do not proceed through a seven coördinated activated complex, that is, by an S_N2 mechanism.

The results are in good agreement with those which would be expected if the intermediate is an activated complex which is penta coördinated such as in S_N1 mechanism. They seem in fact to constitute another example of steric acceleration of reaction velocity such as has been noted in the solvolysis of highly branched tertiary halides.[2]

A significant point is the thirty-fold difference in aquation rates between the *dl*- and *meso*-butylenediamine complexes. It can be seen by the use of molecular models that in the case of the *meso* butylenediamine complex where both methyl groups are on the same side of the five-membered ring there is considerable crowding of these two adjacent groups. This repulsion would not be lessened if the activated complex has a coördination number of seven but could be considerably decreased if this were five. That steric factors are important in accounting for the rapid rate of reaction is further substantiated by the almost instantaneous reaction of the complex which contains the tetramethylethylenediamine.

Other complexes of this type are being studied and in addition activation energies are being determined. These findings will be reported in more detail in the near future.

(2) H. C. Brown, and R. S. Fletcher, THIS JOURNAL, **71**, 1845 (1949).

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RECEIVED APRIL 18, 1952

THE USE OF CARBOXYPEPTIDASE FOR THE IDENTIFICATION OF TERMINAL CARBOXYL GROUPS IN POLYPEPTIDES AND PROTEINS. ASPARAGINE AS A C-TERMINAL RESIDUE IN INSULIN[1]

Sir:

A study of the action of carboxypeptidase on ACTH protein and peptide preparations[2] has led to a reinvestigation of its use as a general method for the identification of C-terminal groups in polypeptides and proteins; results obtained with insulin, fractions A and B derived from insulin by the method of Sanger,[3] and lysozyme, are summarized in Table I.

According to Sanger[4] the insulin molecule (m.w. 12,000) is composed of two pairs of identical polypeptide chains joined together through six S–S linkages; on this basis the insulin molecule should contain four free α COOH groups. Lens[5] reported the liberation of three moles of alanine by the action of carboxypeptidase and concluded that at least one, and possibly three, of the constituent peptide chains in insulin had alanine C-terminal groups. Other investigators using chemical methods found evidence for the presence of both alanine[6,7,8] and glycine[6,7] C-terminal groups in insulin.

TABLE I

ACTION OF CARBOXYPEPTIDASE ON INSULIN AND ITS FRACTIONS, AND LYSOZYME

Substance	Products of carboxypeptidase digestion: C-Terminal groups	Adjacent amino acids
Insulin	Alanine, asparagine	Lysine, leucine, glutamic acid, tyrosine
Fraction B	Alanine	Lysine
Fraction A	Asparagine	Cysteic acid,[a] leucine, tyrosine, glutamic acid
Lysozyme	Leucine	.....

[a] Cystine was not detected from insulin. This is of interest since it suggests that the enzyme proceeded to split peptide bonds further down the A chain, leaving the cystine residue attached only through its –S–S– group to one of the B chains.

In a typical experiment, insulin (Eli Lilly Lot No. 200-18-15; 80 mg., 0.5% solution, *p*H 7.8) was treated with carboxypeptidase[9] (160 μg. N) at 25°; equal aliquots were removed from the digestion mixture at intervals during an eight-hour digestion period. The addition of trichloroacetic acid (TCA) to a final concentration of 5% by volume served to terminate enzyme action, and to precipitate residual protein which was then removed by centrifugation; formation of free amino groups in supernatant fractions was followed by the ninhydrin reaction. For paper chromatographic studies, aliquots of the same supernatant fractions were passed through Amberlite (IR 4B) resin columns to remove TCA. Chromatography in butanol/acetic acid/water (4:1:5) revealed the presence of alanine, asparagine and aspartic acid after only two minutes digestion with the enzyme; after 8 hours of digestion, lysine, glutamic acid, tyrosine and leucine could also be detected. Starch column analysis of the fraction soluble at *p*H 5.4, after a four-hour digestion of insulin with carboxypeptidase under the conditions described above, confirmed the presence of alanine and asparagine.[10] Liberation of alanine was found to be complete after four hours, and amounted to two moles of alanine per mole of insulin. Preliminary studies indicate that proteolysis follows first order kinetics and that the rate of formation of alanine is about eight times that of the asparagine.

Digestion of Fraction B with carboxypeptidase confirmed that alanine[11] is in fact the C-terminal group of the phenylalanine chains in insulin. When Fraction A was treated with the enzyme, asparagine was found to be the initial product of digestion; further digestion led to the formation of cysteic acid, leucine, tyrosine and glutamic acid. It is concluded that, contrary to previous results,[6] asparagine occurs at the carboxyl end of the glycyl chains in the insulin molecule and that the other

(1) This work was supported in part by a grant to Dr. C. H. Li from the Rockefeller Foundation, New York. The author wishes to acknowledge the able assistance of Ning G. Pon.

(2) J. I. Harris and C. H. Li, Abstracts XIIth International Congress of Chemistry, 68 (September, 1951).

(3) F. Sanger, *Biochem. J.*, **44**, 126 (1949).

(4) F. Sanger, *ibid.*, **45**, 563 (1949).

(5) J. Lens, *Biochem. et Biophys. Acta*, **3**, 367 (1949).

(6) C. Fromageot, M. Justisz, D. Meyer and L. Penasse, *ibid.*, **6**, 283 (1950).

(7) A. C. Chibnall and M. W. Rees, *Biochem. J.*, **48**, xlvii (1951).

(8) S. G. Whaley and J. Watson, *J. Chem. Soc.*, 2394 (1951).

(9) The crystalline enzyme (8× recrystallized) used in this work was obtained through the generosity of Prof. H. Neurath.

(10) Some decomposition of asparagine to give aspartic acid was found to occur during the recovery and subsequent chromatography of TCA soluble fractions.

(11) F. Sanger, and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

amino acids are subsequently liberated in an undetermined sequence.

The lysozyme molecule is believed to consist of only one peptide chain having lysine as its N-terminal group.[12,13] Leucine was found to be the only amino acid formed by digestion of lysozyme with carboxypeptidase and it is concluded from this observation that leucine occurs at the carboxyl end of the lysozyme molecule.[14]

(12) F. C. Green and N. A. Schroeder, THIS JOURNAL, **73**, 1385 (1951).

(13) A. R. Thompson, *Nature*, **168**, 390 (1951).

(14) A similar result was recently reported by A. R. Thompson, *Nature*, **169**, 495 (1952).

DEPARTMENT OF BIOCHEMISTRY
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THE BIOLOGICAL ACTIVITY OF ENZYMATIC DIGESTS OF INSULIN[1]

Sir:

It has recently been demonstrated[2] that both alanine and asparagine are liberated simultaneously by the action of carboxypeptidase on insulin, the rate of formation of the alanine being of the order of eight times faster than that of the asparagine. In an experiment designed to correlate biological activity with loss of terminal carboxyl groups, insulin[3] solutions (80 mg. in 16 cc., *p*H 7.8) were treated with carboxypeptidase (160 μg. N); at the appropriate time intervals enzyme action was stopped by adjusting the *p*H to 5.4; the resulting precipitates were collected by centrifugation and subsequently assayed for insulin activity by the mouse convulsion test.[4] Fractions soluble at *p*H 5.4 were analyzed by the ninhydrin reaction[5] and the micro-Kjeldahl procedure. The presence of alanine and asparagine as the main products of digestion was confirmed by chromatography on paper; starch column analysis[6] showed that 1.9 moles of alanine and 0.4 mole of asparagine were present in the *p*H 5.4 soluble fraction after six hours digestion with carboxypeptidase. Results are summarized in Table I.

TABLE I

EFFECT OF CARBOXYPEPTIDASE ON THE HYPOGLYCEMIC ACTIVITY OF INSULIN

Time of digestion, hr.	Soluble N at *p*H 5.4, %	αNH_2-N alanine equiv.,[a] μM	Insulin activity, %
0	0.4	1.0	100
2	1.5	13.4	98
4	1.9	13.8	87
6	2.4	14.3	80
20	2.8	18.8	40

[a] Based on 80 mg. of insulin.

It may be concluded from the assay figures that, contrary to the results reported by Lens,[7] the two alanine C-terminal residues of the constituent phenylalanine chains of the insulin molecule are not essential for its biological activity; it would appear, however, that loss of terminal asparagine leads to a decrease of activity.

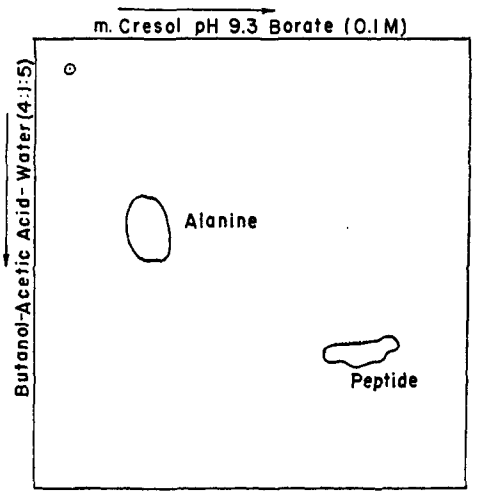

Fig. 1. Two-dimensional chromatogram of the *p*H 5.4 soluble fraction from a tryptic digest of insulin (enzyme/substrate ratio 1:5000 by weight).

Butler, *et al.*,[8,9] studied the action of trypsin on insulin and concluded that the observed diminution of biological potency was due to the splitting of one or two peptide bonds in the insulin molecule. Using lower enzyme concentrations Van Abeele and Campbell[10] demonstrated the formation of a stable trypsin-insulin complex which retained full insulin activity. The finding by Sanger and Tuppy,[11] that alanine and the heptapeptide gly.-phe.phe.tyr.thr.pro.lys are formed by the action of trypsin on Fraction B, led us to reinvestigate the action of this enzyme on insulin. Since no basic amino acids are present in the glycine chains of insulin, it might be predicted in accordance with the postulated specificity of the enzyme that trypsin would split four peptide bonds in the insulin molecule to give the same products as were obtained from Fraction B by Sanger and Tuppy.[11] In a series of preliminary experiments insulin (0.5% solutions) was incubated with crystalline trypsin (enzyme/substrate ratios varied from 1:50–1:5000) at *p*H 8.0 and 25° for 12–18 hours. Precipitates formed by adjusting digestion mixtures to *p*H 5.4 were removed by centrifugation and supernatant fractions were examined for digestion products by chromatography on paper (see Fig. 1). As predicted, the main products of digestion were found to be alanine and a heptapeptide of the same amino acid composition as the one identified by Sanger and Tuppy.[11] Under conditions where Van Abeele and Campbell[10] reported no loss of biological potency we found alanine to be the main

(1) This work has been supported in part by a grant from the Eli Lilly Laboratories.

(2) J. I. Harris, THIS JOURNAL, **74**, 2944 (1952).

(3) We are indebted to Drs. E. D. Campbell and O. K. Behrens of the Eli Lilly Laboratories for the generous supply of the crystalline insulin sample and the assay results.

(4) R. E. Thompson, *Endocrinology*, **39**, 1 (1946).

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

(6) W. H. Stein and S. Moore, *ibid.*, **176**, 337 (1948).

(7) J. Lens, *Biochimica et Biophysica Acta*, **3**, 367 (1949).

(8) J. A. V. Butler, E. C. Dodds, D. M. P. Phillips, and J. M. L. Stephen, *Biochem. J.*, **42**, 116 (1948).

(9) Butler, *et al.*, *ibid.*, **44**, 224 (1949).

(10) F. R. Van Abeele and E. D. Campbell, *Fed. Proc.*, **10**, 263 (1951).

(11) F. Sanger, and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).