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.

ACKNOWLEDGMENT

The authors would like to thank Mrs. Bertha L. Martin and Miss Sharon L. Hayes for aid with the amino acid analyses. We are indebted to Dr. W. Konigsberg for transmitting to us the details of the modified Edman degradation before publication.

REFERENCES

Carpenter, F. H. (1958), *Arch. Biochem. Biophys. 78*, 539.
Carpenter, F. H., and Chrambach, A. (1962), *J. Biol. Chem. 237*, 404.
Carpenter, F. H., and Slobin, L. I. (1962), *Fed. Proc. 21*, 407d.
Davie, E. W., Newman, C. R., and Wilcox, P. E. (1959), *J. Biol. Chem. 234*, 2635.
Edman, P. (1950), *Acta Chem. Scand. 4*, 277, 283.
Fraenkel-Conrat, J., and Fraenkel-Conrat, H. (1950), *Biochim. et Biophys. Acta 5*, 89.
Green, M., and Stahmann, M. A. (1952), *J. Biol. Chem. 197*, 771.
Guidotti, G., Hill, R. J., and Konigsberg, W. (1962), *J. Biol. Chem. 237*, 2184.
Harfenist, E. J. (1953), *J. Am. Chem. Soc. 75*, 5528.
Harfenist, E. J., and Craig, L. C. (1952), *J. Am. Chem. Soc. 74*, 3083.
Harris, J. I. (1952), *J. Am. Chem. Soc. 74*, 2944.
Harris, J. I., and Li, C. H. (1952), *J. Am. Chem. Soc. 74*, 2945.
Hill, R. L., and Smith, E. L. (1957), *J. Biol. Chem. 228*, 577.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Lens, J. (1949), *Biochim. et Biophys. Acta 3*, 367.
Li, C. H. (1957), *Adv. Prot. Chem. 12*, 306.
Li, C. H., and Cummins, J. T. (1958), *J. Biol. Chem. 233*, 73.
Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Neurath, H. (1960), *in* The Enzymes, Boyer, P. D., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., Vol. 4, Part A, Chap. 2.
Nicol, D. S. H. W. (1960), *Biochem. J. 75*, 395.
Nicol, D. S. H. W., and Smith, L. F. (1956), *Biochem. J. 64*, 17P.
Pechère, J. F., Dixon, G. H., Maybury, R. H., and Neurath, H. (1958), *J. Biol. Chem. 233*, 1364.
Polson, A., Mosley, V. M., and Wyckoff, R. W. G. (1947), *Science 105*, 603.
Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.
Sanger, F. (1949), *Biochem. J. 44*, 126.
Smith, K. L. (1950), *in* Hormone Assay, Emmens, C. W., editor, New York, Academic Press, Inc., p. 35.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1960), *J. Biol. Chem. 235*, 64.
Young, J. D. (1959), Ph.D. Thesis, University of California, Berkeley, p. 40.
Young, J. D., and Carpenter, F. H. (1961), *J. Biol. Chem. 236*, 743.

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

LAWRENCE I. SLOBIN AND FREDERICK H. CARPENTER

From the Department of Biochemistry, University of California, Berkeley

Received August 17, 1962

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

---

* This investigation was supported in part by Grant A-608 of the National Institutes of Health and G-19561 of the National Science Foundation. A preliminary report of this work was made before the American Society of Biological Chemists (Carpenter and Slobin, 1962).

commercial samples of crystalline insulin were composed of two or more biologically active components (Carpenter and Hess, 1956; Chrambach and Carpenter, 1960). Amide analyses on two fractions (I and III) which had been isolated by partition column chromatography from commercial samples of insulin indicated that they differed from one another by one amide group (Carpenter and Chrambach, 1962). In this respect the fractions I and III prepared by partition column chromatography appeared identical to insulin-A and desamido-insulin, respectively, of countercurrent distribution and hence we have adopted the latter notation to designate the fractions.

When the main component (insulin-A) of most insulin preparations was isolated and allowed to stand in 0.1 N HCl, it was gradually transformed into a mixture of biologically active components which could be separated by partition column chromatography (Chrambach and Carpenter, 1960). The transformation was accompanied by the liberation of ammonia. The major transformation product possessed the same distribution constant as desamido-insulin (fraction III) and contained one less amide than the original insulin-A (Carpenter and Chrambach, 1962). Therefore, the acid-transformation of insulin appeared to involve the conversion of insulin-A into desamido-insulin and several minor components which also may be deamidated products.

Sanger's proposed structure for insulin contains six amide groups, three as glutamine residues and three as asparagine residues (Sanger *et al.*, 1955; Ryle *et al.*, 1955). Desamido-insulin could be composed of one molecular species in which the amide was missing from the same residue or it could be composed of a mixture of species which were deamidated on different residues. Since desamido-insulin appears to be the major product formed in the acid transformation, a loss of one amide group, rather than a partial loss of six amides, would indicate a preferential reactivity of this group.

In the proposed structure for insulin (Sanger *et al.*, 1955) one of the six amide groups occurs on the carboxyl-terminal asparagine of the A-chain. This particular amide is subject to investigation by the use of carboxypeptidase-A. The relative composition with regard to asparagine and aspartic acid of the amino acids liberated by the action of carboxypeptidase-A on insulin-A and desamido-insulin should be indicative of the differences in their structure. Presumably, in insulin-A the carboxyl-terminal group of the A-chain is entirely asparagine. In desamido-insulin, this group could be (1) all asparagine, which would indicate the loss of an amide from some other location in the molecule, (2) all aspartic acid, which would indicate a loss of amide from the carboxyl-terminal asparagine, or (3) a mixture of aspartic acid and asparagine, which, depending on the relative composition, would indicate some type of indiscriminate loss of amides from the six amides of insulin-A.

The present report describes the results obtained on treatment of a number of samples of crystalline bovine insulin, purified insulin-A, desamido-insulin, and acid-transformed insulin with carboxypeptidase. The work was greatly aided by the availability of several samples of insulin which contained about equal amounts of insulin-A and desamido-insulin as judged by their behavior in countercurrent distribution and partition column chromatography. The interpretation of the results depended upon measuring the relative proportions of asparagine and aspartic acid liberated by carboxypeptidase. In order for this procedure to be valid, it was essential that all of the carboxyl-terminal aspartic acid or asparagine be cleaved by the enzyme. Experiments designed to determine the conditions for such a digestion of insulin by carboxypeptidase are described in the accompanying paper (Slobin and Carpenter, 1963).

As it turned out, the relative rate of release of aspartic acid and asparagine is greatly dependent on *p*H. Advantage was taken of this fact to prepare a sample of desalanine-desamido-insulin by the action of carboxypeptidase on desamido-insulin for a short time at high *p*H, where the carboxyl-terminal aspartic acid was very resistant to the action of the enzyme.

EXPERIMENTAL

Materials.—Crystalline zinc insulin of bovine origin was generously furnished by Eli Lilly and Company (Lots No. PJ 3371, PJ 1531, PJ 3026, PJ 4812, and 535664) or was purchased from the British Drug Houses Ltd. (Batch No. 2189). All insulin samples were converted to their hydrochlorides (Carpenter, 1958) before use. Carboxypeptidase-A (three times crystallized and treated with diisopropyl fluorophosphate) was secured as an aqueous suspension from Worthington Biochemical Corporation (Lots No. 629–30 and 6110).

Amino Acid Analyses.—The amino acids liberated by the action of carboxypeptidase were determined by the method of Spackman *et al.* (1958) with a Beckman/Spinco Amino Acid Analyzer, Model 120. For routine analyses the 50-cm column was used. The amino acid composition of desalanine-desamido-insulin was determined on samples which had been hydrolyzed for 6 hours at 120° (Carpenter and Chrambach, 1962).

Ammonia Analysis.—Free ammonia was determined by a diffusion method similar to that described by Seligson and Seligson (1951). The sample (1 ml containing 0.05 to 0.75 μmole of NH_3) was contained in 30-ml serum bottles in which the rubber caps held a small glass tube containing a cup with a drop of 2 N H_2SO_4. Borate buffer (0.3 ml of a solution prepared by dissolving 20 g of potassium tetraborate in 100 ml of 0.5 N NaOH) was injected by syringe into each sample (final *p*H was about 9.2). The bottles were placed on their sides on a rotating tissue culture drum. Diffusion was allowed to take place at 37° for 41 hours. The glass rods containing the drop of acid were placed in test tubes with 1 ml of water. Ninhydrin color was developed by the method of Moore and Stein (1954) and read in a Zeiss spectrophotometer, Model PMQ, at 570 mμ.

Carboxypeptidase Digestions.—Digestions were performed at 37° and *p*H 7.4 in the 0.05 M buffer and under the conditions described previously for a complete reaction (Slobin and Carpenter, 1963).

Acid Transformation.—Asparagine (1.0 μmole/ml) or insulin hydrochloride (0.5 μmole/ml) in 0.1 N HCl was incubated at 30° with gentle agitation of the flask. At various time intervals aliquots were removed for analyses. The aliquots from the asparagine incubation were diluted several fold with the *p*H 2.2 buffer (Moore *et al.*, 1958) and stored at −10° until submitted for amino acid analysis. Aliquots from the insulin incubation were treated differently depending upon whether the heat precipitate (Blatherwick *et al.*, 1927; Du Vigneaud *et al.*, 1928), designated fibrils by Waugh (1946), had formed. When fibrils were absent the aliquots were adjusted to *p*H 7.4 and submitted to the conditions for digestion by carboxypeptidase. The enzyme digests were acidified to *p*H 2 and kept at −10° until amino acid analyses were performed. Where insulin fibrils were present, they were solubilized by adding 0.5 N NaOH to *p*H 12.5 and then quickly read-

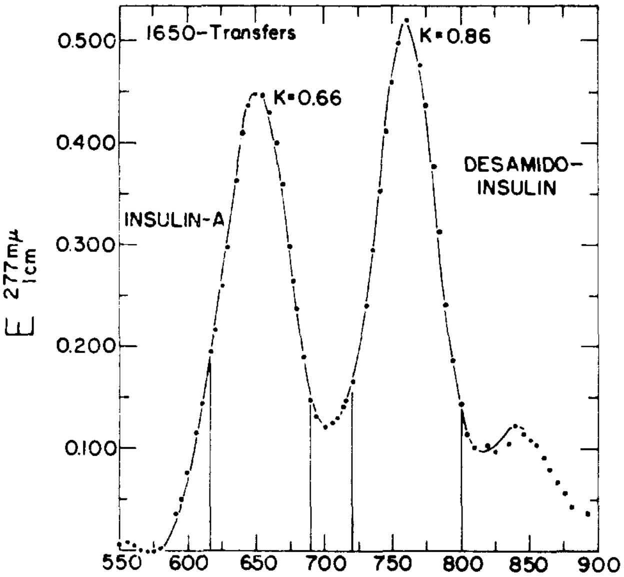

FIG. 1.—Countercurrent distribution of bovine insulin (PJ 3371) between 2-butanol and 1.56% (w/v) dichloroacetic acid. The vertical lines indicate the fractions which were pooled for isolation for insulin-A and desamido-insulin.

justing the *p*H back to 7.4 with 0.1 N HCl (Waugh, 1948) before submitting the sample to the action of carboxypeptidase.

Preparations of acid-transformed insulin were obtained by incubating the insulin hydrochloride in 0.1 N HCl at 30° for 2 weeks. Fibrils generally formed in the flask after about 5 to 7 days. At the end of the incubation period the fibrils were redissolved by the brief alkali treatment noted above. The mixture of transformed insulins was precipitated as the hydrochloride by addition of 18 volumes of acetone to the acid solution (Carpenter, 1958).

Countercurrent Distribution.—Crystalline zinc insulin (1.73 g of Lot No. PJ 3371) was introduced into the lower layers of the first 20 tubes of a 500-tube instrument containing 10 ml of upper and lower layers prepared by equilibrating equal volumes of 2-butanol with 1.56% (w/v) dichloroacetic acid. A total of 1650 transfers was performed by the recycle procedure. The contents of the tubes were pooled as indicated in Figure 1 and the fractions were isolated as their hydrochlorides by a procedure similar to that described for isolation of desalanine-desasparagine-insulin (Slobin and Carpenter, 1963). Insulin-A weighed 0.54 g and desamido-insulin weighed 0.45 g.

Partition Column Chromatography.—The technique was similar to that described by Chrambach and Carpenter (1960). All chromatograms were run at 4°. Three different sized columns were used: *analytical*, 0.83 cm^2 cross-sectional area by 44 cm high prepared from 4 g of Microcel-*C* and 5 g of Celite-545 (Johns-Manville Products Corporation) and 10 ml of stationary phase for loads up to 15 mg; *semipreparative* 3.64 cm^2 cross-sectional area by 44 cm high prepared from 17 g of Microcel-*C* and 23 g of Celite-545 and 38 ml of stationary phase for loads up to 60 mg; *preparative*, 11.4 cm^2 in cross-sectional area and 48 cm high prepared from 54 g of Microcel-*C* and 74 g of Celite-545 and 123 ml of stationary phase for loads up to 300 mg. The solvent systems were mixtures of *n*-butanol–2-butanol–0.1 N HCl in the volume proportions 12:88:100 or 16:84:100. The lower layer was used as the stationary phase. Improved performance of the partition columns has been realized by pressing a tight-fitting disc cut from porous Teflon sheet ($^1/_8$ inch thick, grade 50–55, Fluoro-Plastics Company, 2417 Federal St., Philadelphia) on top of the column. The disc allows compression of the volume of the column and prevents disturbance of the surface of the column during introduction of the sample. The measurement and isolation of components from the eluted fractions were as described previously (Chrambach and Carpenter, 1960). The distribution constant (*K*) was calculated from the relationship between volume of the stationary phase (V_S), the hold-up volume (V_H), and the elution volume of maximum concentration (V_E) (Carpenter and Hess, 1956); $K = V_S/(V_E - V_H)$.

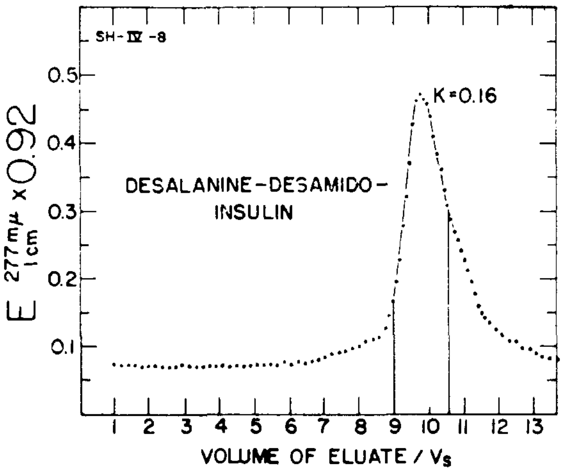

FIG. 2.—Partition column chromatography of desalanine-desamido-insulin (112 mg) in system prepared from *n*-butanol–2-butanol–0.1 N HCl (16:84:100). Volume of stationary phase (V_S) was 123 ml. The fractions between the vertical lines were pooled for isolation of material.

Preparation of Desalanine-Desamido-Insulin.—Desamido-insulin hydrochloride (150 mg, isolated from PJ 3371 by countercurrent distribution) was dissolved in 15 ml of the mixed buffer at *p*H 9.4 (Slobin and Carpenter, 1963) and the *p*H was readjusted to 9.4 with 0.1 N NaOH. Carboxypeptidase (6 mg in 0.8 ml) was added and the resulting solution was incubated at 37° for 15 minutes. The solution was acidified with 1 ml of N HCl, an aliquot was removed for analysis of liberated amino acids, and the remainder was clarified by centrifugation. The clear supernatant was desalted on a dextran gel column, the eluate was concentrated, and the material was isolated as the hydrochloride with all procedures similar to those previously described (Slobin and Carpenter, 1963). The yield of desalanine-desamido-insulin was 120 mg.

A portion (111 mg) of the material was subjected to partition column chromatography at 4° on the *preparative* size column with the solvent system prepared from *n*-butanol–2-butanol–0.1 N HCl (16:84:100). The material in the eluate fractions as indicated in Figure 2 was isolated as the hydrochloride (42 mg) and used for chemical and biological characterization.

RESULTS

Relative Amounts of Carboxyl-Terminal Asparagine and Aspartic Acid in Various Insulin Preparations.—The results obtained upon digestion by carboxypeptidase-A of a variety of samples of insulin (all of bovine origin) are shown in Table I. Since it had been demonstrated previously (Slobin and Carpenter, 1963) that one mole of alanine was rapidly released per mole of insulin, the amount of alanine released upon diges-

TABLE I

AMOUNTS OF CARBOXYL-TERMINAL ASPARAGINE AND ASPARTIC ACID IN VARIOUS INSULIN SAMPLES

Group	Insulin Sample	Asparagine (moles/mole)[a]	Aspartic Acid (moles/mole)[a]
A	PJ 3371	0.51	0.51
	PJ 3026	0.87	0.14
	PJ 4812	0.82	0.16
	PJ 1531-MC	0.79	0.19
	535664	0.82	0.15
	2189	0.79	0.18
B	Insulin-A[b]	0.91	0.09
	Insulin-A[c]	0.87	0.10
	Desamido-insulin[d]	0.17	0.81
	insulin[e]	0.11	0.86
	insulin[f]	0.13	0.87
	insulin[g]	0.11	0.87
C[h]	Ia	0.92	0.11
	I	0.91	0.08
	II	0.76	0.20
	III	0.31	0.67
	IV	0.13	0.89

[a] Moles of asparagine or aspartic acid released per mole of alanine released. [b] Insulin-A isolated from partition column chromatography of PJ 4812 (see Fig. 4, *upper*). [c] Insulin-A isolated from countercurrent distribution of PJ 3371 (see Fig. 1). [d] Desamido-insulin isolated from countercurrent distribution of PJ 3371 (see Fig. 1). [e] Desamido-insulin isolated from partition column chromatography of PJ 5279. [f] Desamido-insulin isolated from partition column chromatography of PJ 3371 (see Fig. 3, *upper*). [g] Desamido-insulin isolated by rechromatography (see Fig. 3, *lower*). [h] Fractions isolated from partition column chromatography of acid-transformed insulin (see Fig. 4, *lower*).

tion with carboxypeptidase could be used as an internal control on the amount of insulin present in the digestion mixture. The values are expressed as moles of aspartic acid or asparagine released per mole of alanine released. The completeness of digestion is indicated by the sum of the moles of aspartic acid plus asparagine. The totals range between 0.97 and 1.02. The results under group A of Table I were obtained from various commercial preparations of bovine insulin. All samples contained some carboxyl-terminal aspartic acid, which varied in amount from 14 to 51% of the carboxyl-terminal alanine. A number of these samples had been investigated for homogeneity by the techniques of partition column chromatography (Figs. 3 and 4, *upper;* and Figs. 2 and 6 of Chrambach and Carpenter, 1960) and countercurrent distribution (Fig. 1). Comparison of the results shows a rough correlation between the amount of fraction III (desamido-insulin) as detected in the separation technique and the amount of carboxyl-terminal aspartic acid in the sample. This was especially true for sample PJ 3371, which contained about an equal mixture of desamido-insulin and insulin-A (Figs. 1 and 3) and which also liberated about an equal amount of aspartic acid and asparagine upon treatment with carboxypeptidase.

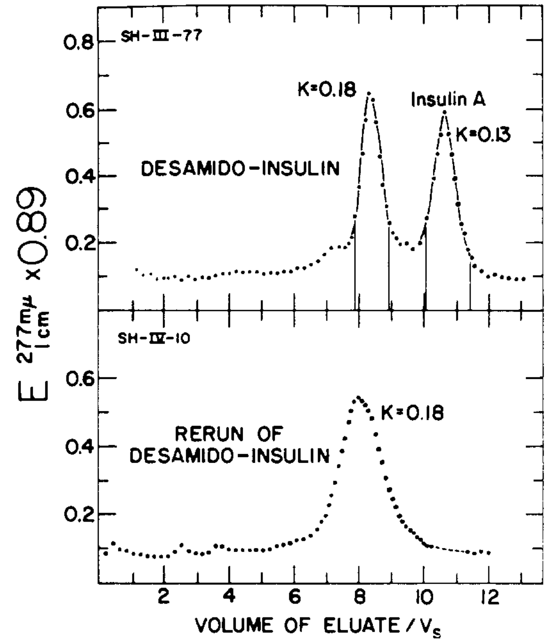

FIG. 3.—Partition column chromatography of various insulin samples in the solvent system prepared from *n*-butanol-2-butanol-0.1 N HCl (12:88:100) in which the stationary phase (V_S) had a volume of 38 ml. *Upper*, insulin hydrochloride prepared from PJ 3371. *Lower*, rerun of desamido-insulin hydrochloride isolated from PJ 3371.

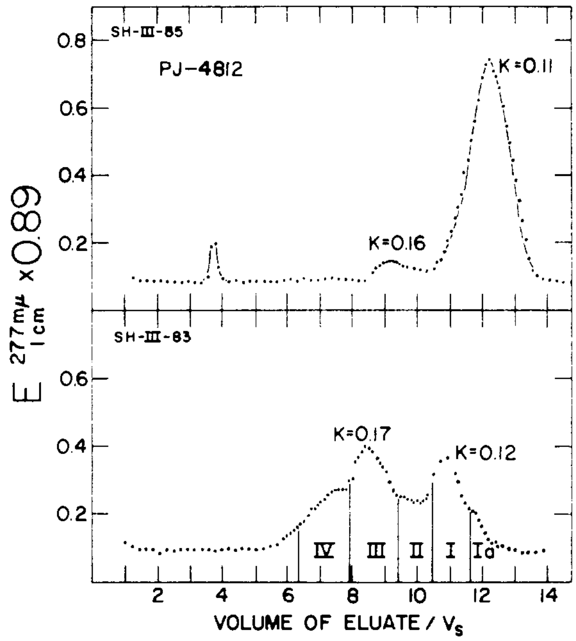

FIG. 4.—Partition column chromatography of various insulin samples in system prepared from *n*-butanol-2-butanol-0.1 N HCl (12:88:100). The stationary phase was 38 ml. *Upper*, insulin hydrochloride prepared from PJ 4812. *Lower*, acid-transformation products of PJ 4812.

The correlation between amount of carboxyl-terminal aspartic acid and the amount of desamido-insulin in the samples was largely confirmed when samples separated by countercurrent distribution and partition column chromatography were submitted to the action of carboxypeptidase. The results under group B of Table I show that desamido-insulin, isolated by either countercurrent distribution or partition column chromatography, consisted for the main part (81–87%) of carboxyl-terminal aspartic acid with small amounts (11–17%) of carboxyl-terminal asparagine. On the other hand, the insulin A isolated by these techniques contained for the main part (87–91%) carboxyl-terminal asparagine with small amounts (9–10%) of aspartic acid.

The above data indicated that the chief difference between insulin-A and desamido-insulin was in the presence of carboxyl-terminal asparagine or aspartic

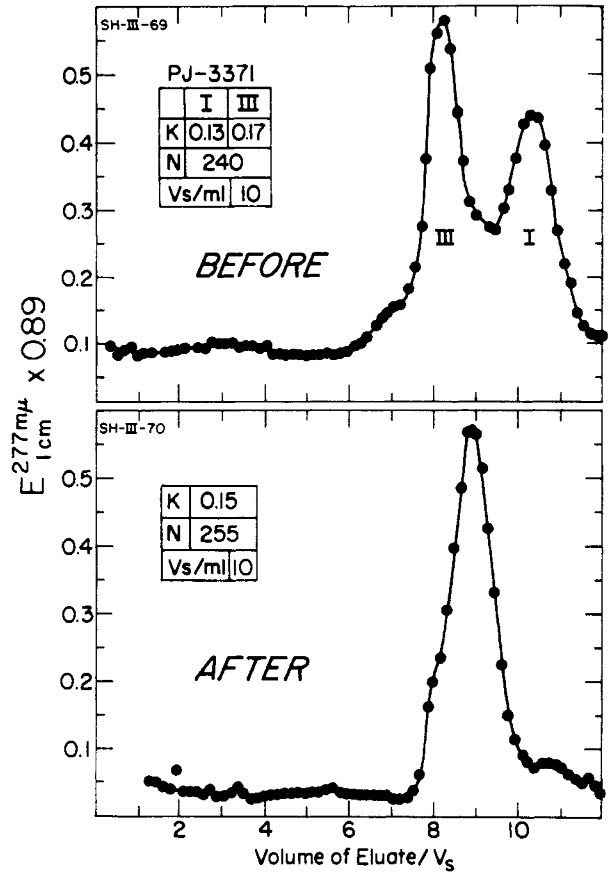

FIG. 5.—Partition column chromatography of insulin samples in solvent system prepared from *n*-butanol–2-butanol–0.1 N HCl (12:88:100). The stationary phase was 10 ml. Insulin hydrochloride prepared from PJ 3371 before (*upper*) and after (*lower*) treatment with carboxypeptidase.

acid. If this were the case, then the separation of mixtures of desamido-insulin and insulin-A as depicted in countercurrent distribution (Fig. 1) or partition column chromatography (Fig. 3) should not be effective on samples which had been digested by carboxypeptidase. Removal of the carboxyl-terminal alanine and asparagine of insulin-A should convert it into a compound identical with that obtained by removal of the carboxyl-terminal alanine and aspartic acid from desamido-insulin. Figure 5 shows the results obtained on partition column chromatography of PJ 3371 before and after treatment with carboxypeptidase. The two components with *K* values of 0.13 (insulin-A) and 0.17 (desamido-insulin) before treatment were replaced by one major component with an intermediate *K* value of 0.15 (desalanine-desasparagine-insulin) after treatment with carboxypeptidase. Both components of PJ 3371 apparently gave rise to the same desalanine-desasparagine-insulin. In countercurrent distribution studies in the solvent system 2-butanol–1.56% dichloroacetic acid, the desalanine-desasparagine-insulin exhibited a distribution constant of 0.74 (Slobin and Carpenter, 1963) which was intermediate to the values for insulin-A (0.66) and desamido-insulin (0.86) for this solvent system.

Acid Transformation.—A sample of insulin which was about 90% insulin-A (Fig. 4, *upper*) was incubated in 0.1 N HCl at 30°. The rate of conversion of carboxyl-terminal asparagine to aspartic acid as well as the rate of release of ammonia was determined. For comparison, the rate of conversion of free asparagine to aspartic acid was measured under the same conditions. The rates were plotted as first order reactions, and the half-life (in days) of the various reactions was calculated. The conversion of free asparagine to aspartic acid at 30° in 0.1 N HCl had a half-life of 60 days. The conversion of carboxyl-terminal asparagine to aspartic acid in insulin under the same conditions took place at a much faster rate, having a half-life of 16 days. If only one of the amide groups in insulin had hydrolyzed, then the release of ammonia under these conditions should have taken place at the same rate as the conversion of carboxyl-terminal asparagine to aspartic acid. This was not the case; when calculated on the basis of one labile-amide group per insulin molecule, the half-life for release of ammonia was 5 days. However, if the ammonia released by the conversion of carboxyl-terminal asparagine to aspartic acid was subtracted from the total ammonia values at each time interval and the remainder was assumed to arise at an equal rate from all five of the other amide groups, then a half-life of 44 days was calculated for the hydrolysis of these groups.

In another series of experiments, insulin was incubated in 0.1 N HCl at 30° for 2 weeks, during which time fibrils formed. After re-solution of the fibrils in alkali, the transformed insulin was isolated as its hydrochloride. During this time, 0.79 mole of NH_3 per mole of insulin was released and 0.32 mole of carboxyl-terminal asparagine had been converted to aspartic acid. No free amino acids had been liberated. The transformed insulin was subjected to partition column chromatography. The starting insulin (Fig. 4, *upper*), had been converted into a rather complex mixture which was isolated in five fractions as indicated in Figure 4, *lower*. Each one of the fractions was analyzed for carboxyl-terminal asparagine and aspartic acid, with the results shown in group C of Table I. The slowest-moving fractions, I and Ia, had *K* values and carboxyl-terminal asparagine content (0.91 moles/mole of insulin) which were quite similar to those of the starting insulin. The faster-moving fractions (II, III and IV) showed a progressive increase in carboxyl-terminal aspartic acid (0.20, 0.67, and 0.89 moles/mole of insulin respectively) and a progressive decrease in asparagine (0.76, 0.31, and 0.13 moles/mole of insulin respectively).

Desalanine - Desamido - Insulin.—Desamido - insulin was subjected to the short action (15 minutes) of carboxypeptidase at high *p*H (9.4), during which time 1.0 mole of alanine, 0.09 mole of asparagine, and 0.02 mole of aspartic acid were released per mole of insulin. The desalanine-desamido-insulin was isolated from the reaction mixture and further purified by partition column chromatography (Fig. 2). The partition constant was 0.16, which is similar to the value of 0.15 found for desamido-insulin on the same column. The amino acid analyses of the desalanine-desamido-insulin (Table II) indicated the loss of one alanine residue. Biological assays were performed at Eli Lilly and Company by the mouse convulsion technique (Smith, 1950). The results with 60 mice gave a value of 15 units/mg.

DISCUSSION

The results of carboxyl-terminal analyses showed that desamido-insulin which had been separated from commercial samples of insulin had 81–87% carboxyl-terminal aspartic acid, whereas insulin-A contained 87–91% carboxyl-terminal asparagine. There can be no doubt that the major difference between these molecules rests in the presence or absence of an amide group on the carboxyl-terminal aspartic acid. This observation is confirmed by the fact that the same compound (as judged by partition column chromatography) was

TABLE II
AMINO ACID COMPOSITION OF DESALANINE-DESAMIDO-INSULIN

Amino Acid	Moles/Mole[a]
Lysine	1.07
Histidine	2.00
Arginine	1.04
Aspartic acid	2.98
Threonine	1.06
Serine	2.87
Glutamic acid	*7.00*
Proline	1.07
Glycine	4.08
Alanine	**2.07**
Valine	4.11
Isoleucine	0.54
Leucine	5.80
Tyrosine	3.97
Phenylalanine	3.01

[a] Moles of amino acid per moles of (glutamic acid/7). Boldface indicates a difference in amino acid composition from that found for insulin under similar conditions (Slobin and Carpenter, 1963). No attempt was made to analyze for cystine.

produced by the digestion of insulin-A or desamido-insulin with carboxypeptidase. However, the analyses on the desamido-insulin indicated the presence of 11–17% carboxyl-terminal asparagine while approximately the reverse was noted for insulin-A (*ca.* 10% aspartic acid).

Several explanations may be invoked for the presence of small amounts of carboxyl-terminal asparagine in desamido-insulin and of aspartic acid in insulin-A. One explanation assumes incomplete separation of the insulin-A and desamido-insulin. Although the possibility of some cross-contamination cannot be ruled out completely, cross-contaminations in the amounts of 10–15% do not appear compatible with the shape and position of the curves obtained in countercurrent distribution (Fig. 1) and the partition column chromatography (Fig. 3, *upper*). Furthermore, upon rechromatography of desamido-insulin (Fig. 3, *lower*), the content of carboxyl-terminal asparagine was not decreased appreciably (Table I). Although it is possible that the small amount of carboxyl-terminal aspartic acid in insulin-A is an artifact caused by the acidic conditions used in the separation and isolation procedures, such an explanation cannot be invoked to explain the small amount of carboxyl-terminal asparagine in desamido-insulin. Further investigation is necessary in order to clarify these results.

The acid transformation experiments showed that the carboxyl-terminal asparagine was much more labile to acid hydrolysis than was free asparagine. It also appeared that the carboxyl-terminal asparagine was much more labile than any of the other amide groups in insulin. If one assumes that the other five amides in insulin were all hydrolyzed at an equal rate, then the rate of release of ammonia from these groups (half-life of 44 days) was in reasonable agreement with that expected from the studies on free asparagine (half-life of 60 days). Comparable agreement of rates would be obtained by making other assumptions such as complete resistance of the amide groups of the three glutamine residues coupled with rates for the amides of all three asparagine residues that were similar in value to that found for the carboxyl-terminal asparagine. Of these two explanations, the first is the most attractive. It is in agreement with the finding that the major components separated by partition column chromatography of acid-transformed insulin contained a relatively large amount (67 to 89%) of carboxyl-terminal aspartic acid. If deamidation occurred at an equal rate on all of the asparagine residues, one would not obtain such high amounts of carboxyl-terminal aspartic acid in separated desamido-insulins. The evidence indicates that the desamido-insulin formed in the acid-transformation of insulin as well as that found in some commercial samples of insulin contains for the main part carboxyl-terminal aspartic acid with perhaps small amounts of other desamido-forms.

Leach and Lindley (1953a,b) have made a study of the rate of hydrolysis in weakly acidic solutions of the amide bonds in asparagine-containing peptides. They found that the hydrolysis of the amide group of peptides containing a carboxyl-terminal asparagine took place more rapidly and by a different mechanism than the hydrolysis of the amide in free asparagine or in peptides containing an amino-terminal asparagine. Our finding that the amide group of the carboxyl-terminal asparagine of insulin is more labile than the other amides of insulin is in agreement with what one would predict from the data secured by Leach and Lindley on model peptides. Grannis (1960) has studied the rate of release of aspartic acid from insulin during hydrolysis at *p*H 1.8 and 105° and found that one aspartic acid residue was released at a much faster rate than the other two residues present in insulin. He reasoned that the release of aspartic acid residues was dependent upon the prior amide bond hydrolysis and invoked a rapid hydrolysis of the amide bond on the carboxyl-terminal asparagine to explain his results. Recently, Schultz *et al.* (1962) reported the results of similar studies on insulin in which use was made of the Amino Acid Analyzer to secure quantitative data. They found that at least one amide residue as well as one aspartic acid residue was released at a faster rate than the other amide and aspartic acid residues of insulin. Our demonstration that the amide residue of the carboxyl-terminal asparagine is more labile to mild acid hydrolysis than the other amides in insulin is compatible with the above results.

Desalanine-Desamido-Insulin.—In the early work of Harris and Li (1952) and later of Nicol (1960) insulin had been treated with carboxypeptidase under conditions which removed most of the alanine and small amounts of the asparagine without serious effect on the biological activity of the digestion mixture. Harfenist and Craig (1952) had found that desamido-insulin was fully active, and we had confirmed this observation with the material isolated by partition column chromatography (Chrambach and Carpenter, 1960). Therefore, it is not surprising that the desalanine-desamido-insulin was found to possess high activity in the mouse convulsion test. The activity of desalanine-desamido-insulin (15 units/mg) was not as high as that of native insulin (23–25 units/mg) in the mouse convulsion assay. However, because of the limited number of samples which have been assayed, it is not possible at this time to place much significance on the small difference in potency between the desalanine-desamido-insulin and native insulin.

On the other hand, the carboxyl-terminal asparagine appears to play an important role in the structure of insulin. It possesses a labile amide group which, however, can be removed without affecting the biological activity. Removal of the carboxyl-terminal asparagine or aspartic acid results in a large decrease in biological activity of the molecules (Slobin and Carpenter, 1963). Perhaps pertinent to this point is the fact that all of the insulins which have been isolated so far from various species contain a carboxyl-terminal asparagine or aspartic acid (Brown *et al.*, 1955; Harris *et al.*, 1956;

Nicol and Smith, 1960; Wilson and Dixon, 1961; Kotaki, 1961).

ACKNOWLEDGMENTS

We are indebted to Dr. E. L. Grinnan of Eli Lilly and Company for generous supplies of insulin and for arranging for the biological assays. We would also like to thank Mrs. Bertha L. Martin for aid with the amino acid analyses and Miss Sharon L. Hayes for aid with the partition column chromatography.

ADDED IN PROOF

Sundby (1962) has recently reported on the acid-catalyzed transformation of insulin in which the transformation products were detected by paper electrophoresis in urea-containing buffers. His results indicated that the acid-transformation involves a progressive liberation of ammonia from the 6 amides of insulin. From a comparison of the rates of travel on paper electrophoresis of the various components in the mixture formed in the early stages of the transformation both before and after treatment of the mixture with carboxypeptidase, Sundy concluded, in agreement with our finding (Carpenter and Slobin, 1962), that the first amide was lost from the carboxyl-terminal asparagine. In view of Sundby's report, it appears probable that the desamido-insulin described in the present paper is a mixture of desamido-forms in which the major portion (85–90%) contains carboxyl-terminal aspartic acid instead of asparagine, and the minor portion (10–15%) has lost an amide from positions other than the carboxyl-terminal asparagine.

REFERENCES

Blatherwick, N. R., Bischoff, F., Maxwell, L. C., Berger, J., and Sahyum, M. (1927), *J. Biol. Chem. 72*, 57.

Brown, H., Sanger, F., and Kitai, R. (1955), *Biochem. J. 60*, 556.

Carpenter, F. H. (1958), *Arch. Biochem. Biophys. 78*, 539.

Carpenter, F. H., and Chrambach, A. (1962), *J. Biol. Chem. 237*, 404.

Carpenter, F. H., and Hess, G. P. (1956), *J. Am. Chem. Soc. 78*, 3351.

Carpenter, F. H., and Slobin, L. I. (1962), *Fed. Proc. 21*, 407d.

Chrambach, A., and Carpenter, F. H. (1960), *J. Biol. Chem. 235*, 3478.

DuVigneaud, V., Geiling, E. M. K., and Eddy, C. A. (1928), *J. Pharmacol. Exp. Therap. 33*, 497.

Grannis, G. F. (1960), *Arch. Biochem. Biophys. 91*, 255.

Harfenist, E. J. (1953), *J. Am. Chem. Soc. 75*, 5528.

Harfenist, E. J., and Craig, L. C. (1951), *J. Am. Chem. Soc. 73*, 877.

Harfenist, E. J., and Craig, L. C. (1952), *J. Am. Chem. Soc. 74*, 3083.

Harris, J. I., and Li, C. H. (1952), *J. Am. Chem. Soc. 74*, 2945.

Harris, J. I., Sanger, F., and Naughton, M. A. (1956), *Arch. Biochem. Biophys. 65*, 427.

Kotaki, A. (1961), *J. Biochem. (Japan) 50*, 256.

Leach, S. J., and Lindley, H. (1953a), *Trans. Faraday Soc. 49*, 915.

Leach, S. J., and Lindley, H. (1953b), *Trans. Faraday Soc. 49*, 921.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.

Nicol, D. S. H. W. (1960), *Biochem. J. 75*, 395.

Nicol, D. S. H. W., and Smith, L. F. (1960), *Nature 187*, 483.

Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.

Sanger, F., Thompson, E. O. P., and Kitai, R. (1955), *Biochem. J. 59*, 509.

Schultz, J., Allison, H., and Grice, M. (1962), *Biochemistry 1*, 694.

Seligson, D., and Seligson, H. (1951), *J. Lab. Clin. Med. 38*, 324.

Slobin, L. I., and Carpenter, F. H. (1963), *Biochemistry 2*, 16 (this issue).

Smith, K. L. (1950), *in* Hormone Assay, Emmens, C. W., editor, New York, Academic Press, Inc., p. 35.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Sundby, F. (1962), *J. Biol. Chem. 237*, 3406.

Waugh, D. F. (1946), *J. Am. Chem. Soc. 68*, 247.

Waugh, D. F. (1948), *J. Am. Chem. Soc. 70*, 1850.

Wilson, S., and Dixon, G. H. (1961), *Nature 191*, 876.

A Study of Actin by Means of Starch Gel Electrophoresis*

MARY E. CARSTEN† AND W. F. H. M. MOMMAERTS

From the Department of Medicine and The Los Angeles County Heart Association Cardiovascular Research Laboratory, The University of California at Los Angeles

Received July 30, 1962

The properties of globular actin prepared by ultracentrifugal isolation of F-actin followed by reversible depolymerization in the presence of ATP have been investigated. By ammonium sulfate fractionation and by starch gel electrophoresis the actin preparations were found to be homogeneous and free of tropomyosin. In the starch gel electrophoresis under a variety of conditions the actin preparations revealed diverse characteristic patterns. Four or five new bands appeared in the starch gel electrophoresis on standing, on removal of free ATP, on substitution of the sulfhydryl groups, or on exposure to *p*H 2, *p*H 10, or 7.2 M urea.

The purification of actin by ultracentrifugal isolation of its polymer (Mommaerts, 1952, 1958) followed by depolymerization has become widely accepted. However, the judgment as to the purity of the product rests on somewhat limited criteria: the complete polymerizability of the product upon the addition of salt, and the homogeneous appearance of the sedimenting boundary of the monomer in the ultracentrifuge. The first criterion is most valuable, but it might not detect moderate amounts of impurity associated with the

---

* This investigation was supported by Research Grant H-3067 of the National Heart Institute, National Institutes of Health, Bethesda, Md., and a grant from the Muscular Dystrophy Associations of America, Inc.

† Established Investigator, Los Angeles County Heart Association.