Insulin and Glucagon Degradation by the Same Enzyme

William C. Duckworth, M.D., and Abbas E. Kitabchi, Ph.D., M.D., Memphis

SUMMARY

An insulin-degrading enzyme has been previously isolated from rat skeletal muscle and highly purified by a series of procedures including affinity chromatography on insulin-agarose. Glucagon-degrading activity was also found in each of the purification steps including the final highly purified material. Insulin- and glucagon-degrading activities were associated with the same band on polyacrylamide gel and both activities were obtained from affinity chromatography on glucagon-agarose.

Insulin- and glucagon-degrading activities could not be differentiated by the effect of sulfhydryl reagents, pH, or by heat inactivation. Insulin served as a competitive inhibitor of glucagon degradation (Ki = 1.5×10^{-8}M) and glucagon was a competitive inhibitor of insulin degradation (Ki = 5.3×10^{-6}M).

On the basis of these studies it appears that insulin and glucagon can be degraded by the same enzyme which is found in the soluble fraction of rat skeletal muscle homogenate. DIABETES *23*:536-43, June, 1974.

The biologic effects of polypeptide hormones such as insulin and glucagon are initiated by binding of the hormones to receptors on the cell membrane[1,2] which then leads to an intracellular response.[3] The subsequent fate of the hormone receptor complex remains unclear. It has been suggested that insulin degradation may be one of the physiologic mechanisms for termination of the cellular response to the hormone.[4] This would make hormonal degradation as important in the control of the cellular response as hormone-receptor binding. It has been shown that hormonal degradation is not an integral part of the hormone-receptor interaction, i.e. the binding of the hormone to the receptor does not necessarily result in degradation of the hormone,[5,6] but in most systems in which biologic activity of the hormone is studied a significant amount of the hormone is degraded.[2,4] Thus, a further understanding of the mechanism of hormonal degradation becomes of great importance.

Although quantitatively the liver is the major site of insulin removal and degradation,[7-9] peripheral tissues such as muscle and fat also have the ability to degrade insulin. Hormonal degradation in any of these tissues could help determine the effectiveness of insulin in that tissue. For these reasons, we have been examining insulin degradation by a proteolytic enzyme isolated from muscle. This enzyme, which rapidly degrades physiologic concentrations of insulin has been purified by affinity chromatography and partially characterized.[10] Further studies on the purified enzyme, reported below, have revealed that this enzyme can also degrade glucagon. This raises the possibility that both hormones may be degraded, at least partially, by the same enzyme. Since it has been suggested that the insulin: glucagon ratio rather than the absolute level of either hormone is the critical factor in controlling glucose homeostasis,[11] it is possible that the tissue handling of these hormones may be related. The preliminary results of these studies have been previously reported.[12]

MATERIALS AND METHODS

Male Holtzman rats (200 to 300 gm.) were used for all enzyme preparations. Porcine insulin, proinsulin, and glucagon were gifts from Dr. R. Chance of Eli Lilly Company, Indianapolis, Indiana. ^{125}I-insulin was purchased from Cambridge Nuclear Corp., Cambridge, Massachusetts, and had 0.3 atom of iodine per mole of insulin; it was 96 per cent trichloroacetic acid 94 per cent immunoprecipitable, and was biologically active in isolated fat cells. Ammonium sulfate, ultrapure grade, was obtained from Schwarz/Mann,

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From the Laboratories of Endocrinology and Metabolism, Research Service, Veterans Administration Hospital and Departments of Medicine and Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38104.

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Orangeburg, New York. All other chemicals were of reagent grade and were purchased commercially. Porcine proinsulin and glucagon were lightly iodinated (less than 1 g-atom of iodine per mole of proinsulin) with ^{125}I Na).[13] As shown previously for insulin,[14] glucagon labeled with greater amounts of iodine is degraded poorly or not at all by the degrading enzyme. Insulin-agarose and glucagon-agarose were synthesized as previously described.[15] Cyanogen bromide-activated agarose was reacted with glucagon in 0.2 M sodium bicarbonate buffer at pH 9 and with insulin in 0.2 M sodium citrate at pH 5. The agarose derivatives were extensively washed prior to use. In spite of the washing, small amounts of insulin could still be eluted from the insulin-agarose during the purification procedure as has been previously reported.[16] This insulin could be removed from the final purified enzyme preparation by passage over a small column (0.5 x 3 cm.) of cellulose (cellulose powder MN300) although the very small quantity of insulin (5 to 10 μU./ml.) present did not interfere with the enzyme studies.

Enzyme Preparation and Purification

Insulin-degrading enzyme was prepared from rat skeletal muscle. The 100,000 x g supernatant of the muscle homogenate was fractionated with ammonium sulfate as described previously.[10] After dialysis to remove the ammonium sulfate, the crude material was stored frozen in 2 ml. aliquots. Further purification procedures were as follows: 2 to 3 ml. of the ammonium sulfate fraction were passed through a Sephadex G-200 column (0.9 x 41 cm.) which had been equilibrated with 20 mM sodium acetate (pH 6.2) containing 0.001 M dithiothreitol; the peak activity was pooled and adsorbed to a QAE-Sephadex column (1.5 x 7 cm.); this column was washed with 20 mM sodium acetate followed by 0.1 M NaCl in 20 mM sodium acetate and then eluted with 0.2 M NaCl in 20 mM sodium acetate; all solutions contained 0.001 M dithiothreitol; the peak activity was again pooled and immediately dialyzed against 20 mM acetate buffer (pH 6.2) without dithiothreitol for three hours with four changes; this material was then adsorbed to either an insulin-agarose or a glucagon-agarose column (0.5 x 6 cm.), washed with 20 mM acetate buffer and eluted with 0.2 M NaCl in 20 mM acetate (pH 6.2); the purified enzyme was then dialyzed and stored in 1 per cent bovine-serum albumin. The term "purified enzyme" as used in this paper refers to material which has been through all of the above steps.

Assay of Enzymatic Activity

Insulin- and glucagon-degrading activity were measured by the conversion of ^{125}I-labeled hormone to trichloroacetic acid soluble products as described previously.[10] Briefly the assay system consisted of ^{125}I-insulin or ^{125}I-glucagon in the specified concentration with 0.5 per cent bovine serum albumin in 0.05 M tris-HCl pH 7.5 in a total volume of 1 ml. After incubation at 37° in a Dubnoff metabolic shaker for the indicated time, 1 ml. of 10 per cent trichloroacetic acid was added and the mixture centrifuged in an International refrigerated centrifuge. The supernatant and precipitate were counted in a Packard auto gamma spectrometer and the per cent degraded calculated from the increase in trichloroacetic acid soluble radioactivity present in the supernatant over control tubes incubated without enzyme. Detailed assay conditions are described in the legends.

Enzyme activity was also assayed by the decrease in immunoassayable insulin or glucagon after incubation with the enzyme. The reaction was terminated with N-ethylmaleimide (0.001 M) as previously described.[10] Insulin was measured by the double antibody immunoassay as described previously.[10] Glucagon was measured using the 30 K antibody of Unger.[11]

RESULTS

Enzyme Purification

Table 1 shows the purification of the rat muscle insulin- and glucagon-degrading enzyme(s). As can be seen, insulin- and glucagon-degrading activities were increased in parallel by all purification procedures except the initial centrifugation. The insulin-degrading activity from the 100,000 x g supernatant through affinity chromatography was increased two hundred and fifty-six fold; glucagon-degrading activity was increased two hundred and fifty-two fold. Total purification for both activities was over a thousand fold.

Enzyme Stabilization

The instability of the purified enzyme has handicapped detailed studies of the properties of the purified material.[10] Various methods to stabilize the enzyme were tested preliminarily and the most promising ones were examined in detail. Insulin-agarose purified enzyme was prepared and assayed for insulin-degrading activity with and without each of the additions listed in table 2. The enzyme was then frozen in separate aliquots with the added materials. At one, and again at two weeks, separate aliquots were thawed and assayed for activity. The percentage activity remaining as compared with original activity can

TABLE 1

Insulin- and glucagon-degrading activities of rat-muscle enzyme during purification

Each preparation was incubated with ^{125}I insulin and ^{125}I glucagon (1×10^{-10}M) in 0.13M borate, pH 7.5, with 0.5 per cent bovine albumin at 37° for ten minutes. Specific activity is expressed as fmol hormone degraded per milligram protein per minute. Protein determined by the method of Lowry.[17]

	Insulin-Degrading Activity		Glucagon-Degrading Activity	
Purification Procedure	Specific Activity	Fold increase over previous step	Specific Activity	Fold increase over previous step
Homogenate	1.2		3.5	
100,000 x g Supernatant	12.8	10.7	16.0	4.5
Ammonium Sulfate	55.8	4.3	68.0	4.3
G-200 Sephadex	88.0	1.6	123.0	1.8
QAE Sephadex	729.0	13.2	861.0	12.6
Insulin-sepharose	3,278.0	4.5	4,006.0	4.6

be seen in table 2. Both dithiothreitol and bovine serum albumin (BSA) prevented loss of activity. Subsequent studies were performed on the enzyme preparation stored in 1 per cent BSA except where indicated. Under these conditions no loss of enzyme activity was seen for up to three months of storage. Bovine serum albumin was chosen instead of dithiothreitol because dithiothreitol can cleave the disulfide bond of insulin. Moreover, BSA was already present in all of the buffers used in the assays of enzyme activity. BSA has no insulin-degrading activity by itself and does not inhibit insulin degradation by the enzyme preparation.

Glucagon Degradation by Purified Enzyme

The degradation of ^{125}I-glucagon by several preparations of highly purified insulin-degrading enzyme was measured. All preparations produced significant increases in the amount of TCA soluble material after incubation of the glucagon with the enzyme. Since it was possible that the highly purified enzyme may still not have been homogeneous, the presence of a second enzyme was checked by slicing unstained polyacrylamide gels and assaying the slices for degrading activity (figure 1). The major portion of both insulin- and glucagon-degrading activity was present in the polyacrylamide gel fraction corresponding with the major band seen in the stained gel. ^{125}I-proinsulin was not degraded by any of the gel fractions.

Glucagon-agarose Chromatography

An enzyme preparation partially purified by ammonium sulfate fractionation and QAE-Sephadex chromatography was divided into two equal portions.

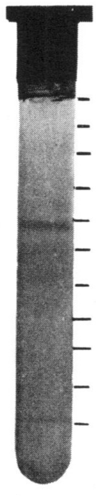
TABLE 2

Activity of purified rat-muscle insulin and glucagon-degrading enzyme(s) after freezing and thawing in the presence of various agents

Activity expressed as percentage of original insulin-degrading activity with each addition. Separate aliquots thawed for each assay. Assay as described under Methods.

Addition	1-week stage per cent	2-week stage per cent
None	49	36
Albumin 1%	90	97
Dithiothreitol 10^{-5}M	89	97
EDTA 10^{-5}M	78	73
Glycerol 4%	77	69

DEGRADATION BY POLYACRYLAMIDE GEL FRACTIONS



Fraction	^{125}I-Insulin	^{125}I-Proinsulin	^{125}I-Glucagon
1	97*	0*	0*
2	51	0	0
3	61	0	0
4	34	0	83
5	782	0	554
6	151	0	13
7	0	0	0
8	27	0	30
9	17	0	0
10	0	0	0

* CPM over control

FIG. 1. Polyacrylamide gels were run at 4° with 1.6 mM dithiothreitol added as described previously.[10] 7.5 per cent gel with pH 8.9 buffer was used. Each gel contained approximately 20 μg protein. Three separate gels were sliced and the fractions minced and incubated in 0.13 M borate with 0.5 per cent bovine albumin with either ^{125}I insulin, ^{125}I proinsulin or ^{125}I glucagon 1×10^{-10}M in a total volume of 1 ml. at 37° for ten minutes. Activities expressed as 5 per cent trichloroacetic acid soluble radioactivity over control. Control tubes contained gel fractions electrophoresed without added protein.

One portion was passed over a column containing glucagon-agarose and the other portion over insulin-agarose as described under Methods. After extensive washing the enzyme was eluted with 0.2 M NaCl in 0.02 M acetate. Each fraction of both columns was assayed for insulin and glucagon degradation. As illustrated in figure 2, the eluted material from both columns yielded identical patterns of insulin and glucagon-degrading activity.

Heat Inactivation and pH Optima

An enzyme preparation purified by affinity chromatography was heated at various temperatures for five or fifteen minutes. After heating, the enzyme was assayed for insulin- and glucagon-degrading activity. The pattern of heat inactivation was identical for both insulin- and glucagon-degrading activities (figure 3).

The effect of pH on insulin and glucagon degradation by highly purified enzyme is depicted in figure 4. Similar curves were obtained for each hormone.

Kinetic Studies

In order to determine whether or not insulin and glucagon could serve as competitive inhibitors of each other's degradation by the purified muscle enzyme, double reciprocal plots were drawn of insulin degradation in the presence and absence of glucagon (figure 5) and of glucagon degradation in the presence and absence of insulin (figure 6). In each case, the effect of two different concentrations of the respective inhibitory hormone was plotted[18] using initial rate for calculation of reaction velocity. The plots demonstrate that insulin and glucagon serve as competitive inhibitors for the degradation of each other by the enzyme. This is to be expected if the two materials serve as alternative substrates for the same enzyme.[19]

The Michaelis constants, calculated from the data in figures 4 and 5,[20] are recorded in table 3. The Ki for glucagon inhibition of insulin degradation is very close to the Km for glucagon degradation and the Ki for insulin inhibition of glucagon degradation is almost identical with the Km of insulin degradation. These results are consistent with the hypothesis that insulin and glucagon are alternative substrates for the same enzyme.[19]

Sulfhydryl Dependence

Both insulin and glucagon degradation are apparently sulfhydryl dependent. N-ethylmaleimide and p-hydroxymercuribenzoate both completely inhibit hormonal degrading activity and dithiothreitol stimu-

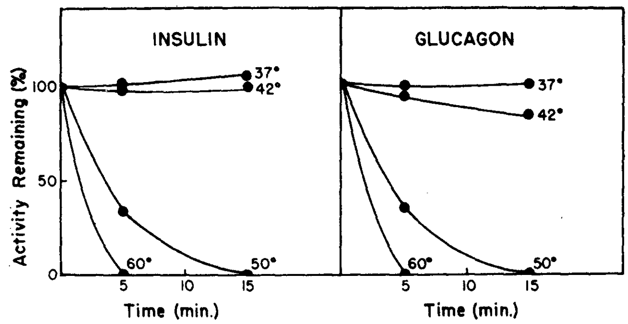


FIG. 3. Heat inactivation of enzyme activity. Aliquots of a purified enzyme preparation were heated at the various temperatures for the specified times. After cooling, aliquots were assayed for insulin and glucagon-degrading activity as described under figure 2. Activity is expressed as percentage-degrading activity remaining after the heating as compared with degrading activity of unheated enzyme from the same preparation.

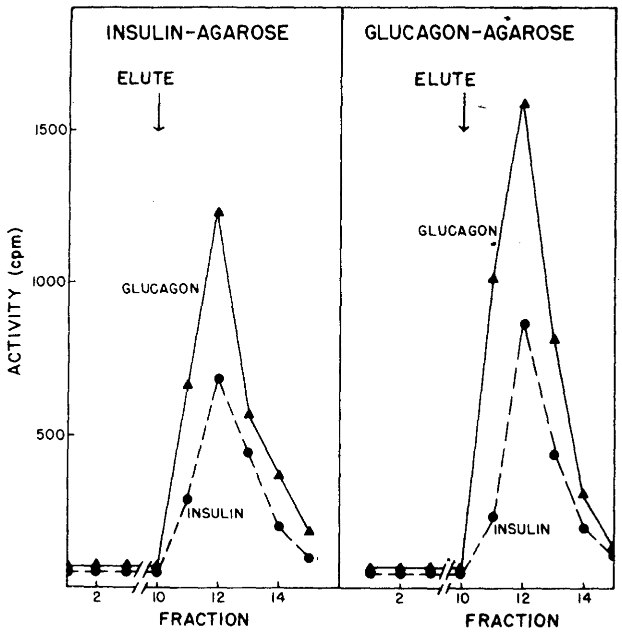


FIG. 2. One milliliter of QAE-Sephadex purified enzyme containing 30μg protein was applied to each of two columns, one containing insulin-agarose and one containing glucagon-agarose. After washing with 0.02 M sodium acetate, enzyme activity was eluted with 0.2 M NaCl in 0.02 M acetate in 1 ml. fraction. 0.025 ml. of each fraction was incubated with ^{125}I-insulin 1×10^{-10}M in 0.13 M sodium borate pH 7.5 with 0.5 per cent bovine albumin and with ^{125}I-glucagon 1×10^{-10}M in 0.13 M borate with 0.5 per cent bovine albumin in a total volume of 1 ml. After ten minutes at 37°, the reaction was stopped by the addition of 1 ml. of 10 per cent TCA. Activity is expressed as increase in TCA soluble radioactivity over control tubes with no enzyme.

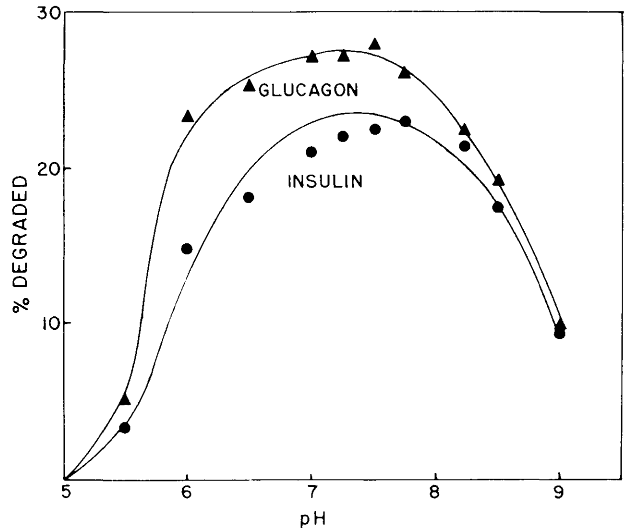


FIG. 4. Effect of pH on insulin- and glucagon-degrading activity. Activity is expressed as percentage ^{125}I-labeled hormone degraded over control. Assay conditions as described under table I except acetate buffer 0.1 M was used for pH 5.5, phosphate buffer 0.1 M was used for pH 6.0 to 6.9 and tris buffer 0.1 M was used for pH 7.0 to 9.0. Assay mixtures prepared in duplicate with one set used for activity assay and one set used for pH determination.

lates both insulin degradation (113 per cent of control) and glucagon-degrading activity (103 per cent of control).

Degradation of Immunoreactive Insulin and Glucagon

When incubated with physiological concentrations of insulin and glucagon, separately and together, the enzyme produced a decrease in immunoreactivity of

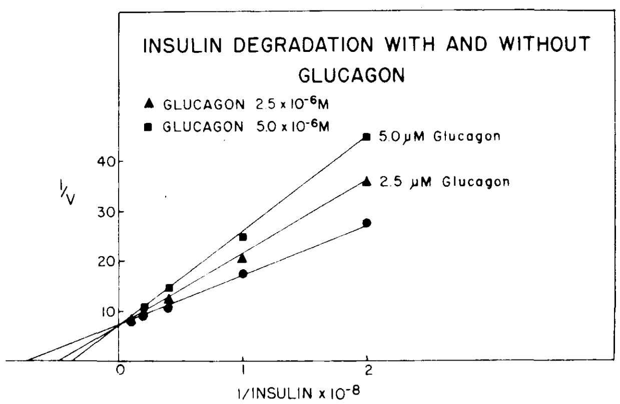


FIG. 5. Effects of insulin concentration on the rate of insulin degradation in the absence or presence of 2.5 μM or 5 μM glucagon. Incubations were in 0.05 M tris-HC1 pH 7.5 with 0.5 per cent bovine albumin for three minutes at 37°. Velocity is expressed as picomole insulin degraded per minute per μg. protein.

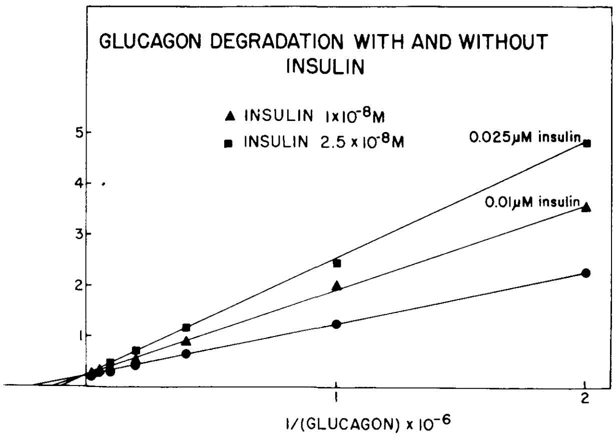


FIG. 6. Effects of various concentrations of glucagon on the rate of glucagon degradation in the absence or presence of 0.025 or 0.01 μM insulin. Assay as in figure 5. Velocity expressed as picomoles glucagon degraded per minute per μg. protein.

both materials, demonstrating that under these conditions both hormones can be degraded by the same enzyme with no apparent inhibition (table 4).

DISCUSSION

The ability of tissue homogenates to degrade insulin was first extensively examined by Mirsky.[8] He and his co-workers investigated various properties of the insulin-degrading system (called insulinase) which he found in numerous tissues with the highest activity in the liver. The system was partially purified and believed to be proteolytic[21] and relatively specific for insulin.[22,23] Subsequent studies have shown proteolytic insulin degradation by fat[24,25] and muscle.[26] The muscle enzyme has been partially purified and characterized.[26] A similar enzyme from the liver[27] and kidney[9] has been studied and a thousand fold purification of the muscle enzyme has been achieved by a series of steps including affinity

TABLE 3

Summary of Michaelis constants calculated from figures 5 and 6

Insulin degradation	Km	1.4 x 10^{-8}M
Insulin inhibition of glucagon degradation	Ki	1.5 x 10^{-8}M
Glucagon degradation	Km	4.5 x 10^{-6}M
Glucagon inhibition of insulin degradation	Ki	5.3 x 10^{-6}M

TABLE 4

Degradation of glucagon and insulin to nonimmunoreactive materials

Time (min)		A	B	C
0	Insulin μU.	48	—	54
	Glucagon $\mu\mu$g.	—	200	210
5	Insulin μU.	5	—	7
	Glucagon $\mu\mu$g.	—	<10	<10
10	Insulin μU.	5	—	5
	Glucagon $\mu\mu$g.	—	<10	<10
20	Insulin μU.	5	—	5
	Glucagon $\mu\mu$g.	—	<10	<10

Experiment A represents incubation of excess enzyme (approximately 10 ugm protein) with insulin alone, B glucagon alone, and C insulin plus glucagon. After incubation, N-ethylmaleimide (0.001M) was added to stop the reaction, and the material was assayed by specific immunoassay. Control tubes (zero time) contained enzyme preincubated with N-ethylmaleimide.

chromatography.[10] The latter studies have shown that a single enzyme from rat skeletal muscle can degrade physiologic levels of insulin at a physiologic pH. The enzyme is relatively specific in that a closely related material, proinsulin, was not degraded as measured by TCA solubility[10,26] or by immunoassay.[10,28] Various proinsulin intermediates have also been evaluated for degradation by this enzyme.[28]

Insulin degradation can also occur by the reductive cleavage of the disulfide bonds.[29,30] This process is mediated by an enzyme called glutathione-insulin transhydrogenase in the presence of reduced glutathione. The physiologic significance of this enzyme has been questioned[31] but a recent series of experiments suggests that it may be of importance especially with supraphysiologic levels of insulin.[32-34]

Glucagon degradation can also occur in a wide variety of tissues.[35] In some studies insulin has acted as a competitive inhibitor.[35] One mode of glucagon degradation is by cleavage of a dipeptide from the amino terminal end of the molecule.[36] The cathepsin C-like enzyme which mediates this process has an absolute requirement for a sulfhydryl compound and has no activity in phosphate buffer alone,[37] which is in contrast to the enzyme studied in the present investigation.

From studies reported here it appears that insulin- and glucagon-degrading activities were found in the same enzyme. A number of physical separation technics including ammonium sulfate fractionation, molecular sieve chromatography, ion exchange chromatography, and polyacrylamide gel electrophoresis have failed to separate the activities. Although the enzyme does not bind to plain agarose,[10] it does bind to glucagon agarose, as well as to insulin agarose and the eluted material from these columns behaves identically. Heating the enzyme produces parallel effects on its ability to degrade insulin and glucagon. It is of interest to note that the heat inactivation pattern of this enzyme is identical with that of the heat labile fraction of the insulinase system of Mirsky.[22]

Insulin- and glucagon-degrading activity of the highly purified enzyme cannot be separated by use of sulfhydryl reagents or by changes in pH. Finally, each hormone serves as a competitive inhibitor for the degradation of the other hormone which is consistent with our hypothesis that the two materials serve as alternative substrates for the same enzyme.[19] Since the likelihood of all these factors occurring coincidentally is exceedingly remote, it must be concluded from our data that insulin and glucagon are degraded by the same enzyme. The relative affinity for insulin, however, is greater than that for glucagon since the Km for insulin is three hundred fold less than for glucagon but at physiologic concentrations (10^{-9}M or less) both hormones can be degraded by this enzyme.

A comparison of insulin and glucagon degradation by liver-cell fractions revealed that at pH 7, without glutathione, both insulin and glucagon were degraded primarily by the cytosol.[38] It is highly likely that a significant portion of that degradation was by the enzyme described here, since parallel purification of insulin- and glucagon-degrading activity from the cytosol was achieved.

This enzyme has many of the characteristics of the insulinase system studies by Mirsky including similar heat inactivation patterns, pH effects and Km's and, as pointed out previously,[10] appears to be identical to the enzyme designated insulin specific protease. Thus this enzyme has been considered to be relatively specific for insulin and to play an important role in the degradation of physiologic quantities of insulin. The demonstration now that this enzyme can also degrade glucagon suggests several possibilities. One is that the degradation of insulin is nonspecific. Another is that the inactivation of insulin and glucagon are related in some complex manner and the control of this inactivation influences the level of active hormone. Much additional investigation will be required to resolve this question.

The interrelationship of insulin and glucagon degradation should also be examined in other tissues such as fat and liver. Such studies are necessary to investigate the physiologic role of the degrading enzyme. Although glucagon has been reported to increase cAMP levels in skeletal and cardiac muscle,[39] a direct effect of physiologic concentrations of glucagon on muscle has not been convincingly demonstrated.

The relationship of this enzyme to the glucagon-degrading system of liver-cell membranes is unclear. In one study insulin inhibited glucagon degradation,[6] while in another, no effect was seen.[39] It is possible that several different processes are involved. The same may be true for the insulin-degrading system of the membrane since the addition of glucagon did inhibit insulin breakdown.[5] Further studies will be required to evaluate the various degradation mechanisms and degradation products of this enzyme system. As has been pointed out previously, the degradation of these hormones by partially purified cell fractions is not specific if supraphysiologic concentrations of the hormone are used.[40] This limits the study to concentrations in the range of 10^{-9}M or less which is below the sensitivity of most analytic technics for detection of degradation products.[40] The recent development of more sensitive technics using fluorescamine for amino acid analysis may answer some of the questions as to the mechanism of action of the degrading enzyme.[41]

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