine	1	0.97	1.00	0.60	0.91
Histidine	2	1.99	1.98	1.67	1.95
Arginine	1	1.02	0.99	0.99	1.03
Aspartic acid	3	2.94	**2.00**	2.90	**2.06**
Threonine	1	0.95	0.96	1.00	1.01
Serine	3	2.63	2.62	2.70	2.75
Glutamic acid	7	*7.00*	*7.00*	*7.00*	*7.00*
Proline	1	1.05	0.94	1.01	1.02
Glycine	4	4.00	4.03	**3.06**	**3.26**
Alanine	3	2.94	**1.92**	2.88	**2.00**
Valine	5	4.52	4.22	3.82	4.10
Isoleucine	1	0.60	0.57	0.40	0.49
Leucine	6	5.90	5.79	5.60	5.79
Tyrosine	4	3.84	3.77	3.12	3.16
Phenylalanine	3	2.97	3.03	**1.89**	**2.09**

[a] Calculated on basis of moles of amino acid per (moles of glutamic acid/7). Italics indicate the assumed value of 7 residues per mole for glutamic acid. Bold face indicates a change in the amino acid composition from that found in insulin. Cystine was not determined.

Effect of pH.—By making use of the sample of insulin which contained about equal amounts of carboxyl-terminal asparagine and aspartic acid, it was possible to measure the effect of *p*H on the rate of release of these amino acids relative to one another and also relative to the release of alanine. In agreement with the early reports of Harris (1952), it was found (see Fig. 1) that the rate of release of alanine was much greater than that of asparagine or aspartic acid. This was true at all *p*H values which were tested (6.8, 7.4, 8.4, and 9.4). Actually at the enzyme concentrations which gave

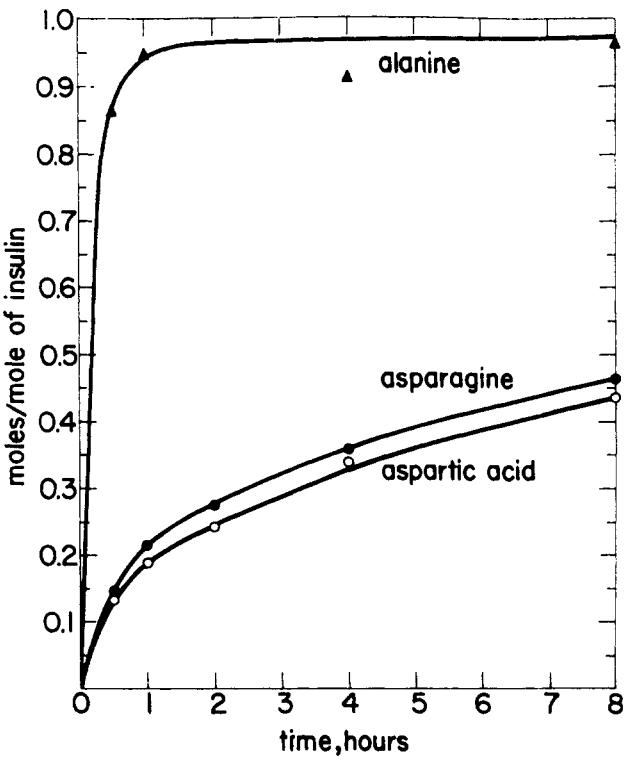

FIG. 1.—Relative rates of release of carboxyl-terminal amino acids by the action of carboxypeptidase-A on insulin: insulin hydrochloride prepared from PJ 3371 at 3 mg/ml, carboxypeptidase at 0.15 mg/ml, solution adjusted to *p*H 7.4 on *p*H-Stat, 25°; alanine (▲); asparagine (●); and aspartic acid (○).

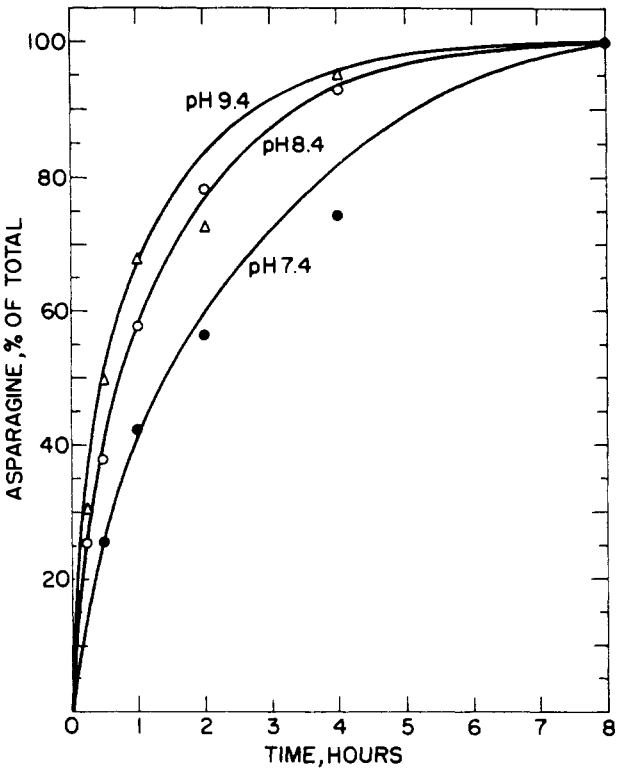

FIG. 2.—Effect of *p*H on rate of release of asparagine by the action of carboxypeptidase on insulin at 25°. Conditions were the same as in Figure 1 except for *p*H: *p*H 7.4 (●); *p*H 8.4 (○); *p*H 9.4 (△).

reasonable rates of hydrolysis of asparagine or aspartic acid, the alanine was released at a rate too fast to measure, the reaction being essentially complete in 15 minutes. Asparagine and aspartic acid were released at about the same rate at *p*H 7.4 (Fig. 1). However, when the *p*H was increased from 7.4 to 9.4, the rate of release of asparagine increased (Fig. 2), while the rate of release of aspartic acid decreased substantially (Fig. 3). The effect of *p*H on the release of aspartic acid was most pronounced from *p*H 7.4 to 8.4, with a further decrease in rate up to *p*H 9.4. During the first 60 minutes of hydrolysis, the release of asparagine and aspartic acid approximately followed first-order kinetics. The half-lives in minutes for the release of asparagine and aspartic acid, respectively, at 25° and the various *p*H values were as follows: at *p*H 7.4 (72 and 60); at *p*H 8.4 (56 and 450); at *p*H 9.4 (32 and >1000). In another set of experiments performed at 37° and *p*H 6.8 it was found that aspartic acid was released at a somewhat faster rate than asparagine, the half-lives being 10 and 19 minutes respectively.

Effect of Salt.—The relative rate of release of asparagine and aspartic acid at *p*H 7.4 and 37° was determined in concentrations of NaCl varying from 0.05 to 0.5 M (Fig. 4). An increase in salt concentration brought about a decrease in the rate of release of both asparagine and aspartic acid. However, the rate of release of aspartic acid appears to be more affected by salt concentration than that of asparagine. This is evident even at a concentration of 0.05 M and is even more pronounced at 0.5 M. Again under all these conditions, alanine was released at a rate too fast to determine, more than 90% of the alanine being liberated in the first 15 minutes.

Stability of Carboxypeptidase.—The stability of carboxypeptidase in the digestion mixture was determined at the various temperatures and *p*H values. At 25° the enzyme became progressively more labile from *p*H 7.4 to 9.4. Even so, 80% of the initial activity remained after 4 hours of digestion at *p*H 9.4 and 25°. At 37° the enzyme was somewhat more labile than at 25°. At *p*H 7.4 and 37°, the activity dropped to approximately 50% of the initial activity in four hours.

Conditions for Digestion.—From the above observations on the effect of *p*H, salt, and temperature on the rate of the reaction and on the enzyme stability, the following conditions were selected to obtain a complete reaction. Insulin hydrochloride (6 mg/ml) was treated with carboxypeptidase-A (weight ratio of 25:1) at 37° in low salt solution (0 to 0.05 ionic strength) at *p*H 7.4. After 4 hours, another aliquot of enzyme (weight ratio of 50:1) was added and the digestion allowed to proceed for an additional 4 hours. Under these conditions 0.95 to 1 mole of alanine and 0.96 to 1 mole of asparagine plus aspartic acid were liberated from one mole of insulin. Digestion mixtures frequently became turbid after 30 minutes to one hour. Most of this turbidity disappeared upon acidification of the reaction mixture at the end of the digestion.

Desalanine-Desasparagine-Insulin.—Insulin hydrochloride, prepared from a sample of zinc-insulin which was nearly pure insulin-A (Carpenter and Chrambach, 1962), was subjected to the action of carboxypeptidase under conditions designed to bring about complete reaction. The desalanine-desasparagine-insulin was isolated as the hydrochloride as described in the experimental part. The hydrochloride was subjected to a 2350-transfer countercurrent distribution in the solvent system prepared by equilibrating 2-butanol with an equal volume of 1.56% (w/v) dichloroacetic acid with the results shown in Figure 5. The shape of the main peak was very close to a theoretical curve for a component with a partition constant of 0.74. The mate-

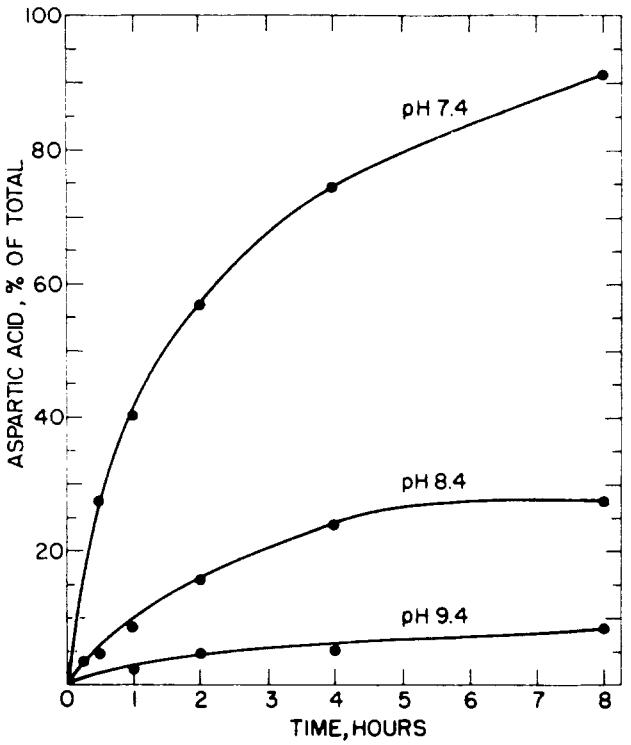

FIG. 3.—Effect of *p*H on rate of release of aspartic acid by the action of carboxypeptidase on insulin at 25°. Conditions were the same as for Figure 1 except for *p*H.

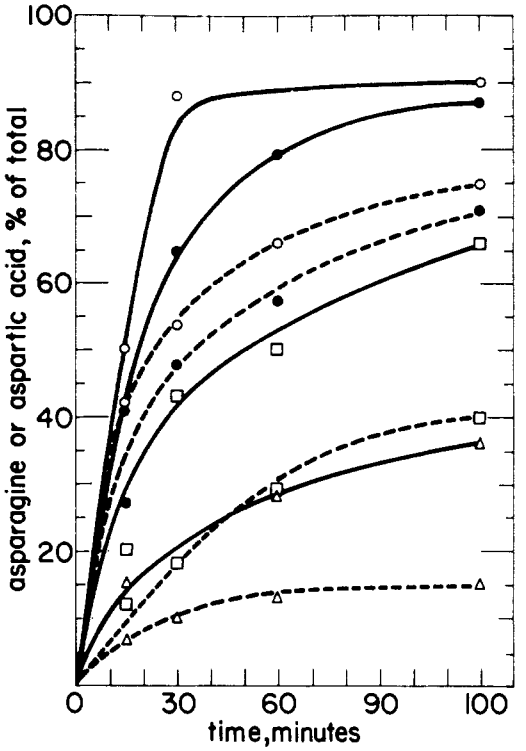

FIG. 4.—Effect of sodium chloride concentration on the rate of release of asparagine (——) and aspartic acid (- - -) by the action of carboxypeptidase on insulin hydrochloride prepared from PJ 3371. The insulin concentration was 2.2 mg/ml, carboxypeptidase was 0.05 mg/ml, *p*H was maintained at 7.4 on *p*H-Stat, temperature was 37°, and the following concentrations of sodium chloride were used: 0.05 M (○); 0.10 M (●); 0.25 M (□); and 0.5 M (△).

rial under A as indicated on Figure 5 was pooled, and the desalanine-desasparagine-insulin was isolated as its hydrochloride. Amino acid composition (Table I) and biological activity were determined for this sample. In previous studies (Carpenter and Chrambach, 1962), the effect of hydrolysis at 120° for various lengths of time on the amino acid composition of insulin had been determined. In the present instance the composition of desalanine-desasparagine-insulin was compared with that of insulin after a 6-hour hydrolysis at 120°. The results in Table I show that the only significant difference between the two samples occurs in the loss of one alanine and one aspartic acid residue from the carboxypeptidase-treated sample. The desalanine-desasparagine-insulin was subjected again to the action of carboxypeptidase under conditions similar to those applied to insulin. After an 8-hour digestion period, the small amounts of amino acids present in the digestion mixture were similar in kind and amounts to those obtained on self-digestion of carboxypeptidase.

Amino-Terminal Residues.—The amino-terminal groups in the desalanine-desasparagine-insulin were reacted with phenylisothiocyanate and cyclized to the thiohydantoins in trifluoroacetic acid (Guidotti *et al.*, 1962). The amino-terminal residues were identified by two methods: (1) by determining the difference in amino acid composition before and after the hydantoins were split off (Table I) and (2) by determining the amino acids released upon hydrolysis of the hydantoins. The results in Table I show that, upon being subjected to one Edman degradation, insulin and desalanine-desasparagine-insulin both lost about one residue of glycine, phenylalanine, and tyrosine. Glycine and phenylalanine are the amino-terminal amino acids of the A and B chains and would be expected to be removed in the Edman degradation. The decrease in tyrosine took place in both insulin and desalanine-desasparagine-insulin and is probably attributable to partial destruction of this amino acid during the manipulations. When the hydantoins which were released by the action of trifluoroacetic acid on the derivatized desalanine-desasparagine-insulin were subjected to hydrolysis at 150° for 24 hours and the hydrolysate was submitted to amino acid analyses, only glycine (0.22 residues per mole) and phenylalanine (0.07 residues per mole) were detected. After standard samples of the phenylthiohydantoins of glycine, phenylalanine, and tyrosine had been subjected to the same hydrolytic conditions, the free amino acids were detected in yields of 75, 24, and 69% respectively. If the values obtained from desalanine-desasparagine-insulin are corrected for the recoveries upon hydrolysis of the model compounds, glycine and phenylalanine were detected in equal amounts (0.29 residues per mole).

Oxidation of Desalanine-Desasparagine-Insulin.—Harris (1952) had detected lysine, leucine, glutamic acid, and tyrosine in addition to alanine and asparagine in carboxypeptidase hydrolysate of insulin. He had suggested that the enzyme cleaved past the tyrosyl-cystine bond on the A-chain, leaving the cystine residue attached through its disulfide to the B-chain. To test this possibility, desalanine-desasparagine-insulin was oxidized with performic acid in order to convert the cystine residues to cysteic acid residues. The oxidation mixture was divided into two parts of which one part was placed directly on the Amino Acid Analyzer, while the other part was treated with carboxypeptidase and then subjected to amino acid analyses. No cysteic acid was detected in the oxidized desalanine-desasparagine-insulin. However, after treatment of the oxidized desalanine-desasparagine-insulin with carboxypeptidase, 0.78 moles of cysteic acid, as well as smaller amounts of a number of other amino acids

(including tyrosine, asparagine, and glutamic acid), were detected.

Biological Activity.—Through the kindness of Dr. Edward L. Grinnan of Eli Lilly and Company, Indianapolis, the desalanine-desasparagine-insulin was tested in the mouse convulsion assay (Smith, 1950). In two assays involving 140 and 160 mice, values of 1.1 and 1.2 units/mg were reported.

Discussion

Degree of Hydrolysis.—Several pieces of evidence indicate that the action of carboxypeptidase-A ceases after the removal of alanine and asparagine (or aspartic acid). No lysine, cystine, or tyrosine was detected in the free amino acids. If the tyrosyl-cystine bond of the A-chain (Ryle *et al.*, 1955) had been cleaved, one would have expected further hydrolysis with the release of tyrosine even though the half-cystine residue would have remained bound to the B-chain through the disulfide bond. In addition, the oxidation experiments conducted on the desalanine-desasparagine-insulin eliminated the possibility of a half-cystine residue being bound through disulfide bonds to the B-chain. Hydrolytic splitting of other bonds in the insulin not involving the release of amino acids was discounted by the fact that no new amino-terminal residues were detected. Furthermore, the good agreement between the theoretical and the found amino acid composition of the desalanine-desasparagine-insulin eliminates the loss of any large fragment of the molecule.

In view of the results obtained on synthetic small substrates (for a recent review see Neurath, 1960), the failure of the carboxypeptidase-A to cleave the prolyllysyl bond on the B-chain is not unexpected. Apparently the action of carboxypeptidase on model peptides containing carboxyl-terminal cystine has not been investigated. However, Cummins and Li (quoted in Li, 1957) have noted that lactogenic hormone was resistant to the attack of carboxypeptidase-A. Later work (Li and Cummins, 1958) indicated the presence of a carboxyl-terminal half-cystine residue in the hormone. The present results indicate that the carboxyl-terminal half-cystine residue of the A-chain is resistant to the action of carboxypeptidase-A. The fact that Harris (1952) detected amino acids other than alanine and asparagine (or aspartic acid) in his digestion of insulin can probably be attributed to other enzymatic impurities (such as carboxypeptidase-B) in the carboxypeptidase preparations available at that time.

Rates of Hydrolysis.—The observation that alanine is released at a much faster rate than asparagine (or aspartic acid) is in agreement with previous findings (Harris, 1952; Harris and Li, 1952; Nicol and Smith, 1956; and Nicol, 1960).

The difference in the effect of *p*H on the rate of release of aspartic acid as contrasted with asparagine deserves mention. It is known that the presence of charged groups in the vicinity of the carboxyl-terminal group may change the *p*H optimum of carboxypeptidase. The *p*H optimum, which is ordinarily quoted as 7.5 (Neurath, 1960) for synthetic substrates, decreases to *p*H 5.5 for action of carboxypeptidase on polyglutamic acid (Green and Stahmann, 1952) and increases to above *p*H 8.5 for the removal of histidine from β-lactoglobulin (Davie *et al.*, 1959). The latter effect was attributed to a change in the protein substrate. In the case of insulin the rate of release of asparagine increases from *p*H 7.4 to 9.4. Previous studies with trypsin had noted similar effects in the rate of cleavage of the susceptible bonds from *p*H 7.4 to 9.4. These effects may well be attributed to a change in the charge,

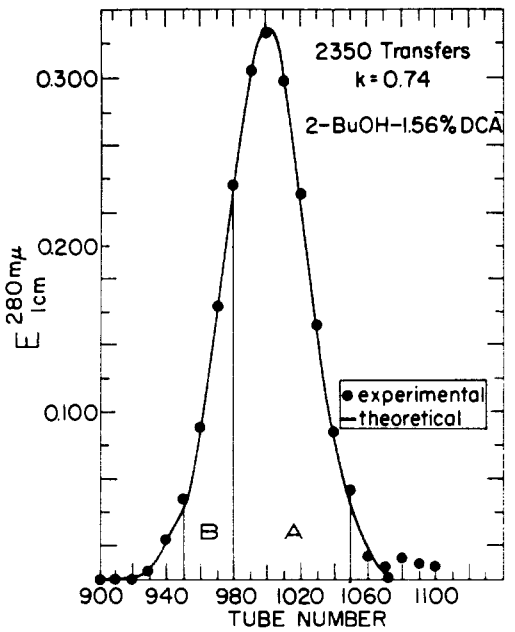

FIG. 5.—Results of 2350 transfer countercurrent distribution of desalanine-desasparagine-insulin between 2-butanol-1.56% (w/v) dichloroacetic acid: experimental (●), theoretical curve (——). Material under A was pooled for isolation.

aggregation, or conformation of the insulin. However, it is unlikely that the opposite effect noted for aspartic acid, that is, the decrease in its rate of release by carboxypeptidase from *p*H 7.4 to 9.4, can be attributed to changes in the insulin. The carboxyl groups of the aspartic acid and asparagine are completely ionized throughout this range, so that the contrasting *p*H effect cannot be attributed to a difference in the degree of ionization between the two carboxyl-terminal residues. A change in the charge on the enzyme could explain the results. The carboxypeptidase may contain a unique positively charged group that is essential for the hydrolysis of aspartic acid but is relatively unimportant for the release of asparagine. Raising the *p*H discharges the group and prevents the action of the enzyme on carboxyl-terminal aspartic acid without having any effect on the release of asparagine. Since the effect of *p*H on the release of aspartic acid is most pronounced between *p*H 7.4 and 8.4, the positively charged group on the enzyme could be attributed to an α-amino group. Further studies, especially on model peptides, are needed to illuminate this *p*H effect.

An increase in ionic strength resulted in a lowering of the rate of hydrolysis of the insulin; this is in contrast to what one normally finds for the action of carboxypeptidase on synthetic substrates (Lumry *et al.*, 1951). However, Davie *et al.* (1959) made a similar observation on the effect of salt on the rate of the hydrolysis of the carboxyl-terminal groups in β-lactoglobulin. In unpublished work, we have noted that the rate of hydrolysis of insulin by trypsin decreases with increasing salt concentration. It is possible that these salt effects may reflect changes in the state of aggregation of the substrate.

Biological Activity.—The results of the bioassay by the mouse convulsion test indicate that the desalanine-desasparagine-insulin possesses at most only about 5% of the activity of native insulin. Since the particular sample had been purified by countercurrent distribution to a point where it should have been free of unreacted insulin, the low activity of the desalanine-desasparagine-insulin is probably an inherent property of the molecule

rather than the result of a trace contamination with native insulin. Harris and Li (1952) reported very little loss in biological activity accompanying the rapid release of alanine from insulin, but a slow loss of biological activity accompanying the slow release of asparagine. Similar results were noted by Nicol (1960). These observations, combined with our present data on the very low activity of the desalanine-desasparagine-insulin, indicate the profound effect that the removal of just one amino acid may have on the biological activity of the molecule.

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The Labile Amide in Insulin: Preparation of Desalanine-Desamido-Insulin*

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When insulin is treated with mild acid it is transformed into several biologically active components of which the major component is a desamido-insulin. The present study of the action of carboxypeptidase-A on various insulin preparations was undertaken in an attempt to locate the labile amide(s) involved in the transformation reaction. Desamido-insulin which had been isolated by countercurrent distribution or partition column chromatography gave primarily *aspartic acid* (and alanine) when treated with carboxypeptidase. Similar treatment of insulin-A gave primarily *asparagine* (and alanine). Insulin-A and desamido-insulin were converted by the action of carboxypeptidase into the same compound as judged by partition column chromatography. During the acid transformation of insulin-A, the hydrolysis of the amide group of the carboxyl-terminal asparagine took place at a much faster rate than the hydrolysis of the amide group in free asparagine or in the other five amides of insulin. These results all indicate that insulin-A contains an acid-labile amide group on the carboxyl-terminal asparagine of the A-chain and that this amide group is the one which is primarily involved in the acid-transformation reaction of insulin. Cleavage of the carboxyl-terminal alanine of the B-chain of insulin without removal of appreciable amounts of the carboxyl-terminal aspartic acid from the A-chain was realized by treating desamido-insulin with carboxypeptidase at *p*H 9.4. The resulting desalanine-desamido-insulin was quite potent (15 units/mg) in the mouse convulsion test.

In 1951, Harfenist and Craig reported on the separation of a sample of bovine insulin into two biologically active components by countercurrent distribution. Later studies (Harfenist and Craig, 1952; Harfenist, 1953) indicated that the two components differed in the number of amide groups: one contained six amides (insulin-A) and the other contained five amides (desamido-insulin). In partition column chromatographic studies on insulin, this laboratory had noted that most

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