Effects on Mammalian Adipose Tissue of Fragments of Bovine Insulin and of Certain Synthetic Peptides*

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ABSTRACT: Insulin causes at least two different effects on adipose tissue *in vitro:* acceleration of the metabolism of glucose and suppression of the lipolytic response to adrenocorticotropin, epinephrine, or other lipase-activating hormones. The present study investigated the structural basis for these two actions of the hormone. The following insulin derivatives furnished by Dr. F. H. Carpenter were assayed both for activity in stimulating the oxidation of glucose to CO_2 (on rat epididymal adipose tissue) and for antilipolytic activity (on hamster adipose tissue): desalanine insulin, desalanine-desasparagine insulin, desoctapeptide insulin, and the heptapeptide B23–29. Desalanine insulin showed the same glucose oxidation and antilipolytic potencies as intact insulin. Desalanine-desasparagine insulin and desoctapeptide insulin were active in both assays but were considerably less potent than insulin: minimal effective dose in the glucose oxidation assay was 100 times and 1000 times greater than that of intact insulin, respectively, and the maximal response was only one-half as great. The minimal effective dose for antilipolytic potency of these two derivatives, however, was only ten times greater than that of insulin and the maximal response was not curtailed. The heptapeptide was inactive in both assays. Intact insulin and all four insulin derivatives failed to produce either a glucose oxidation or antilipolytic effect on rabbit adipose tissue. A series of synthetic di- and tripeptides, including several corresponding to various sequences in the insulin structure, were also assayed. The following peptides showed antilipolytic but not glucose oxidation activity on adipose tissue of hamster, rabbit, and rat: *N*-carbobenzoxy-Leu-Tyr-NH_2, Leu-Tyr-NH_2, *N*-carbobenzoxy-Gly-Tyr-NH_2, and Gly-Tyr-NH_2. The minimal effective close for antilipolytic activity of these peptides was 1000 times or more greater than that of insulin but the magnitude of the effect was the same.

Insulin acts in at least two different ways on adipose tissue: it stimulates transport of glucose into the fat cell, with resulting accelerated conversion of the hexose into CO_2 and triglyceride (Winegrad and Renold, 1958; Crofford and Renold, 1965) and it also suppresses the cell's response to lipolytic hormones (Jungas and Ball, 1963). The structural basis for the hormone's capacity to stimulate the metabolism of glucose by rat adipose tissue has been considered by several workers. Mirsky (1965) found negligible activity in "desoctapeptide insulin" produced by tryptic removal of B23–30, or in "desalanine-desasparagine insulin" lacking B30 and A21; he concluded that virtually the entire insulin molecule is essential for this action. Meek and Bolinger (1966), however, have described a weak glucose oxidation effect of the reduced insulin A chain on rat adipose tissue. The fact that oxytocin also stimulates the metabolism of glucose by rat adipose tissue *in vitro* (Mirsky and Perisutti, 1961; Pittman *et al.*, 1961) has suggested that this biological effect may be related to the 20-membered disulfide ring present in both oxytocin and in the A chain of insulin. The question of the structural basis for the antilipolytic property of insulin has not yet been considered in the literature.

Our interest in the structural requirement for insulin's action on adipose tissue stemmed from work on an insulin-cleaving peptidase system present in the adipose tissue of rat, mouse, and hamster (Di Girolamo *et al.*, 1965; Rudman *et al.*, 1966, 1968). While the mode of cleavage has not been conclusively established, the available evidence suggests initial hydrolyses in the regions of A13–14 (Leu-Tyr), A18–19 (Asn-Tyr), B11–12 (Leu-Val), B15–16 (Leu-Tyr), B24–25 (Phe-Phe), and B25–26 (Phe-Tyr); the resulting five peptides then probably undergo stepwise removal of NH_2- and COOH-terminal residues. The degradation of insulin in these experiments was found to abolish the hormone's antilipolytic property, but allowed persistence of a weak activity in stimulating the oxidation of glucose to CO_2 (Rudman *et al.*, 1968). This finding suggested different structural bases for the two actions of insulin on the fat cell. To gain further information on this problem, we have now measured the *in vitro* glucose oxidation activity and the antilipolytic activity on adipose tissue of the following derivatives of insulin furnished by Dr. F. H. Carpenter: desalanine insulin, desalanine-desasparagine insulin, desoctapeptide insulin, and the heptapeptide B23–29. The resulting data, together with those of the preceding paper (Rudman *et al.*, 1968) on insulin frag-

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ments produced by the insulin-cleaving peptidases of rat adipose tissue, suggested the possible importance of the Leu-Tyr (A13–14, B15–16) bond for the antilipolytic action of insulin; accordingly Leu-Tyr and its derivatives, together with a variety of other synthetic di- and tripeptides, were then also tested for biological activity on adipose tissue.

Material and Methods

Substances Tested for Glucose Oxidation and Antilipolytic Activity. Bovine insulin (24 units/mg) was furnished by Dr. J. A. Galloway of Lilly. Desalanine insulin, desalanine-desasparagine insulin, desoctapeptide insulin, and the synthetic B23–29 heptapeptide were generously donated by Dr. F. H. Carpenter, University of California (see review by Carpenter, 1966). A series of 52 synthetic di- and tripeptides with and without various blocking groups on the amino and carboxyl groups, listed in Table V, was obtained from Cyclo Chemical Co. Optically active amino acids contained in these peptides were of the L configuration in all cases. All the peptides which possessed either a free NH_2 group, or a tyrosine, histidine, or arginine moiety, were demonstrated to move as a single spot during chromatography on Whatman No. 4 paper in butanol–acetic acid–H_2O (12:3:5) and in 1-butanol–pyridine–H_2O (1:1:1), with staining by ninhydrin, Pauly's reagent, or Sakaguchi reagent.

Assay Methods. With the methods described in the accompanying paper (Rudman et al., 1968), the substances listed above were assayed for the capacity to stimulate oxidation of glucose C-1 to CO_2 by the epididymal adipose tissue of the Wistar rat (120–160 g) and by the perirenal adipose tissue of the male albino rabbit (3.5–4.5 kg); and for the capacity to suppress the response to lipolytic hormones by the epididymal adipose tissue of the rat, the epididymal adipose tissue of the golden hamster (150–170 g), and the perirenal adipose tissue of the rabbit. In the antilipolytic assays, 1 μg/ml of oxycellulose-purified porcine ACTH (100 units/mg, Wilson), 1 μg/ml of L-epinephrine (Mann), or 1 μg/ml of bovine β-MSH[1] (1.5×10^{10} units g, Dr. A. V. Schally) served as the lipolytic agent. Activity in the glucose oxidation assay was calculated as μatoms of glucose C-1 converted into CO_2/g of tissue per 2 hr in response to the specified concentration of test material in the incubation medium. Activity in the antilipolytic assay was calculated as reduction in FFA concentration (microequivalents per gram of tissue) in tissue slices exposed to 1 μg/ml of lipolytic hormone plus the specified concentration of test material, as compared with the FFA concentration in tissue slices exposed only to 1 μg/ml of lipolytic hormone.

Results

Insulin and Carpenter's Derivatives of Insulin. With the assay techniques employed, the minimal effective dose for insulin's glucose oxidation effect on the rat tissue was 0.001 μg/ml and the maximal response an increase of 8 μatoms/g of tissue per 2 hr in conversion of C-1 of glucose into CO_2. The minimal effective dose for the hormone's antilipolytic effect on the hamster tissue was 0.01 μg/ml, the maximal response (R_{max}) being a reduction of 6–7 μequiv/g of tissue in the concentration of FFA in adipose tissue slices exposed to 1 μg/ml of either epinephrine or ACTH. Representative assays are summarized in numerical form in Tables I and II and in graphic form in Figure 1. Insulin, as reported previously (Di Girolamo and Rudman, 1966; Rudman and Shank, 1966), did not cause a detectable glucose oxidation or antilipolytic effect on rabbit adipose tissue (Table III and Figure 2). The desalanine derivative showed the same potency as insulin in both glucose oxidation and antilipolytic assays upon the rat and hamster tissues, respectively, both in terms of minimal effective dose and R_{max} (Figure 1). In contrast, the desalanine-desasparagine and desoctapeptide derivatives were less active than insulin in both assays. For glucose oxidation activity on the rat tissues, the minimal effective dose of these peptides was 100 and 1000 times greater than that of insulin, respectively; R_{max} in each case was approximately one-half as great (Figure 1). In the antilipolytic assay on hamster adipose tissue, these two derivatives showed a minimal effective dose only ten times higher than that of insulin, and the magnitude of the effect was equal to that of insulin (Figure 1). The heptapeptide B23–29 was totally inactive in both assays. The combination of desoctapeptide insulin with the heptapeptide exhibited the same potency in both assays as the desoctapeptide derivative alone. All of these derivatives of insulin were inactive in both the glucose oxidation and antilipolytic assays on the rabbit tissue, like insulin itself (Table III).

The increased minimal effective dose and the reduced

TABLE I: Bioassays of Insulin, Desalanine Insulin, and Desoctapeptide Insulin for Glucose Oxidation Activity on Rat Adipose Tissue.[a]

Dose (μg/ml)	μatoms of Glucose C-1 Converted into CO_2/g of Tissue per 2 hr		
	Insulin	Desalanine Insulin	Desoctapeptide Insulin
0	1.9 ± 0.2	2.3 ± 0.3	1.7 ± 0.5
0.0001	2.0 ± 0.2	2.1 ± 0.3	
0.001	5.0 ± 0.5	5.8 ± 0.6	1.4 ± 0.3
0.01	6.7 ± 0.6	9.2 ± 1.0	2.0 ± 0.5
0.1	9.7 ± 1.0	9.7 ± 0.8	6.0 ± 0.8
1	9.4 ± 0.8	9.6 ± 0.7	5.9 ± 0.7
10	9.7 ± 0.7	10.3 ± 0.6	6.2 ± 0.9

[a] Each value represents average plus and minus standard error for four observations.

[1] Abbreviations used are as stated in *Biochemistry 5*, 1445 (1966), are: MSH, melanophore-stimulating hormone; FFA, free fatty acid.

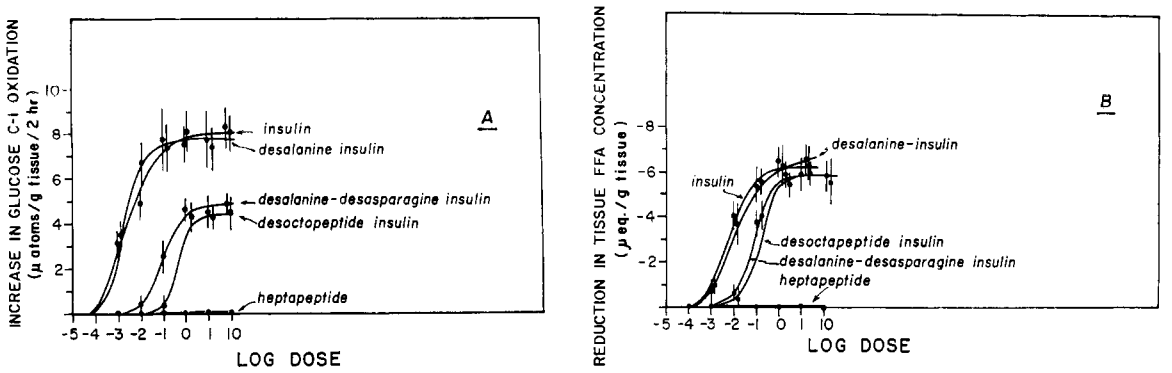

FIGURE 1: Dose–response curves for (A) glucose oxidation effect of insulin and four insulin derivatives upon the rat's epididymal adipose tissue *in vitro* and (B) antilipolytic effect of these peptides on the hamster's epididymal adipose tissue *in vitro*. Abscissa shows logarithm (base 10) of concentration (micrograms per milliliter of peptide) in the incubation medium. Ordinate in graph A shows response calculated as increase in μatoms of glucose C-1 converted into CO_2/g of tissue per 2 hr. In graph B, response represents reduction in concentration of FFA (microequivalents per gram of tissue) in tissue slices exposed for 2 hr to 1 μg/ml of ACTH. Each point represents average of nine observations in curve A and 12–16 observations in curve B; standard errors of each mean are shown.

TABLE II: Bioassays for Antilipolytic Action of Insulin, Desalanine Insulin, and Desalanine-desasparagine Insulin on Hamster Adipose Tissue.

Addition to Medium (μg/ml)	μequiv of FFA/g of Tissue		Desalanine-desasparagine Insulin
	Insulin	Desalanine Insulin	
None	1.0 ± 0.1	1.5 ± 0.1	1.2 ± 0.1
ACTH (1)	8.5 ± 0.9	7.8 ± 0.5	9.2 ± 0.1
ACTH (1) + test substance (0.0001)	7.7 ± 0.6	6.8 ± 0.5	
ACTH (1) + test substance (0.001)	7.5 ± 0.3	7.0 ± 0.5	8.8 ± 0.6
ACTH (1) + test substance (0.01)	3.3 ± 0.4	2.9 ± 0.5	7.0 ± 0.6
ACTH (1) + test substance (0.1)	3.3 ± 0.2	2.8 ± 0.3	3.7 ± 0.5
ACTH (1) + test substance (1)			3.8 ± 0.5

[a] Values represent average plus and minus standard error for four observations.

TABLE III: Summary of Assay Experiments.

	Glucose Oxidation Act. (rat) MED (R_{max})[a]	Antilipolytic Act. (MED)[b]		
		Hamster	Rat	Rabbit
Insulin	0.001 (8 μatoms)	0.01	0.001	Inactive at 100[c]
Desalanine insulin	0.001 (8 μatoms)	0.01	Not tested	Inactive at 25[c]
Desalanine-desasparagine insulin	0.1 (4–5 μatoms)	0.1	Not tested	Inactive at 25[c]
Desoctapeptide insulin	1 (4–5 μatoms)	0.1	Not tested	Inactive at 25[c]
Heptapeptide B23–29	Inactive at 100[c]	Inactive at 100[c]	Not tested	Inactive at 100[c]
N-Cbz-Leu-Tyr-NH_2	Inactive at 150[c]	10	20	10
Leu-Tyr-NH_2	Inactive at 150[c]	100	Not tested	50
N-Cbz-Gly-Tyr-NH_2	Inactive at 150[c]	100	Not tested	100
Gly-Tyr-NH_2	Inactive at 150[c]	300	Not tested	200

[a] R_{max} represents maximal increase in μatoms of glucose C-1 converted into CO_2/g of tissue per 2 hr. MED = minimal effective dose. All units in micrograms per milliliter. [b] The lipolytic agent was ACTH (1 μg/ml). [c] Highest dose tested.

R_{max} for the glucose oxidation effect of the desoctapeptide and desalanine-desasparagine insulin preparations, compared with native insulin, could represent the weak residual biologic potency of these two insulin derivatives. Alternatively, the attenuated glucose oxidation effect could result from the presence in those preparations of a small proportion of intact insulin, the maximal effect of which was reduced through some type of inhibiting influence by the insulin derivative. To investigate the latter possibility, rat adipose tissue slices were exposed to 1 μg/ml of insulin in the absence and in the presence of 10 μg/ml of the desoctapeptide or desalanine-desasparagine derivative. Neither derivative caused a statistically significant reduction in the glucose oxidation effect produced by 1 μg/ml of native insulin (Table IV).

TABLE IV: Glucose Oxidation Activity of 1 μg/ml of Insulin upon Rat Epididymal Adipose Tissue in the Absence and Presence of 10 μg/ml of the Desoctapeptide and Desalanine-desaparagine Derivatives.

Addition to Medium (μg/ml)	Conversion of Extracellular Glucose C-1 into CO_2 (μatoms/g of tissue per 2 hr)
None	1.1 ± 0.2 (8)[a]
Insulin (1)	10.4 ± 1.1 (8)
Desoctapeptide insulin (10)	5.9 ± 0.4 (8)
Insulin (1) plus desoctapeptide insulin (10)	10.7 ± 0.9 (8)
None	0.8 ± 0.1 (7)
Insulin (1)	10.3 ± 1.1 (7)
Desalanine-desasparagine insulin (10)	6.5 ± 0.5 (7)
Insulin (1) plus desalanine-desasparagine insulin (10)	10.1 ± 0.8 (7)

[a] Average plus and minus standard error (number of observations). Four tissue slices were taken from each 120–160-g rat; one slice was incubated in each of the four media specified under either the desoctapeptide or desalanine-desasparagine experiment. A total of eight rats was employed in the assays with the desoctapeptide derivative, and seven rats with the desalanine-desasparagine preparation.

Assay of Synthetic Dipeptides. Since the previous study had suggested that cleavage of insulin in the region of the four tyrosyl residues abolished antilipolytic activity but allowed weak glucose oxidation activity to persist (Rudman *et al.*, 1968), a series of synthetic di- and tripeptides including various tyrosyl compounds were now assayed for antilipolytic activity on hamster adipose tissue. In all, 52 compounds were tested at a concentration of 500 μg/ml; these compounds are listed in Table V. Among these, four were active, in the order of potency *N*-Cbz-Leu-Tyr-NH_2 > Leu-Tyr-NH_2 = *N*-Cbz-Gly-Tyr-NH_2 > Gly-Tyr-NH_2. The minimal effective doses were 10, 100, 100, and 300 μg/ml, respectively (Figure 2 and Table III). *N*-Cbz-Leu-Tyr-NH_2 was also active as an antilipolytic agent on the rabbit tissue at 10 μg/ml, and upon the rat tissue at a concentration of 20 μg/ml. Among the 48 inactive peptides were *N*-Cbz-Leu-Tyr, Leu-Tyr, and *N*-Cbz-Gly-Tyr (showing the requirement for a blocked carboxyl group), and a variety of blocked peptides containing either Leu, Gly, or Tyr (suggesting the specificity of the Leu-Tyr or Gly-Tyr sequence). *N*-Cbz-Leu-Tyr-NH_2, Leu-Tyr-NH_2, and Gly-Tyr-NH_2 were all inactive at a concentration of 150 μg/ml in the glucose oxidation assay upon adipose tissue slices from rat or rabbit (Table III).

All the antilipolytic assays with the synthetic peptides up to this point utilized ACTH (1 μg/ml) as the lipolytic stimulus. The capacity of *N*-Cbz-Leu-Tyr-NH_2 to suppress the lipolytic response of the hamster tissue to epinephrine (1 μg/ml) and of the rabbit tissue to β-MSH (1 μg/ml) was now examined. In each instance an antilipolytic effect was observed similar in magnitude to that observed with ACTH as the lipolytic agent (Figure 2B,-D).

Discussion

The results of all the assays are summarized in Table III, in terms of the minimal effective dose for the glucose oxidation and antilipolytic effects. The minimal effective dose for insulin's glucose oxidation action is 0.001 μg/ml on the rat tissue, and 0.01 μg/ml for its antilipolytic action on the hamster tissue. Removal of alanine from the COOH terminus of the B chain of insulin does not cause a detectable reduction of either activity (as expected from earlier work on this preparation, reviewed by Carpenter, 1966).

In contrast, removal of the octapeptide B23–30 from the B chain, or the COOH-terminal asparagine from the A chain, increases the minimal effective dose for glucose oxidation activity 1000 or 100 times, respectively, and reduces R_{max} by about 50%. The antilipolytic activity is somewhat less impaired by these structural alterations, minimal effective dose being increased only ten times with R_{max} unchanged. Before accepting these conclusions, however, the possibility must be considered that the apparent weak activity of these two derivatives could result from contamination with a small amount of unaltered insulin. Since the antilipolytic activity of both derivatives seems to be about one-tenth that of native insulin, such contamination would need to be roughly 10%. Tending against such an explanation is the altered ratio of antilipolytic and glucose oxidation potencies of the two derivatives compared with this ratio for insulin, and the altered shape of the dose–response curve for the glucose oxidation effect. If the glucose oxidation effect of the desoctapeptide and desalanine-desasparagine preparations resulted from the presence of a small amount of intact insulin, then the dose–response curve should ordinarily be characterized by an increased minimal effective dose but unchanged R_{max}, compared with

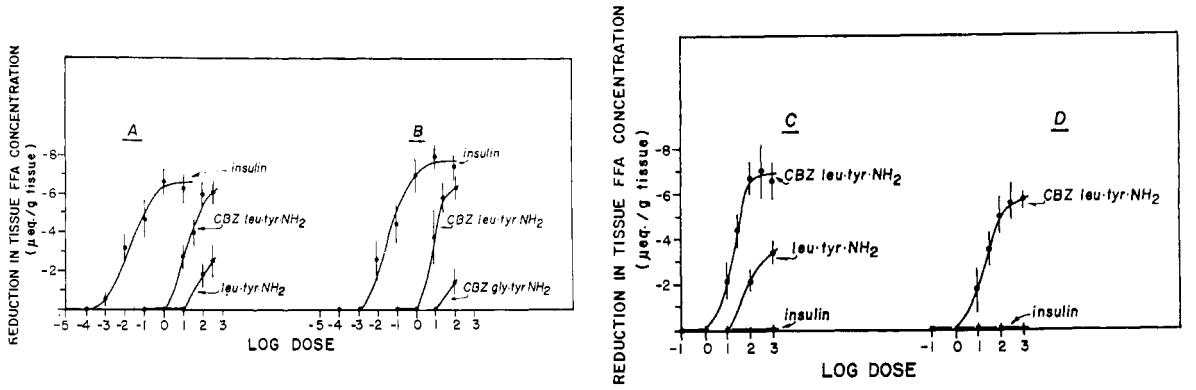

FIGURE 2: Dose–response curves for antilipolytic action of various peptides upon hamster (A, B) and rabbit (C, D) adipose tissues. Symbols as in Figure 1. In graphs A and C, the lipolytic agent was 1 μg/ml of ACTH. In B, 1 μg/ml of epinephrine was employed to stimulate lipolysis, and in D 1 μg/ml of β-MSH. Values represent average plus and minus standard error for eight observations.

TABLE V: Synthetic Peptides Tested for Capacity to Reduce the *in Vitro* Lipolytic Response of Hamster Adipose Tissue to 1 μg/ml of ACTH.[a]

Antilipolytic		
N-Cbz-Leu-Tyr-NH_2 (MED 10 μg/ml)		
Leu-Tyr-NH_2 (MED 100 μg/ml)		
N-Cbz-Gly-Tyr-NH_2 (MED 100 μg/ml)		
Gly-Tyr-NH_2 (MED 300 μg/ml)		
No Antilipolytic Effect at 500 μg/ml		
Tyrosyl Peptides	Glycyl Peptides	Other Peptides
N-Cbz-Ser-Tyr-NH_2	*N*-Cbz-Gly-Phe	*N*-Benzoyl-Phe-NH_2
Leu-Tyr	*N*-Cbz-Gly-Tyr	*N*-Benzoyl-Phe
N-Cbz-Leu-Tyr	*N*-Cbz-Gly-Trp	*N*-Cbz-Phe-Phe-NH_2
Tyr-Gly	*N*-Cbz-Gly-NH_2	*N*-Cbz-Glu-Phe
N-Benzoyl-Tyr-Gly-NH_2	*N*-Benzoyl-Gly-Gly-NH_2	*N*-Cbz-Ala-Asn-NH_2
N-Acetyl-Tyr-NH_2	*N*-Cbz-Ala-Gly-NH_2	*N*-Cbz-*S*-benzoyl-Cys-Ala
Tyr	*N*-Cbz-Gly-Ser-NH_2	*N*-Benzoyl-Gly-Lys
Leucyl Peptides	*N*-Cbz-Gly-Phe-NH_2	*N*-Benzoyl-Gly-Arg
N-Cbz-Leu-Val-NH_2	*N*-Cbz-*S*-benzoyl-Cys-Gly-NH_2	*N*-Benzoyl-Arg-ethyl ester
N-Cbz-Leu-Gly-NH_2	*N*-Cbz-Gly-Ala-NH_2	*N*-Benzoyl-Arg-NH_2
N-Cbz-Ala-Leu-NH_2	*N*-Benzoyl-Gly-Gly	
N-Cbz-Gly-Gly-Leu-NH_2	*N*-Cbz-Gly-Glu	
N-Cbz-Phe-Leu-NH_2	*N*-Lys-Gly-NH_2	
N-Cbz-Gly-Leu	Gly-NH_2	
Leu-Gly	Gly-Ser	
N-Cbz-Leu-NH_2	Gly-His	
N-Benzoyl-Gly-Leu-NH_2	Lys-Gly	
N-Cbz-Ser-Leu-NH_2	Gly-Phe	
N-Cbz-His-Leu-$NHNH_2$	Lys-Gly-NH_2	

[a] Optically active amino acids were of the L configuration in all cases. MED = minimal effective dose.

a pure preparation of the native hormone. A reduced R_{max}, however, could result if the desoctapeptide and desalanine-desasparagine derivatives partially inhibited the glucose oxidation effect of the intact hormone. Since such a reduction of the R_{max} of insulin's glucose oxidation effect by the two derivatives could not be demonstrated (Table IV), it seems likely that the desoctapeptide and desalanine-desasparagine derivatives themselves possess a weak degree of biologic activity on adipose tissue.

The data thus suggested that both the glucose oxidation and antilipolytic activities probably reside within

the desoctapeptide-desasparagine structure,[2] but are greatly enhanced by the presence of B23–30 and A21. The earlier assays (Rudman *et al.*, 1968) on the products of the degradation of insulin by the insoluble fraction of rat adipose tissue homogenate had shown total disappearance of the antilipolytic activity, but persistence of a weak degree of glucose oxidation activity, with the dose–response curves for the latter activity being similar in R_{max} and minimum effective dose to those of the desoctapeptide and desalanine-desasparagine derivatives. The available data on the free amino acids and peptide fragments released during the degradation of insulin in these experiments suggested that the desoctapeptide-desasparagine region of the insulin molecule underwent cleavage in the region of A13–14 (Leu-Tyr), A18–19 (Asn-Tyr), B11–12 (Leu-Val), and B15–16 (Leu-Tyr). Since the mixture of cleavage products retained glucose oxidation activity comparable with that of the desoctapeptide-desasparagine structure, but had lost the latter's antilipolytic activity, it was hypothesized that the antilipolytic but not the glucose oxidation property was dependent on the integrity of these leucyl and/or tyrosyl bonds.

Some support for this conclusion was then provided by the finding that the amides of Leu-Tyr or Gly-Tyr possess antilipolytic but not glucose oxidation activity. Consideration of the results with all 52 synthetic di- and tripeptides indicates that in addition to the Leu-Tyr or Gly-Tyr sequence, the carboxyl group must be blocked in order for antilipolytic activity to be present; the activity is further enhanced by carbobenzoxylation of the amino group. The specificity of the Leu-Tyr or Gly-Tyr sequence is indicated by the total inactivity of blocked dipeptides containing a variety of other combinations of two amino acids.

The various observations on peptide fragments of insulin and on synthetic peptides described in the previous (Rudman *et al.*, 1968) and present study can be drawn together under the following hypothesis. Both glucose oxidation and antilipolytic properties of insulin are present within the desoctapeptide-desasparagine structure, but are markedly amplified by the addition of B23–30 and A21. The desoctapeptide-desasparagine structure can be cleaved in the region of its tyrosyl residues with little effect on the glucose oxidation activity remaining after removal of B23–30 and A21, but with total disappearance of the residual antilipolytic activity. The glucose oxidation property seems to reside in a portion of the desoctapeptide-desasparagine structure containing one or more disulfide bridges, while the antilipolytic activity appears to stem from regions surrounding the tyrosyl residues. Since the two effects of insulin on the fat cell seem to depend on separate regions of the hormone molecule, they presumably occur through different mechanisms.

[2] The desoctapeptide-desasparagine derivative was not actually studied. The statement is based on the observations that detectable glucose oxidation and antilipolytic activities are retained after removal of *either* B23–30 or A21 and on the assumption that similar degrees of activity would persist after removal of *both* B23–30 and A21.

Before such a hypothesis can be accepted, however, the following requirements will need to be met. (a) More rigorous proof will be needed that the apparent weak glucose oxidation and antilipolytic activities of the desoctapeptide and desalanine-desasparagine derivatives do not result from contamination of these preparations with intact insulin. (b) It must be shown that the antilipolytic activity of the synthetic amides of Leu-Tyr and Gly-Tyr results from the same mechanism as the antilipolytic action of insulin, which is believed by some investigators (Butcher *et al.*, 1966) to involve a reduction in the intracellular concentration of 3,5-cyclic adenosine monophosphate. (c) The peptide fragment(s) produced during the degradation of insulin by the insoluble lipid-containing fraction of rat adipose tissue homogenate, which retain weak glucose oxidation but no antilipolytic activity, must be isolated and the structure(s) determined.

Bromer and Chance (1967) have recently shown that desoctapeptide insulin possesses only 1% of the antigenic reactivity of native insulin. Accordingly examination of the effect of antiinsulin serum on the apparent biological activities of the desoctapeptide derivative will be useful in investigating question (a) above. Bromer and Chance also reported that desoctapeptide insulin was less than 1% as potent as insulin in the mouse convulsion assay. Nicol (1960), however, concluded that the desoctapeptide derivative possessed 15% the potency of insulin in stimulating glucose uptake by rat diaphragm *in vitro*. The present *in vitro* assay showed that the desoctapeptide preparation was 0.1% as potent as native insulin in stimulating glucose oxidation by rat adipose tissue, but 10% as potent as an antilipolytic agent on hamster adipose tissue. These differing estimates of the biological activity of the desoctapeptide derivative emphasize the need for employing a variety of assay methods, both *in vitro* and *in vivo*, in future investigations on the structural basis of insulin's biological actions.

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The *in Vivo* Metabolism of 16α-Hydroxylated C_{19} Steroids in Late Pregnancy*

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ABSTRACT: Following the intravenous administration of ^{3}H- and ^{14}C-labeled 16α-hydroxylated C_{19} steroids to subjects in the third trimester of pregnancy, labeled 3β,16α-dihydroxyandrost-5-en-17-one, 16α-hydroxyandrost-4-ene-3,17-dione, 3α,16α-dihydroxy-5α-androstan-17-one, 3α,16α-dihydroxy-5β-androstan-17-one, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3β,16α,17β-triol, and estra-1,3,5(10)triene-3,16α,17β-triol (estriol) were isolated from the urine in either the sulfate or glucosiduronate fraction or in both. In other to determine the origin of these neutral metabolites and estriol, the following pairs of substrates were injected into pregnant subjects in the third trimester: (a) [7-^{3}H]3β,16α-dihydroxyandrost-5-en-17-one (16D) and [4-^{14}C]16α-hydroxyandrost-4-ene-3,17-dione (16Δ^4), (b) [7-^{3}H]16D and [4-^{14}C]17-oxoandrost-5-ene-3β-yl sulfate (DS), and (c) [7-^{3}H]16D-3 sulfate (16DS) and [4-^{14}C]DS. From the percentage conversion of the injected radioactivity, the specific activities, and the $^{3}H/^{14}C$ ratios in the urinary metabolites, it was possible to conclude that 16D and 16Δ^4 were equally well converted into estriol (E_3), but no β,γ-unsaturated alcohols were formed from 16Δ^4; DS was converted into 16D and androst-5-ene-3β, 16α,17β-triol (DT) and both 16D and DS gave rise to E_3; 16DS and DS were not efficiently converted into 16Δ^4 or 3α,16α-dihydroxyandrost-5-en-17-one but formed urinary E_3 in good yield. In two studies, [^{14}C]3-hydroxyestra-1,3,5(10)-trien-17-one was isolated while in one study [^{14}C]3β-hydroxyandrost-5-en-17-one and [^{14}C]3α-hydroxy-5α-androstan-17-one were isolated in crystalline form. While all the injected steroids were good precursors of E_3, 16DS and DS seemed to be the most efficient. Moreover, the metabolite possessing the 3α-hydroxy-5-ene structure was derived mainly from 16D. From the specific activities of the isolated E_3 it was evident that all the precursors studied were not the sole source of E_3 in late pregnancy. When labeled 16D was injected intravenously to a normal male and a normal female in the luteal phase of the menstrual cycle, most of the above-mentioned neutral urinary metabolites were isolated but no labeled E_3 was found, indicating that E_3 in the normal female is not formed from the aromatization of a 16α-hydroxy neutral steroid.

It has been well documented that in man 16α-hydroxylated C_{19} steroids serve as precursors of estriol[1] during pregnancy (Ryan, 1958, 1959; Magendantz and Ryan, 1964; Siiteri and MacDonald, 1966; Dell'Acqua *et al.*, 1967a,b). The metabolism of these steroids in the maternal and fetal compartments has not, to date, been extensively investigated. Siiteri and MacDonald (1966) reported that 16α-hydroxydehydroisoandrosterone administered to subjects in the third trimester of pregnancy was converted into androst-5-

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[1] The following trivial names of steroids have been employed in the text: 16α-hydroxydehydroisoandrosterone = 3β,16α-dihydroxyandrost-5-en-17-one; deoxycorticosterone = 21-hydroxypregn-4-ene-3,20-dione; 16α-hydroxyetiocholanolone = 3α,16α-dihydroxy-5β-androstan-17-one; 16α-hydroxyandrosterone = 3α,16α-dihydroxy-5α-androstan-17-one; dehydroisoandrosterone = 3β-hydroxyandrost-5-en-17-one; androsterone = 3α-hydroxy-5α-androstan-17-one; androstenedione = androst-4-ene-3,17-dione; 16α-hydroxyandrostenedione = 16α-hydroxyandrost-4-ene-3,17-dione; dehydroisoandrosterone sulfate = 17-oxoandrost-5-ene-3β-yl sulfate; 16α-hydroxydehydroisoandrosterone sulfate = 16α-hydroxy-17-oxoandrost-5-ene-3β-yl sulfate; estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol = estra-1,3,5(10)-triene-3,17β-diol; estriol = estra-1,3,5(10)-triene-3,16α,17β-triol.