Further Observations on the Cleavage of Bovine Insulin by Rat Adipose Tissue*

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ABSTRACT: A previous investigation showed that the water-insoluble, lipid-rich fraction of the aqueous homogenate of rat adipose tissue contains a system of peptidases which cleave insulin into numerous fragments. In this study, bovine insulin was incubated for 2 hr at pH 7.5 with this fraction of rat adipose tissue homogenate and the mixture of insulin cleavage products was then fractionated by gradient elution chromatography on DEAE-cellulose columns followed by high-voltage electrophoresis and paper chromatography. In the first experiment 13 peptide fragments of insulin, and in the second experiment 17 peptide fragments, were isolated and their quantitative amino acid compositions were determined. These data, as well as those previously reported on the quantitative composition of the mixture of free amino acids released, are compatible with the hypothesis that the cleavage process involves initial hydrolyses in the regions of A 13–14 (Leu-Tyr), A 18–19 (Asn-Tyr), B 11–12 (Leu-Val), B 15–16 (Leu-Tyr), B 24–25 (Phe-Phe), and B 25–26 (Phe-Tyr); the resulting five peptides then undergo stepwise removal of COOH- and NH_2-terminal residues. The cleavage of insulin in this manner abolishes the hormone's antilipolytic activity on rat and hamster adipose tissue but allows persistence of a weak degree of activity in stimulating the oxidation of glucose to CO_2 by the rat tissue. The structure of the peptide fragments responsible for the residual glucose oxidation activity was not established. These observations nevertheless indicate separate structural bases for the *in vitro* glucose oxidation and antilipolytic actions of insulin on adipose tissue slices.

Earlier work in several laboratories (Goodridge, 1964; Goodridge and Ball, 1965, 1966; Steinke *et al.*, 1965; Di Girolamo and Rudman, 1966) has revealed pronounced species differences in the *in vitro* responsiveness of adipose tissue slices to insulin. This hormone exerts at least two effects upon the fat cells: acceleration of glucose transport into the cell (Winegrad and Renold, 1958; Crofford and Renold, 1965) and suppression of the cell's response to lipolytic hormones (Jungas and Ball, 1963). Rat and mouse adipose tissues are highly sensitive to both actions of insulin; the hamster tissue only to the antilipolytic property; the tissues of guinea pig, rabbit, and several birds show little or no response to either action of insulin (Rudman and Di Girolamo, 1967). Possibly related to these species differences in the tissue's responsiveness to insulin is the fact that adipose tissues of rat, mouse, and hamster contain an insulin-cleaving system of peptidases, which is absent in the adipose tissues of rabbit and guinea pig (Di Girolamo *et al.*, 1965; Rudman *et al.*, 1966). These observations led to the questions: what is the chemical nature of the cleavage process; and what is the relationship (if any) between the cleavage of insulin and the tissue's response to the two actions of the hormone. Since amoung the five species studied the insulin-cleaving enzymes are found in adipose tissues highly responsive to insulin (rat, mouse, or hamster), but not in tissues with little or no responsiveness (rabbit and guinea pig), one possibility is that the cleavage process serves an "activating" function by releasing biologically active fragments of the hormone molecule; alternatively, the cleavage process might serve the function of terminating the biological response of the sensitive tissue by cleaving the hormone with the formation of inactive products.

In approach to these questions, an earlier study was carried out on the cleavage of bovine insulin by rat adipose tissue (Rudman *et al.*, 1966). The localization of the insulin-cleaving system in the aqueous-insoluble, lipid-rich fraction of the tissue homogenate, from which all soluble nitrogenous material could be removed by repeated washing prior to incubation with insulin, made possible preparation of the cleavage products uncontaminated by tissue peptides or amino acids. Analysis of these preparations for sulfhydryl groups, free amino and carboxyl groups, and trichloroacetic acid soluble nitrogen demonstrated the proteolytic nature of the cleavage process. That portion of the preparation soluble at pH 3.6 was fractionated by ion-exchange chromatography on phosphocellulose; the separated free amino acids were measured by the quantitative analyzer, while the separated acid-soluble peptides were hydrolyzed and their amino acid composition was studied by two-dimensional paper chromatography. The resulting data were compatible with the following type of cleavage

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process: (a) initial cleavage of insulin at bonds A 13–14, A18–19, B11–12, B15–16, B14–25, and B25–26, by one or more endopeptidases, followed by (b) stepwise removal by exopeptidases of NH_2- and COOH-terminal residues from the peptides initially produced.

The present report continues along these lines and describes the following additional data. (a) Fractionation of the entire system of cleavage products by ion-exchange chromatography at pH 9.0 on DEAE-cellulose; (b) quantitative analysis of the amino acid composition of the peptide fragments thus isolated; and (c) assay of the cleavage products for the capacity to stimulate oxidation of glucose to CO_2 and to suppress lipolysis in the adipose tissues of rat, hamster, and rabbit.

Materials and Methods

Animals. Male albino rabbits (3.5–4.5 kg) were fed Purina rabbit pellets *ad lib;* male golden hamsters (130–150 g) and male Wistar rats (either 130–170 or 500–600 g) were fed Purina Laboratory Chow Checkers *ad lib.*

Hormones and Reagents. Bovine insulin (24 units/mg) (Lilly), L-epinephrine bitartrate (Mann), oxycellulose-purified ACTH (100 units/mg) (Wilson), and DEAE-cellulose powder DE-11 (Reeve-Angel) were used.

Digestion of insulin by the washed insoluble lipid-rich fraction of rat adipose tissue homogenate was performed as previously described (Rudman *et al.*, 1966). Perirenal and epididymal adipose tissue (15 g) from male rats (500–600 g) was homogenized with a Waring Blendor in 100 ml of 0.15 M ammonium acetate buffer (pH 7.5) for 30 sec and spun at 0°, 1000*g*, for 15 min. The solid supernatant floating layer, containing nearly all the adipose tissue lipid as well as certain cytoplasmic organelles (Strand *et al.*, 1964), and which will henceforth be referred to as the "insoluble fraction" of the homogenate, was removed, resuspended in the original volume of buffer, and centrifuged again. After three such washings, the insoluble fraction was resuspended in 1000 ml of the buffer containing 1 mg/ml of insulin. This mixture was incubated with gentle stirring for 2 hr at 25°. The insoluble tissue fraction was then removed by centrifugation and filtration; the solution's pH was lowered to 5.4 with acetic acid and after 2 hr at 0° the precipitate (undigested insulin) was removed, and the supernatant was then freeze dried with removal of the ammonium acetate. This material could be dissolved at 10 mg/ml in 0.05 M ammonium acetate buffer (pH 9.0); 20 ml of this solution was applied to the DEAE-cellulose column.

Ion-Exchange Chromatography. A column 0.9 × 30 cm was packed with DEAE-cellulose equilibrated with 0.05 M ammonium acetate (pH 9.0). After introduction of the sample, 400–500 ml of this buffer was passed through the column; effluent was collected in 10-ml fractions. A gradient system with four mixing chambers was then attached: chamber 1, 200 ml of the initial 0.05 M buffer; chamber 2, 200 ml of 0.1 M buffer; chamber 3, 200 ml of 0.2 M buffer; and chamber 4, 200 ml of 0.3 M buffer, all at pH 9.0. After 800–1000 ml of effluent had been collected, the buffers in the four chambers were changed to 0.3, 0.4, 0.5, and 1.0 M, respectively, and an additional 1000 ml of effluent was collected. Finally, the influent was changed to 2.0 M buffer and 400 ml of eluate was collected. The column was operated at room temperature and flow rate was maintained at 100 ml/hr with a peristalsis pump. Each fraction (1 ml) was dried *in vacuo* at 80°; 2 ml of water was added to each tube and the evaporation was repeated. Each tube was then subjected to alkaline hydrolysis and colorimetric ninhydrin reaction (Hirs *et al.*, 1956). Peaks of ninhydrin-reactive material thus located were pooled and lyophilized; these samples are designated "column fractions" with numbers corresponding to the tubes pooled.

Analysis of Fractions from the DEAE Column. Each column fraction (3–5 mg) was subjected to high-voltage electrophoresis for 2 hr at pH 5.3 on Brinkmann MN paper in a Brinkmann Pherograph II apparatus (3000 V, 75 mA; the buffer consisted of 40 ml of pyridine, 20 ml of acetic acid, and 4000 ml of H_2O). Guide strips were stained with ninhydrin (0.2% in acetone) and the zones thus located were eluted with 10% acetic acid, lyophilized, and then chromatographed on No. 4 Whatman paper in the system 1-butanol–acetic acid–water (12:3:5) ("BuA") (descending technique, 18–24-in. run). Components located by spraying guide strips with ninhydrin were again eluted and lyophilized in two or three aliquots. One aliquot was used to verify purity of the peptide on MN paper by high-voltage electrophoresis at pH 5.3 in the first dimension followed by chromatography in solvent BuA in the second dimension. A second aliquot was hydrolyzed in 6 N HCl at 110° under nitrogen for 24 hr. In the case of cystine-containing peptides, a third aliquot was oxidized with performic acid (Moore, 1963) and lyophilized before acid hydrolysis. The acid hydrolysates were dried over KOH *in vacuo* and then analyzed quantitatively for amino acid composition in the Beckman–Spinco Model 120C instrument.

Bioassay of Column Fractions. Each fraction was assayed on epididymal adipose tissue of the 150-g rat for capacity to stimulate oxidation of glucose to CO_2, on hamster or rat epididymal tissue for the antilipolytic activity, and on the rabbit perirenal tissue for both effects. In the glucose oxidation assay, seven slices of rat epididymal adipose tissue (40–70 mg) were placed individually in seven incubation flasks, each containing 1 ml of Krebs–Ringer phosphate buffer (KRP),[1] 30 mg of albumin, 0.5 mg of glucose, 0.1 μCi of glucose-1-^{14}C, and serially increasing concentrations (0–100 μg) of the sample under test. Seven slices from each of three rats were incubated simultaneously. At the end of incubation, the microatoms of C-1 of extracellular glucose converted into CO_2 by the slice in each

[1] In preliminary experiments, dose–response curves for the glucose oxidation effect of insulin on the rat tissue were compared in KRP and in Krebs–Ringer bicarbonate buffer. Because the curves obtained in KRP showed less day-to-day variation in minimal effective dose and maximal response, this buffer was chosen for the experimental assays. Abbreviations used are as given in *Biochemistry 5*, 1445 (1966). FFA, free fatty acid.

TABLE I: Amino Acid Compositions of Peptides Isolated in Expt I.[a]

	Column Fraction 7–13	Column Fraction 50–62				
	Peptide I	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Peptide 6
Lysine	0.72 (1.00) (1)	—	—	—	—	—
Histidine	—	—	—	—	—	—
Arginine	—	—	—	—	0.11 (1.37) (1)	0.19 (0.83) (1)
Half-cystine	—	—	0.48 (2.00) (2)	0.25 (1.92) (2)	0.17 (2.11) (2)	0.47 (2.04) (2)
Aspartic acid	—	0.35 (0.92) (1)	0.22 (0.92) (1)	0.13 (1.00) (1)	0.08 (1.00) (1)	—
Threonine	—	—	—	—	—	—
Serine	—	—	—	—	—	—
Glutamic acid	—	0.32 (0.84) (1)	—	—	0.07 (0.88) (1)	0.23 (1.00) (1)
Proline	0.50 (0.70) (1)	—	—	—	—	—
Glycine	—	—	—	0.10 (0.77) (1)	0.10 (1.25) (1)	0.42 (1.83) (2)
Alanine	0.55 (0.76) (1)	—	—	—	—	—
Valine	—	—	—	—	0.06 (0.75) (1)	0.15 (0.65) (1)
Isoleucine	—	—	—	—	—	—
Leucine	—	0.38 (1.00) (1)	—	—	—	—
Tyrosine	—	—	—	—	—	—
Phenylalanine	—	—	—	—	—	—
EM (cm)[b]	−9.5	7.6	−1.1	−1.1	−1.1	−3.0
R_F[c]	0.42	0.53	0.05	0.12	0.29	0.15
Proposed structure	B28–30	A17–18	A20–21 S S B19	A20–21 S S B18–19	A20–21	A20 S S B18–19–23

	Column Fraction 63–70		Column Fraction 85–94		Column Fraction 96–111		
	Peptide 7	Peptide 8	Peptide 9	Peptide 10	Peptide 11	Peptide 12	Peptide 13
Lysine	—	—	—	—	—	—	—
Histidine	—	—	—	0.21 (1.10) (1)	0.51 (1.89) (2)	—	0.25 (1.73) (2)
Arginine	—	—	—	—	—	—	—
Half-cystine	—	—	0.59 (4.90) (4)	0.59 (3.10) (4)	1.27 (4.70) (4)	0.82 (2.10) (2)	0.64 (4.41) (4)
Aspartic acid	—	0.47 (1.00) (1)	—	0.13 (0.68) (1)	0.27 (1.00) (1)	—	—
Threonine	—	—	—	—	—	—	—
Serine	—	—	0.24 (2.00) (2)	0.16 (0.84) (1)	0.76 (2.82) (3)	—	0.29 (2.00) (2)
Glutamic acid	0.52 (1.00) (1)	0.74 (1.57) (2)	0.23 (1.92) (2)	0.51 (2.68) (3)	0.46 (1.70) (2)	0.39 (1.00) (1)	0.29 (2.00) (2)

Proline	—	—	—	—	—	—	—	—
Glycine	—	—	0.16 (1.33) (1)	0.21 (1.10) (1)	0.22 (0.82) (1)	0.35 (0.90) (1)	0.17 (1.17) (1)	
Alanine	0.73 (1.40) (1)	—	0.12 (1.00) (1)	0.19 (1.00) (1)	0.35 (1.29) (1)	—	0.12 (0.83) (1)	
Valine	0.42 (0.81) (1)	—	0.11 (0.92) (1)	0.30 (1.58) (2)	0.43 (1.59) (2)	0.31 (0.80) (1)	0.24 (1.66) (2)	
Isoleucine	—	—	—	—	—	—	—	
Leucine	—	0.39 (0.83) (1)	—	0.19 (1.00) (1)	0.46 (1.70) (2)	—	0.16 (1.10) (1)	
Tyrosine	—	—	—	—	—	—	—	
Phenylalanine	—	—	—	—	—	—	—	
EM (cm)[b]	3.4	3.4	−3.0	3.4	−1.9	3.4	−1.9	
R_F[c]	0.29	0.47	0.29	0.38	0.38	0.35	0.17	
Proposed structure	B12-14	A15-18	A3-7-12	A3(4)-7-11	A5-7-12(13)	A20	A5-7-11	
			S	S	S	S	S	
			S	S	S	S	S	
			B7-8	B2(3)-7-8	B2(3)-7-11(12)	B18-19-21	B2(3)-7-11(12)	

[a] No correction was made for destruction or incomplete liberation of amino acids during 6 N HCl hydrolysis. Analyses are reported as micromoles. First value in parentheses represents observed molar ratio relative to the amino acid specified as 1.00, 2.00, or 4.00. Second value in parentheses indicates number of residues per molecule of peptide with the proposed structure. Dash indicates that amino acid was either undetectable or present in amount less than 0.01 μmole. [b] EM = electrophoretic mobility. (−) indicates migration toward cathode. [c] R_F in BuA solvent system.

flask were determined as described previously (Di Girolamo and Rudman, 1966). Each column fraction was assayed in this way on a total of six to nine rats. The glucose oxidation effect of each concentration of the material under assay was calculated as increase in μatoms of glucose C-1 converted into CO_2/g of tissue per 2 hr. In assays on rabbit adipose tissue, the same technique was used but four slices of perirenal adipose tissue were tested at each dose; in each experiment the tissue from a single animal was used.

Assay for antilipolytic effect (Rudman and Shank, 1966): The incubation medium consisted of 2 ml of KRP (four tissue slices in four individual flasks), KRP containing 1 μg/ml of ACTH (four slices), and KRP with 1 μg/ml of ACTH plus varying concentrations (0–100 μg/ml) of the material under test (four slices at each concentration). After a 90-min incubation, the tissue slices were removed and analyzed for FFA concentration. The lipolytic effect of ACTH was calculated as increase in the concentration of FFA in the tissue slice (microequivalents per gram of tissue) produced by the hormone. The antilipolytic effect of each concentration of the test material was calculated as reduction in the tissue concentration of FFA (microequivalents per gram of tissue) in the slices incubated with ACTH plus test material, as compared with the slices incubated with ACTH alone.

Results

Ion-Exchange Chromatography of the Mixture of Cleavage Products. For standardizing purposes, the cumulative volumes at which the various amino acids present in insulin, and at which the intact hormone, were eluted from the DEAE column, were determined. The results are shown in Figure 1A: the basic and neutral amino acids appeared in fraction 6–18, the acidic amino acids in fraction 28–40, and insulin in fraction 140–190. In a control experiment, the washed insoluble fraction from 15 g of rat adipose tissue was incubated without insulin for 2 hr, the tissue preparation was removed, and the supernatant was lyophilized and run through the column; no ninhydrin-reactive peaks were detected in the column effluent. The elution diagram from chromatography of 100 mg of the cleavage product preparation is given in Figure 1B (expt I); the results of a repeat expt II, with a preparation of cleavage products made under the same conditions on another day, are given in Figure 1C.

Purification and Analysis of the Column Fractions. The various ninhydrin-reactive peaks from the column in expt I and II were resolved into their individual components by high-voltage electrophoresis and paper chromatography (Table I). In expt I, from 8 column fractions, a total of 32 components were isolated. Of these, 6 proved to be free amino acids while 26 proved to be peptides. Of these 26, 13 were obtained in pure form and in sufficient yield for quantitative analysis of amino acid composition, the results of which are given in Table I. Photographs of typical two-dimensional electropherogram chromatograms documenting the purity of isolated peptides are reproduced in Figure 2.

TABLE II: Amino Acid Compositions of Peptides Isolated in Expt II.[a]

	Column Fraction 8–12			Column Fraction 17–20
	Peptide 1	Peptide 2	Peptide 3	Peptide 4
Lysine	0.09 (1.00) (1)	0.27 (1.00) (1)	0.15 (1.00) (1)	–
Histidine	–	–	–	–
Arginine	–	–	–	–
Half-cystine	–	–	–	–
Aspartic acid	–	–	–	–
Threonine	–	–	0.11 (0.76) (1)	0.28 (0.92) (1)
Serine	–	–	–	–
Glutamic acid	–	–	–	–
Proline	–	0.34 (1.26) (1)	0.13 (0.89) (1)	0.30 (1.00) (1)
Glycine	–	–	–	–
Alanine	0.13 (1.40) (1)	0.26 (0.95) (1)	0.18 (1.18) (1)	–
Valine	–	–	–	–
Isoleucine	–	–	–	–
Leucine	–	–	–	–
Tyrosine	–	–	0.11 (0.71) (1)	0.25 (0.85) (1)
Phenylalanine	–	–	–	–
EM (cm)	−12.4	−9.2	−5.7	−1.6
R_F	0.45	0.37	0.34	0.23
Proposed structure	B29–30	B28–30	B26–30	B26–28

	Column Fraction 75–85	Column Fraction 86–99		
	Peptide 10	Peptide 11	Peptide 12	Peptide 13
Lysine	–	–	–	–
Histidine	0.29 (2.42) (2)	–	–	0.18 (1.80) (2)
Arginine	–	0.15 (1.00) (1)	–	–
Half-cystine	0.49 (4.00) (4)	0.26 (1.73) (2)	0.36 (1.80) (2)	0.29 (2.90) (3)
Aspartic acid	0.11 (0.87) (1)	0.12 (0.80) (1)	0.23 (1.15) (1)	0.09 (0.90) (1)
Threonine	–	–	–	–
Serine	0.41 (3.30) (3)	–	–	0.24 (2.40) (3)
Glutamic acid	0.41 (3.38) (3)	0.18 (1.17) (1)	0.24 (1.20) (1)	0.21 (2.10) (2)
Proline	–	–	–	–
Glycine	0.16 (1.32) (1)	0.20 (1.32) (1)	0.20 (1.00) (1)	0.11 (1.10) (1)
Alanine	0.16 (1.32) (1)	–	–	0.08 (0.80) (1)
Valine	0.42 (3.44) (3)	–	0.22 (1.10) (1)	0.17 (1.70) (2)
Isoleucine	–	–	–	–
Leucine	0.23 (1.85) (2)	–	–	0.20 (2.00) (2)
Tyrosine	–	–	–	–
Phenylalanine	–	–	–	–
EM (cm)	−2.9	−1.9	1.5	−0.95
R_F	0.36	0.30	0.35	0.15
Proposed structure	A3–7–12 S S B2–7–11	A20–21 S S B19–22	A20–21 S S B18–19–21	A5–7–12 S S B2–7–11

[a] See caption of Table I for explanation of symbols.

Column Fraction 75-85				
Peptide 5	Peptide 6	Peptide 7	Peptide 8	Peptide 9
–	–	–	–	–
–	–	–	–	–
–	–	–	0.19 (0.81) (1)	0.25 (0 89) (1)
–	–	–	0.46 (2.00) (2)	0.44 (1.57) (2)
–	0.09 (1.00) (1)	–	0.18 (0.78) (1)	–
–	–	0.39 (1.00) (1)	–	–
–	–	–	–	–
0.28 (0.80) (1)	0.18 (2.00) (2)	–	0.28 (1.21) (1)	0.34 (1.21) (1)
–	–	–	–	–
–	–	–	0.54 (2.35) (2)	0.27 (0.97) (1)
–	–	–	–	–
–	–	–	0.17 (0.76) (1)	0 28 (1.00) (1)
–	–	–	–	–
0.35 (1.00) (1)	0.08 (0.91) (1)	–	0.22 (0.95) (1)	0.31 (1.11) (1)
–	–	0.33 (0.84) (1)	–	–
–	–	–	–	–
6.0	2.0	−1.9	−2.1	−0.95
0.42	0.52	0.50	0.15	0.20
A16–17	A15–18	B26–27	A20–21 S S B17–19–23	A20 S S B17–19–22

Column Fraction 114–120		Column Fraction 120-130	
Peptide 14	Peptide 15	Peptide 16	Peptide 17
–	–	–	–
–	0.17 (1.10) (1)	–	0.96 (2.40) (2)
–	–	–	–
0.18 (1.63) (2)	0.55 (3.65) (4)	0.48 (1.60) (2)	1.53 (3.84) (4)
0.11 (1.00) (1)	0.15 (1.00) (1)	0.27 (0 90) (1)	0 40 (1 00) (1)
–	–	–	–
–	0.42 (2.77) (3)	–	–
0.11 (1.00) (1)	0.48 (3.20) (3)	0.30 (1.00) (1)	1.55 (3.86) (4)
–	–	–	–
0.11 (1.00) (1)	0.18 (1.21) (1)	0.31 (1.03) (1)	0.56 (1.00) (1)
–	0.17 (1.12) (1)	–	0.81 (2.02) (2)
0.10 (0.91) (1)	0.45 (2.97) (3)	–	1.29 (3.22) (3)
–	–	–	–
0.10 (0.91) (1)	0.17 (1.16) (1)	–	0.90 (2.24) (2)
–	–	–	–
–	–	–	0.34 (0.86) (1)
1.2	−2.0	1.2	−2.5
0.28	0.20	0.20	0.10
A20–21 S S B17–19–21	A3–7–12 S S B2–7–9	A20–21 S S B19–21	A4–7–12 S S B1–7–14

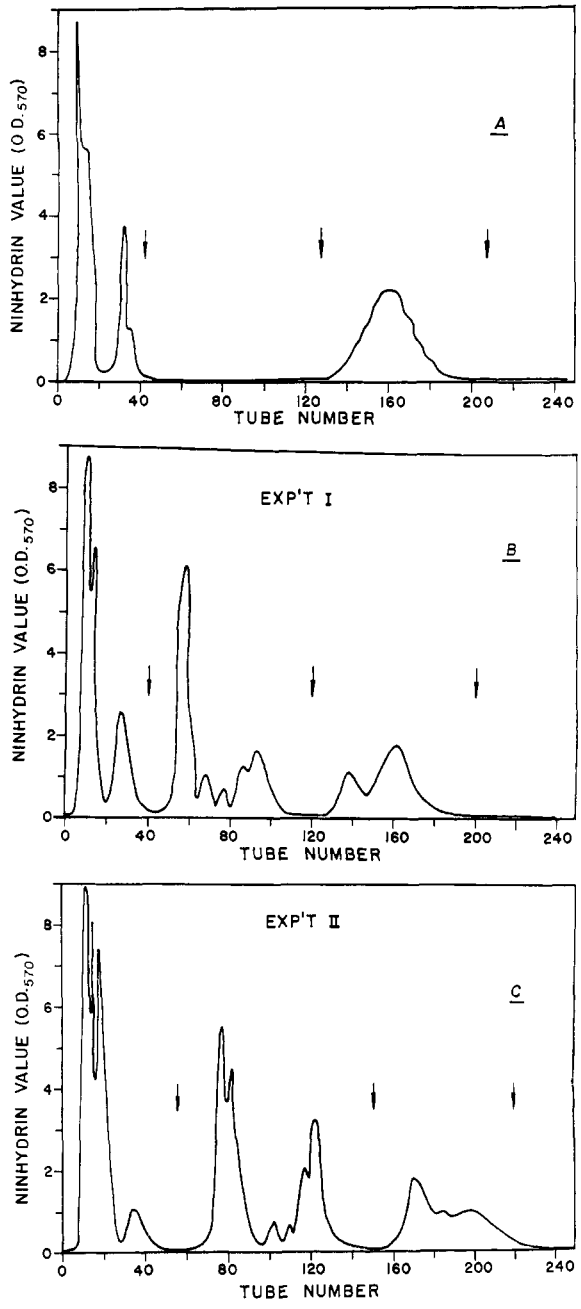

FIGURE 1: Column chromatography on DEAE-cellulose of: (A) mixture of 3 mg each of the 17 amino acids present in insulin and 30 mg of intact bovine insulin; (B and C) 200 mg of cleavage products produced by incubating insulin with the washed insoluble fraction of rat adipose tissue. Details of chromatographic procedure are given in text. The initial influent solution was 0.05 M (pH 9.0); vertical arrows indicate changes to various four-solvent gradient systems described in text.

The results in expt II generally paralleled those of expt I. Thirty six components were visualized of which eight were free amino acids and the remainder peptides; seventeen of the latter were analyzed quantitatively (Table II). The quantitative composition of the mixture of free amino acids released during the cleavage of insulin by the rat adipose preparation was reported in the previous study (Rudman *et al.*, 1966).

Consideration of the amino acids present in each peptide generally indicated the region of the insulin molecule (Figure 3) from which it was derived. Thus, peptide 3 in expt I, containing only half-cystine and aspartic acid, could only represent

A20–21
S
S
B19

while peptide 4 in expt I, containing these amino acids and valine, must represent

A20–21
S
S
S
B18–19

These postulated structures (Tables I and II) could be tested by comparing the molar ratios calculated from the amino acid analysis, after assigning an appropriate residue a value of 1, 2, or 4, with the molar ratios dictated by the proposed structure. In general, the calculated ratios were consistent with the postulated structures, although several deviations from the theoretical ratio were encountered (for example, half-cystine in peptides 9–11 and 13 in expt I). These inconsistencies could have resulted from destruction or incomplete liberation of amino acids during acid hydrolysis of the peptide, or from presence of traces of contaminating peptides despite apparent homogeneity on electrophoresis and paper chromatography. The latter possibility is favored because the nature of the cleavage process (see Discussion) probably leads to accumulation of groups of structurally similar peptides, with some members of each group differing from each other only in the presence or absence of a single terminal amino acid. Such pairs of peptides might in some cases possess the same electrophoretic and chromatographic mobilities and therefore would be eluted together in the purification process, with resulting deviation from the theoretical molar ratios.

In both expt I and II, the last peaks from the column [130–180 in expt I and 160–220 in expt II (corresponding to the general position of elution of insulin)] did not move from the origin on high-voltage electrophoresis. These fractions were therefore analyzed at pH 8.6 by low-voltage (200 V) electrophoresis on cellulose acetate strips, in comparison with intact insulin. While Carpenter and Hayes (1963) carried out this procedure in buffer containing 7 M urea, we found that urea could be omitted when the cellulose acetate strips furnished by Millipore Corp. were employed as supporting medium. Insulin itself moved as a major band followed closely by one or (in some electropherograms) two minor bands. Fractions 153–166 from expt I and 190–220 from expt II showed the same pattern as intact insulin. Fractions 130–150 of expt I and 160–180 of expt II, however, consisted largely of the slowest of the three bands visualized in uncleaved insulin (Figure 4). Qualitative anal-

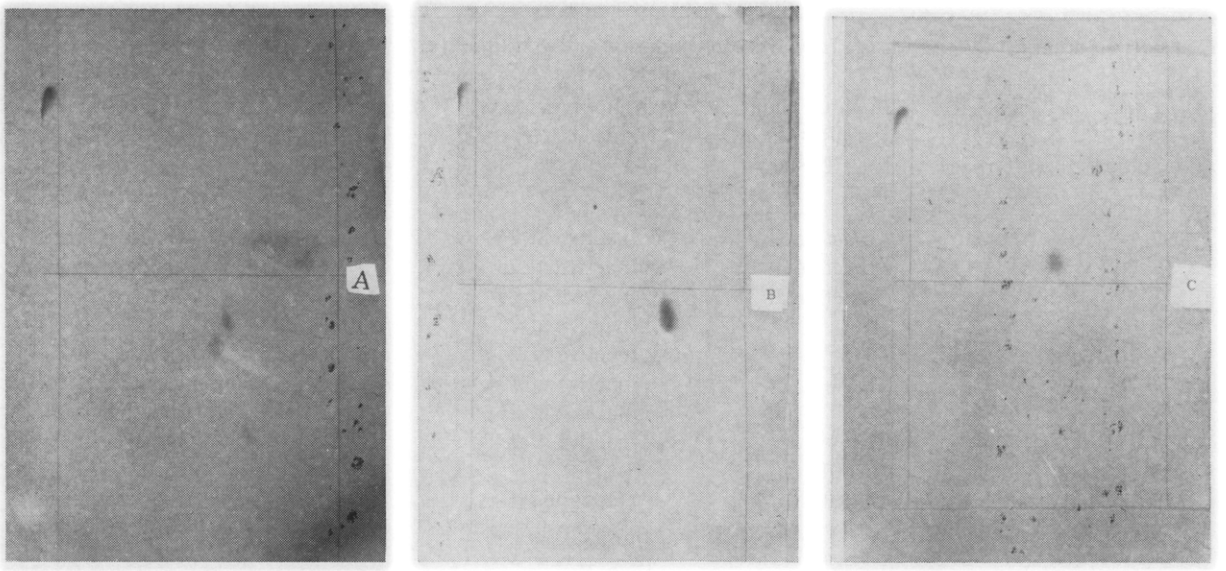

FIGURE 2: Two-dimensional electropherogram chromatogram of column fraction 50–62 from expt I (panel A), and of two purified peptides: peptide 14, expt II (panel B); and peptide 8, expt II (panel C). The point of sample application was at the junction of the righ-hand vertical line and the horizontal line (just to the left of the labeling letter). Electrophoresis at pH 5.3 was carried our for 2 hr at 3000 V in the vertical dimension, the cathode being above. Ascending chromatography in solvent BuA was then performed for 18 hr, the right-hand margin of the paper being immersed in the solvent tray, the solvent front ascended to within 1 in. of the vertical line on the left. The spot in the upper left of each paper is arginine, which was applied at the left end of the horizontal line as a marker for electrophoresis. The chromatographic solvent front was not allowed to reach the arginine marker

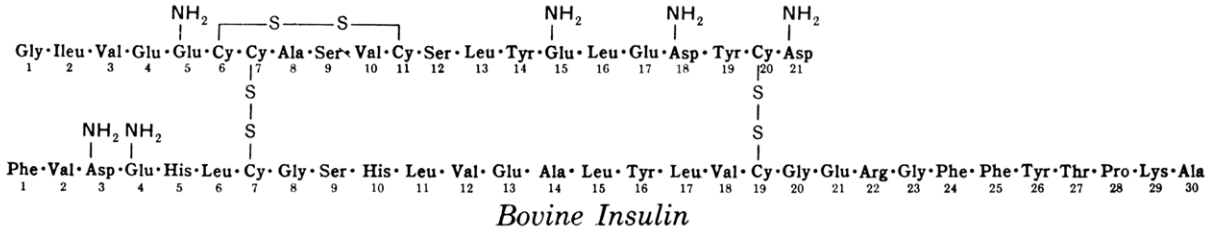

FIGURE 3: Structure of bovine insulin.

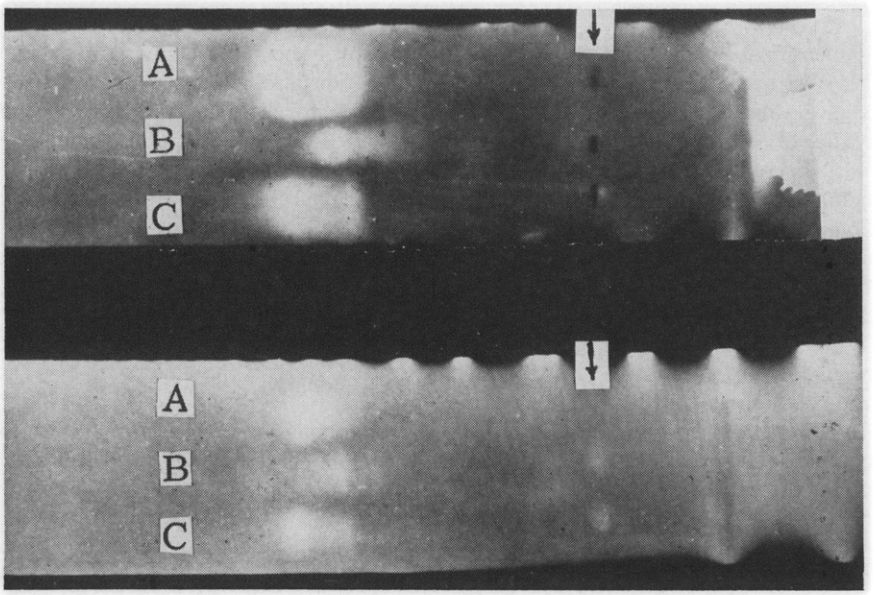

FIGURE 4: Electrophoresis of insulin and experimental fractions on 1.5 × 6.5 in. strips of cellulose acetate (Millipore) at pH 8.6 in sodium Veronal buffer (ionic strength, 0.1) for 2 hr at 200 V (2 mA/strip). Arrow marks site of sample application (10 μl of solution containing 50 μg of sample). Cathode to the right. (A) Insulin; (B) fraction 130–150, expt I; (C) fraction 153–166, expt I. Upper strip was stained with 0.001% nigrosin in 2% acetic acid. Lower strip was fixed in 20% sulfosalicylic acid and stained with 0.25% Coomassie blue. The strips were photographed by transmitted light as contact prints.

TABLE III: Summary of Assays for *in Vitro* Glucose Oxidation and Antilipolytic Activity on Adipose Tissue.[a]

Column Fraction	Glucose Oxidation Assay Rat Adipose Tissue	Antilipolytic Assay Hamster Adipose Tissue	Antilipolytic Assay Rat Adipose Tissue	Antilipolytic Assay Rabbit Adipose Tissue
		Expt I		
7–13	Inactive at 25	Inactive at 25		Inactive at 25
11–20	Inactive at 25	Inactive at 25		Inactive at 25
21–27	Inactive at 25	Inactive at 25		Inactive at 25
50–62	0.1	Inactive at 25	Inactive at 25	Inactive at 25
63–70	0.1	Inactive at 25	Inactive at 25	Inactive at 25
71–78	1	Inactive at 25	Inactive at 25	Inactive at 25
85–94	1	Inactive at 25		Inactive at 25
96–111	1	Inactive at 25		Inactive at 25
153–166	0.001	0.01		Inactive at 25
Mixture of cleavage products	Not tested	Not tested		Inactive at 100
		Expt II		
8–12	Inactive at 25	Inactive at 25		Not tested
17–20	Inactive at 25	Inactive at 25		Not tested
30–40	Inactive at 25	Inactive at 25		Not tested
75–85	1.0	Inactive at 25		Inactive at 25
86–99	0.1	Inactive at 25		Inactive at 25
114–120	1	Inactive at 25		Inactive at 25
120–130	1	Inactive at 25		Inactive at 25
160–200	0.001	0.01		Inactive at 25
Mixture of cleavage products	Not tested	Not tested		Inactive at 100
Insulin	0.001	0.01		Inactive at 100

[a] Results are given as minimum effective dose where activity was present, and as highest concentration tested where no activity was found. Units in micrograms per milliliter.

ysis with two-dimensional paper chromatography (Rudman *et al.*, 1966) of the 6 N HCl hydrolysates of all of these fractions gave a pattern identical with that of the acid hydrolysate of uncleaved insulin. Further studies on these various components in the final peaks from the DEAE columns are in progress.

Bioassays of Column Fractions. The results in expt I and II were similar and are summarized in Figure 5 and Table III. Materials contained in the column effluent before the first change in buffer system (see Figure 1), *e.g.*, fractions 6–13, 11–20, and 21–27 in expt I, were inactive in both glucose oxidation and antilipolytic assays at 25 μg/ml, the highest dose tested. Fractions eluted between the first and second buffer changes (within the range 50–111 in expt I and 75–130 in expt II) exhibited weak glucose oxidation activity on the rat tissue (minimal effective dose 0.1–1 μg/ml compared with 0.001 μg/ml for insulin; maximal response of 2.5 μatoms increase in amount of glucose C-1 converted into CO_2/g of tissue per 2 hr compared with 6.5 μatoms for insulin) but were devoid of antilipolytic activity on either the hamster or rat tissue. Fractions eluted after the second change in buffer system, thus corresponding in position of elution to uncleaved insulin (fractions 152–166 in expt I and 160–200 in expt II), were equipotent with intact insulin in both types of assay.

The entire cleavage product preparation in both expt I and II, as well as the individual column fractions in both experiments, was totally inactive in both types of assay on rabbit adipose tissue at a concentration of 25–100 μg/ml; uncleaved insulin was also inactive in both types of assay on the rabbit tissue, as previously reported (Di Girolamo and Rudman, 1966; Rudman and Shank, 1966).

Discussion

Nature of the Cleavage Process. In the previous study (Rudman *et al.*, 1966), quantitative analyses were done at various time intervals on the mixture of free amino acids released during the cleavage of insulin by the aqueous insoluble fraction of rat adipose tissue. Amino acids were found to be released in constant molar proportions during the course of the cleavage: tyrosine, 27%; leucine, 26%; phenylalanine, 16%; asparagine plus glutamine, 8%; alanine, 7%; valine, threonine, and serine, 3–4%; and glycine and isoleucine, 1–3%. These findings, supported by qualitative data on the amino

acid composition of 14 peptide cleavage products, and lack of appearance of free SH groups during the degradation, led to the hypothesis that the initial sites of cleavage were in the regions of A13–14, A18–19, B11–12, B15–16, B24–25, and B25–26, and therefore liberated five peptides (structures I–V). These, it was proposed, then underwent stepwise removal of NH_2- and COOH-terminal residues, releasing a mixture of free amino acids in characteristic proportions to give five groups of related peptides. If one assumed hydrolysis of the six specified internal bonds, and a yield of the three amino acids closest to the NH_2 and COOH terminus of each of the five peptides initially formed in a ratio of 1:0.5:0.25, than the resulting mixture of free amino acids would have a molar composition closely similar to that which was found experimentally.

A1–7–13			A19–20–21	
S			S	
S			S	
B1–7–11	B12–15	A14–18	B16–19–25	B26–30
I	II	III	IV	V

In the present study, quantitative amino acid analyses have been performed on 17 of the peptide cleavage products. These data indicate structures for these peptides which are consistent with the hypothesis presented above. Thus in expt II peptides 10, 13, 15, and 17 can be assigned to group I; peptides 5 and 6 to group II; peptides 8, 9, 11, 12, 14, and 16 to group IV; and peptides 1, 2, 3, and 4 to group V. Similar relationships are evident for the 13 peptides isolated in expt I.

Two limitations of the evidence gathered in this study must be emphasized. Since the yields of the peptides isolated were not determined, it is not known whether they represent major or minor products of the degradation of insulin. Furthermore, about 15 peptides in each experiment were not recovered in sufficient purity for amino acid analysis. Thus the possibility of additional modes of cleavage cannot be excluded. Nevertheless, the composition of the mixture of free amino acids released, and the fact that no peptide was encountered which did not correspond to one of the five predicted groups, suggest that the proposed model probably describes the major cleavage reactions.

Effect of the Cleavage Process on Activities of the Insulin Molecule. Piazza *et al.* (1959) have shown that slices of rat adipose tissue incubated in insulin-containing medium progressively degrade the hormone to trichloracetic acid soluble fragments. It is not yet known whether this degradation is carried out by the proteolytic enzyme system located in the aqueous-insoluble fraction of the tissue homogenate, the action of which on insulin was examined in the present study. Assuming that this may be the case, then examination of the biological activities of the cleavage products produced in the present experiments might indicate the physiological function of the cleavage reaction. If the cleavage process served a physiological "activation" function, then one would expect one or more cleavage products to retain or gain biological activity, especially for the rabbit tissue which lacks the cleavage enzymes and is virtually unresponsive to intact insulin. However the data do not indicate such an effect. The cleavage preparation, like insulin, is totally inactive upon the rabbit tissue; and every column fraction (with the exception of that eluted in the position of intact insulin (153–166) in expt I) is either inactive or considerably less active than insulin upon the rat and hamster tissues. The high glucose oxidation and antilipolytic activities of fractions 153–166 in expt I and of 160–200 in expt II are presumably due to the content of intact insulin in these fractions. Thus the data seem to indicate that if the

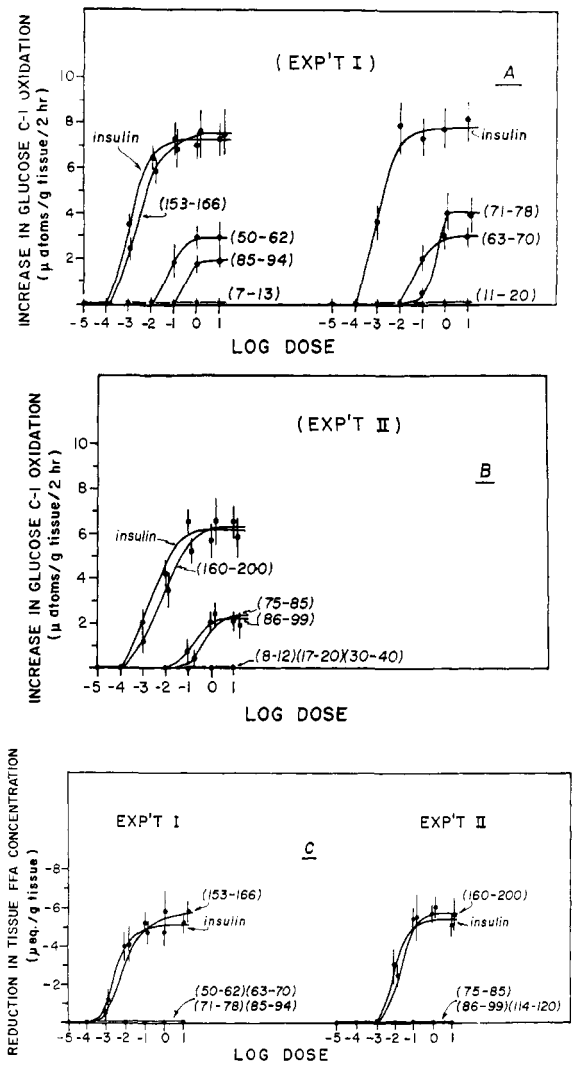

FIGURE 5: Dose–response curves for glucose oxidation activity (A and B) and antilipolytic activity (C) of insulin, and of various column fractions (designated by numbers within parentheses) from expt I and II. Glucose oxidation activity was measured on epididymal adipose tissue of the rat, and antilipolytic activity was determined on the epididymal adipose tissue of the hamster. Abscissa: logarithm (base 10) of concentration of material under assay (micrograms of salt-free preparation per milliliter of incubation medium). In the glucose oxidation assay, response (ordinate) represents increase in μatoms of glucose C-1 converted into CO_2/g of tissue per 2 hr. In the antilipolytic assay, response represents reduction of FFA concentration (microequivalents per gram of tissue) in slices exposed to 1 μg/ml of ACTH. Each point represents mean of six to nine observations; standard error is also shown.

cleavage of insulin studied in the present experiments occurs physiologically in the fat cells of rat, mouse, and hamster, it serves the function of terminating rather than initiating the biological responses.

The weak glucose oxidation activity of fractions eluted between the first and second buffer changes in both expt I and II cannot be attributed to contamination with uncleaved insulin because of the total absence of antilipolytic activity; furthermore the shape of the dose–response curve for the effect of these fractions on glucose oxidation is different from that of intact insulin (Figure 5).[2] It appears that a weak capacity to stimulate glucose oxidation is retained by one or more of the cleavage products. Since the column fractions under consideration which retain weak glucose oxidation activity contain principally peptides of groups I and IV, it may be suspected that one or more peptides in one of these groups is the active fragment, but the biologic activity could also reside in one of the unidentified peptides in these fractions. The fact that the cleavage process abolishes the antilipolytic but not the glucose oxidation property of insulin suggests that the structural bases for these two activities are not identical. The question of different structural bases for the glucose oxidation and antilipolytic actions of insulin is further investigated in the accompanying report (Rudman *et al.*, 1968).

Acknowledgment

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[2] If the glucose oxidation activity of these fractions were due to the presence of traces of uncleaved insulin, then the dose–response curves for this activity would be expected to show a higher minimal effective dose but the same maximal response as the intact hormone. As shown in Figure 5, however, the maximal response was only 40% as great as that of uncleaved insulin. This type of evidence is not conclusive, nevertheless, since the presence in the eluted fraction of a small amount of intact insulin together with a peptide which partially inhibited the hormone's glucose oxidation effect, and totally inhibited its antilipolytic activity, could also lead to the assay results observed.

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