Degradation of Insulin and Proinsulin by Various Organ Homogenates of Rat

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SUMMARY

Degradation of biologically active and immunoreactive insulin and proinsulin was studied in the particulate and soluble fractions of homogenates of liver, brain, fat, lung, kidney, spleen, heart, muscle, pancreas, and testis of four groups of male rats at various time intervals. In addition, the above studies were also conducted in the ovaries, muscles, and adrenals of one group of female rats. Approximately 90 per cent of the degradative activity for immunoreactive insulin and immunoreactive proinsulin was located in the supernatant fraction with the remaining in the particulate fraction. The rate of degradation of immunoreactive insulin in the supernatant fraction expressed as picomoles destroyed per minute per gram of tissue was 53.8, 27.2, 26.6, 26.6, 25.0, 24.3, 14.1, 13.0, 11.5, 8.0, 5.9, and 1.7 for liver, pancreas, kidney, testis, adrenal, spleen, ovary, lung, heart, muscle, brain, and fat, respectively.

With the exception of pancreas and kidney, no organ extract exhibited greater than 10 per cent of immunoreactive proinsulin degradation as compared to immunoreactive insulin. The rate of immunoreactive proinsulin degradation (picomoles per minute per gram of tissue) in the supernatant fraction was 5.0, 5.0, 5.0, 2.4, 2.0, 2.0, 1.6, 1.6, 1.0, 1.0, 0.6, and 0.2 for liver, pancreas, kidney, adrenal, testis, spleen, ovary, lung, heart, muscle, brain, and fat, respectively.

Studies on partially purified insulin protease of kidney indicated (a) an approximately tenfold greater rate for degradation of insulin than proinsulin and no appreciable degradation of intermediate II; (b) degradation of intermediate I was approximately one-third as much as that of insulin; (c) addition of other hormones such as ACTH, glucagon, and growth hormone did not affect the degradation rate of insulin; (d) an inhibition of the enzyme by the sulfhydryl group inhibitor and by heating at 60° for five minutes; (e) a lack of effect of glutathione or trasylol; (f) a pH optimum of 7.4 to 7.6; and (g) a K_m for insulin of 2×10^{-8} M. These properties all agree with similar findings of enzyme systems in liver and muscle, and suggest a similar degree of selectivity for degradation of insulin. DIABETES *21*:1091-1101, November, 1972.

Although degradative enzymes for radioiodinated insulin have been described by many investigators,[1-7] studies on the metabolism of the precursor of this hormone, proinsulin, have been limited in vitro.[8-10] With the recent demonstration of proinsulin in circulation, it became of interest to know the extent of degradation of this prohormone by various tissues. Recent reports from our laboratories on the presence of a protease with greater ability for degradation of insulin than proinsulin in muscle[11] and liver[12] of rats, has prompted us to investigate the ability of the other organs to degrade proinsulin. The purpose of the present studies was threefold: (1) to assess the contribution of each organ to the degradation of immunoreactive insulin (IRI) and immunoreactive proinsulin (IRP); (2) to study the subcellular localization of this degradative activity; and (3) to study the biochemical properties of purified insulin and/or proinsulin-degrading enzyme in the kidney, an organ which has been implied to play an important role in proinsulin metabolism.[13] The studies reported here suggest that rapid degradation of insulin occurs in extracts of many organs in the rat, which confirms the observations of Mirsky and others. It is concluded from these studies that—with the exception of pancreas and kidney—none of the organs is able to degrade proinsulin more than 5 to 10 per cent of the rate at which insulin is degraded. Furthermore, these studies indicate that, with insulin concentration

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in the physiological range (0.1 to 1 nM), the degradation of insulin and/or proinsulin occurs in the 100,000 x g supernatant fraction with the precipitate consisting of debris, nuclei, mitochondria and microsomes having less than 5 per cent of this degradative activity. Further, it has been found that the purified kidney enzyme possesses a specificity for insulin very similar to that of liver and muscle.

MATERIALS AND METHODS

Animals. Male Holtzman rats weighing 180 to 250 gm. were used in these studies. The rats were fed Purina Laboratory Chow and had access to food and water up to the time of their sacrifice.

Chemicals. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Company, Chicago. Glucagon-free pork insulin (lot PJ 5589) and pork proinsulin (lot 615-1039B-45-C) were the gifts of Dr. R. Chance of Eli Lilly and Company, Indianapolis. Beef proinsulin, intermediate I and intermediate II were gifts of Dr. D. Steiner of the University of Chicago. Insulin-I-125 was purchased from Cambridge Nuclear Corporation, Cambridge, Massachusetts. The TCA precipitability of all lots exceeded 95 per cent and only lots with greater than 90 per cent immunoprecipitability were used in these studies. Rabbit antiserum globulin to guinea pig serum was purchased from Cappell Laboratories, Downington, Pennsylvania. All other chemicals were of reagent grade and were obtained commercially.

Preparation of tissue homogenates. Animals were sacrificed by decapitation and organs were removed, rinsed in cold 0.33 M sucrose and homogenized in an all-glass conical homogenizer immersed in ice. The homogenate was diluted in 0.33 M sucrose to a ratio of 1 gm. tissue/4 ml. sucrose solution. The homogenate was then centrifuged in a Model 265B Spinco ultracentrifuge at 100,000 x g for sixty minutes at 4°. The pellet was rinsed with sucrose, recentrifuged as above and resuspended in an equal volume of 0.33 M sucrose. All homogenates and fractions were then quickly frozen in a dry ice acetone bath and stored at —20° for later assay. To circumvent day-to-day variability of the assay, all organs from the same rats were assayed on the same day. In general, organs in each experiment were pooled from three to four rats. A total of four preparations consisting of a total of sixteen rats were studied and are reported here.

Preparation of purified insulin protease from kidney. Kidneys from seven to eight male Holtzman rats (weighing between 200 and 250 gm.) were used. The tissues were cleaned of fat and connective tissue and cut into small pieces with scissors; the homogenate was centrifuged for ten minutes at 10,000 x g in a refrigerated Sorvall centrifuge. The debris was discarded and the supernatant solution, which contains more than 90 per cent of the total insulin-degrading activity of the homogenate, was used for further purification by the procedures previously described[11,12,14] as follows: The supernatant was dialyzed for twenty hours against at least 20 vol. of glass-distilled water with three changes. The precipitate formed was removed by centrifugation at 10,000 x g for twenty minutes.

The total volume of the supernatant was measured. The supernatant was lyophilized and then dissolved in 0.01 M sodium acetate buffer, pH 6.2, in one-eighth to one-sixth of the original volume of the supernatant. Undissolved materials were removed by centrifugation at 17,000 x g for twenty minutes. The protein concentration of this solution was then estimated by ultraviolet absorption and the volume was adjusted with pH 6.2 acetate buffer so that the protein concentration was 10 mg./ml. The total volume at this point is designated as "v". Calcium phosphate gel, prepared according to the method of Keilin and Hartree,[15] was used, preferably within three months of the preparation.

For each batch of gel, an optimum ratio of dry gel per milligram of protein must be established. This ratio should be such that 90 per cent of the activity is adsorbed to the gel, and 80 per cent of the activity is finally eluted from it. This ratio for kidney, in our laboratory, has been found to be 1.0 mg. dry gel per milligram protein. Once the optimal amount of gel was established, the gel was added to the enzyme solution in the cold and stirred for twenty minutes, then centrifuged at 5,000 x g for ten minutes in a Sorvall centrifuge. The supernatant was discarded and the gel was resuspended in "v" ml. of 0.01 M acetate buffer (pH 6.2). Following stirring and recentrifugation as above, the adsorbed enzyme was eluted by stirring with "v" ml. of 0.05 M phosphate buffer, pH 7.5, for twenty minutes. After removal of the gel by centrifugation at 10,000 x g for ten minutes at 4°, the supernatant, which was approximately three- to fivefold purified, was frozen at —70° in small aliquots. The enzyme evaluation was carried out by studying the degradation of exogenous porcine insulin and proinsulin (0.44 nM) in pH 7.5 borate buffer. The incubation times used were 0, 2, 5, 10, 15, 20, and 30 minutes.

Method of assay for insulin and proinsulin degradation. Studies of degradation of insulin or proinsulin in

crude homogenates of organs or purified enzyme from kidney were carried out in 0.13 M borate buffer (0.5 per cent bovine serum albumin) pH 7.5 containing 25 μl. of the enzyme source and 0.1 picomole insulin or proinsulin. In every case, the total volume of the incubation mixture was 225 μl. The reaction was carried out in 75 x 12 mm. Pyrex tubes at 37° in a metabolic incubator at various time intervals. The reaction was stopped by addition of 25 μl. of 0.01 M NEM. The incubation tubes were then directly assayed by the immunoassay method for insulin or proinsulin content, and compared to the control tubes containing enzyme which were inactivated at zero time with NEM. The results were reported as picomoles of insulin or proinsulin degraded per minute per gram of wet weight of the tissue or milligram of protein (table 1). Determination of protein in the purified enzyme preparation was done by the method of Lowry et al.[16]

Calculation of enzyme activity. In order to calculate the rate of degradation of insulin and proinsulin in crude extract of each organ, a particular amount of tissue extract was selected so that the degradation of the substrate in that extract for the first five minutes would follow first-order kinetics. From these experiments, rate of destruction was then calculated as picomoles substrate destroyed/min./gm. tissue (table 1).

The enzyme kinetic constant (K_m) for insulin in purified kidney enzyme was determined by using an incubation mixture consisting of 0.1 M Tris-EDTA buffer pH 7.6, containing bovine serum albumin (3 mg./ml.), I-125-insulin (approximately 24,000 dpm) and varying concentrations of nonradioactive insulin for final substrate concentration range of 10^{-5}-10^{-10} M in the reaction mixture. The reaction was started by addition of the enzyme and incubation was carried out for five minutes at 37° in total volume of 1 ml. The reaction was stopped by addition of 1 ml. of 10 per cent TCA. The percentage of insulin degradation was determined as the net amount of radioactive TCA soluble material.

Method of immunoassay. The modified[17] double antibody immunoassay method of Morgan and Lazarow[18] is used. This immunoassay can detect insulin and proinsulin, but insulin is more immunoreactive than proinsulin. C-peptide is not immunoreactive in this assay (figure 1).

RESULTS

In order to establish the optimal concentration of tissue where comparative studies could be carried out between various tissues, the experiment depicted in figure 2 was carried out where different amounts of tissue from 5 to 50 μg. in total volume of 225 μl. were incubated with immunoreactive insulin. Degradation of IRI was measured in ten minutes in 100,000 x g supernatant solution of liver, muscle, kidney, adrenal, and testis. As can be seen from this figure, degradative activity was proportional to the amount of tissue used. Figure 2 also clearly demonstrates that, of the five

TABLE 1

Degradation of immunoreactive insulin (IRI) and immunoreactive proinsulin (IRP) by 100,000 x g supernatant and precipitate fractions of rat organ homogenates*

	Picomoles IRI or IRP destroyed/min./gm. tissue			
	Supernatant		Precipitate	
Organs	IRI Avg. ± S.E.M.	IRP Avg. ± S.E.M.	IRI Avg. ± S.E.M.	IRP Avg. ± S.E.M.
Liver	53.8 ± 3.40	5.0 ± 0.45	0.28 ± 0.020	0.014 ± 0.002
Pancreas	27.2 ± 0.41	5.0 ± 0.18	0.34 ± 0.022	0.016 ± 0.004
Kidney	26.6 ± 1.47	5.0 ± 0.40	0.28 ± 0.010	0.012 ± 0.002
Testis	26.6 ± 1.25	2.0 ± 0.26	0.18 ± 0.01	0.010 ± 0.003
Spleen	24.25 ± 1.49	2.0 ± 0.18	0.14 ± 0.011	0.008 ± 0.001
Lung	13.0 ± 1.49	1.6 ± 0.05	0.32 ± 0.018	0.013 ± 0.001
Heart	11.5 ± 0.92	1.0 ± 0.16	0.12 ± 0.010	0.008 ± 0.001
Muscle	8.0 ± 0.31	1.0 ± 0.30	0.28 ± 0.016	0.011 ± 0.002
Brain	5.9 ± 0.99	0.6 ± 0.07	0.06 ± 0.005	0.007 ± 0.000
Fat	1.65 ± 0.07	0.2 ± 0.05	0.02 ± 0.004	0.0001±0.000
Adrenal†	24.96	2.4	0.32	0.015
Ovary†	14.08	1.6	0.24	0.014
Muscle†	6.56	0.8	0.24	0.012

* For experimental conditions, see *Materials and Methods* in the text.
† Pooled tissue from eight female rats.

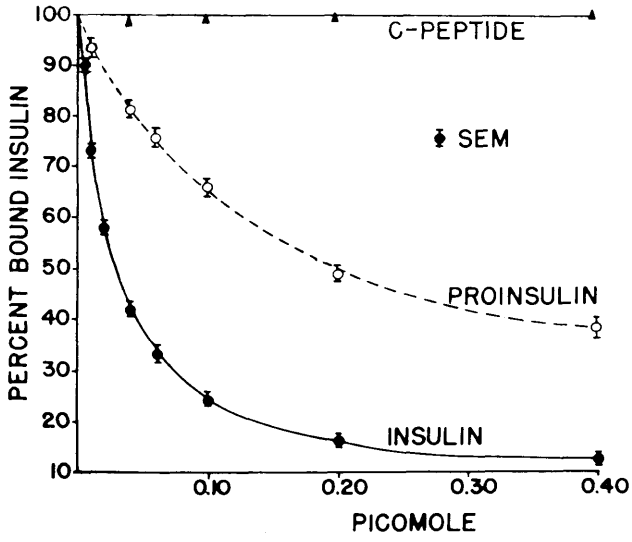

FIG. 1. Immunoprecipitability of porcine insulin, proinsulin, and C-peptide by the insulin double antibody immunoassay. The final dilution of insulin antiserum for 50 per cent immunoprecipitability of labeled insulin is 1:300,000.

organs tested, liver is the most active organ for insulin degradation, confirming the similar earlier findings in many laboratories but extending these findings to non-iodinated insulin.

Kinetics of insulin and proinsulin degradation. In order to study the destruction of insulin and proinsulin in various organs, the supernatant fractions of these organs were tested for their ability to degrade IRI and IRP at 0, 2, 5, 10 and 15 minutes. Figures 3 and 4 depict a timed study of the degradation of insulin and proinsulin by these organs, respectively. As can be seen, with the use of 0.1 picomole of insulin, liver essentially has degraded more than 90 per cent of it in five minutes when comparable amounts of the other tissue were relatively less active. In other experiments (data not shown) insulin degradation was correlated with protein concentration in the supernatant fractions. Essentially similar results to that of figure 3 were obtained when degradation was expressed per milligram of protein, i.e. liver was still the most active and fat the least. Proinsulin degradation was less than 20 per cent of insulin for the first five minutes in all the tissues tested. There was no change in the rate of degradation of proinsulin in the supernatant fractions of kidney, liver or muscle homogenate when a shorter period of incubation than five minutes was used (figure 4).

In order to calculate the rate of degradation of insulin and proinsulin by each organ, a proper dilution of supernatant and precipitate fraction of each organ was selected, so that the rate of degradation for the first five minutes for each sample followed the first-order kinetics. From these data, the rate of degradation was then calculated per minute per original gram of wet weight of the tissue in both the supernatant and particulate fractions and is depicted in table 1. This table depicts degradative activity of IRI and IRP of ten organ homogenates of four groups of male rats with three to four rats in each group. In addition, in order to compare the degradative activity of male and female gonads, ovaries of a group of eight female rats were studied and compared to muscle and adrenal tissues of these rats. As can be seen, the order of the most to the least active organ for insulin degradation in the supernatant is liver > pancreas > kidney > testis > adrenal > spleen > ovary > lung > heart > muscle > brain > fat.

The proinsulin degradation activity in the supernatant fraction is slightly different than for insulin in that three organs—liver, kidney, and pancreas—show the same level of activity, followed by testis, adrenal, spleen, ovary, lung, muscle, heart, brain and fat. The organs least active for both insulin and proinsulin are brain and fat. Organs having the most active proinsulin/insulin-degrading ratio are kidney and pancreas, with 20 per cent, whereas all other organs exhibit a ratio of proinsulin/insulin of approximately 10 per cent. Table 1 also presents data from the same animals, but on the 100,000 x g precipitate fraction, consisting of cell debris, nuclei, mitochondria and microsomes. As can be seen from this table, the precipitate fraction contains less than 10 per cent of the total degradative activity of the supernatant both for insulin and proinsulin degradative ability.

Studies on purified insulin protease from kidney. Because kidney homogenates exhibited a relatively higher ratio of proinsulin/insulin degradative activity than liver and muscle, the enzyme was further purified before being characterized. The enzyme purification was achieved by adsorption and elution from calcium phosphate gel as stated under *Methods,* but in contrast to muscle and liver, the ratio of dry gel to milligrams protein for optimal protein adsorption and elution was 1.0 instead of 0.4. Table 2 depicts the purification steps and fold purification for the kidney enzyme. Approximately fifteenfold purification is obtained by calcium phosphate gel method. Figure 5 depicts the rate of degradation of beef insulin, proinsulin, as well as proinsulin intermediate I (desdipeptide proinsulin with residues 59 and 60 missing) and proinsulin intermediate II (desdipeptide proinsulin with residues 31 and 32 missing) by purified kidney enzyme. As can be seen, kidney enzyme,

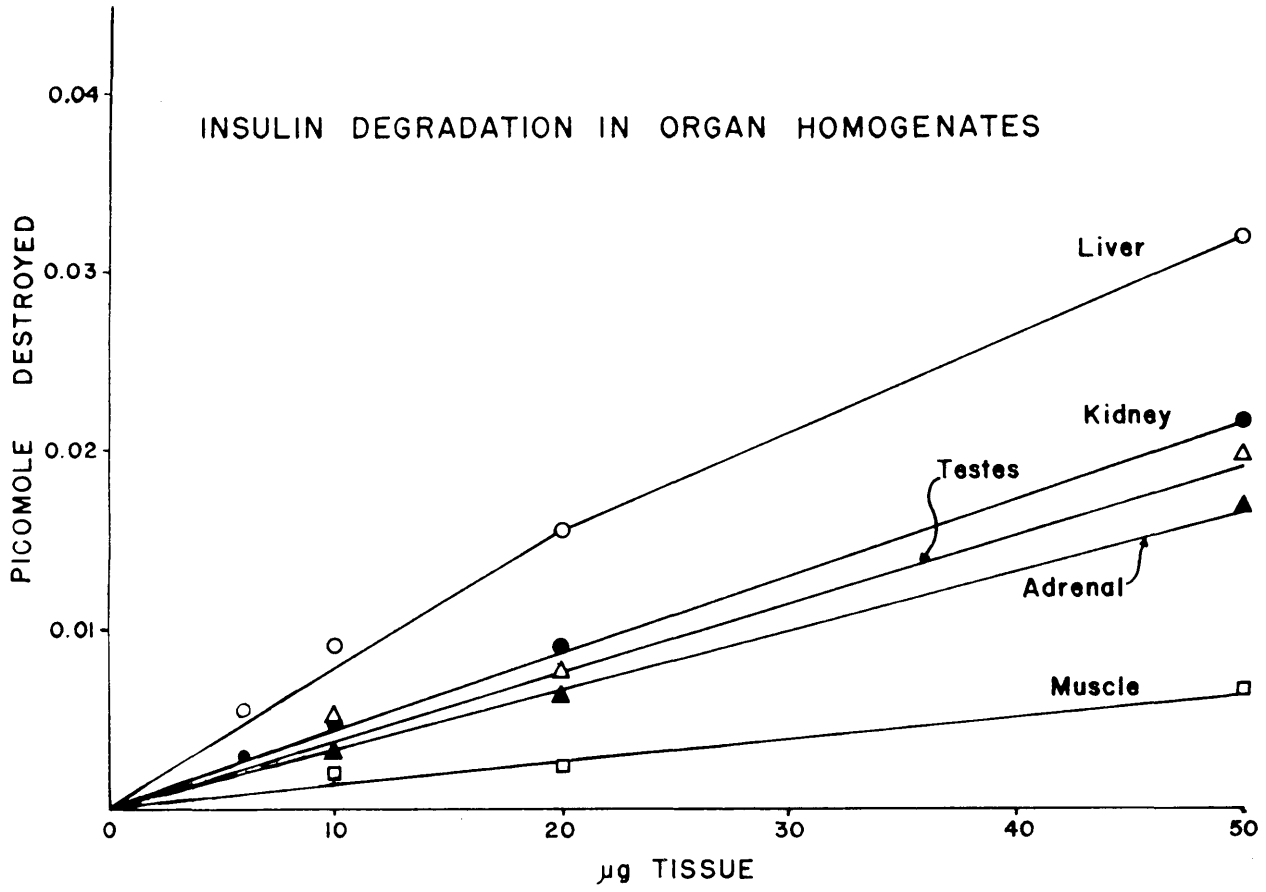

FIG. 2. The relationship of insulin degradation with tissue concentration in 100,000 x g supernatant fractions of five organs is demonstrated in this figure. The incubation mixture consisted of 0.1 ml. of 1 nM insulin, 0.133 M borate buffer (0.5 per cent BSA), pH 7.5, and tissue supernatant in a volume of 0.225 ml.

similar to liver and muscle degrading enzymes,[11,12] is capable of degrading insulin and intermediate I, but not proinsulin or intermediate II. Degradation rate of proinsulin intermediate I is between the rate of insulin and proinsulin degradation.

Additional biochemical studies showed pH optimum for insulin (figure 6) and proinsulin (data not shown) to be between 7.4 and 7.6 and K_m for insulin to be in the range of 2 x 10^{-8} M (figure 7). Because of the low level of activity with proinsulin, a satisfactory calculation of K_m for proinsulin could not be obtained. Table 3 compares the properties of the insulin-degrading enzyme of kidney, muscle, and liver, which demonstrates the extremely close similarity between the K_m of the three enzymes. Table 4 also demonstrates other properties of the kidney enzyme such as inhibition by NEM, sulfhydryl group inhibitor, p-hydroxymercuribenzoate, heat lability at 60° for five minutes, and the lack of effect of proteolytic inhibitor, trasylol, and of glutathione, on the enzyme activity. This table also shows the inhibitory effect of proinsulin on insulin degradation which is noted only when proinsulin is present in 1,000-fold excess of molar concentration of insulin. These properties all resemble the insulin-specific protease of Brush for muscle[11] and of Burghen et al. for liver.[12] Additional studies (data not shown) on degradation of insulin by purified kidney enzyme in the presence of other hormones such as ACTH, glucagon and growth hormone showed no change in rate of degradation of insulin similar to our earlier finding with muscle enzyme.[14]

DISCUSSION

The major method by which insulin degradation is accomplished in the body has been a subject of controversy. Both insulinase and glutathione insulin transhydrogenase (GIT) have been claimed to be major systems for insulin degradation. GIT, however, has not

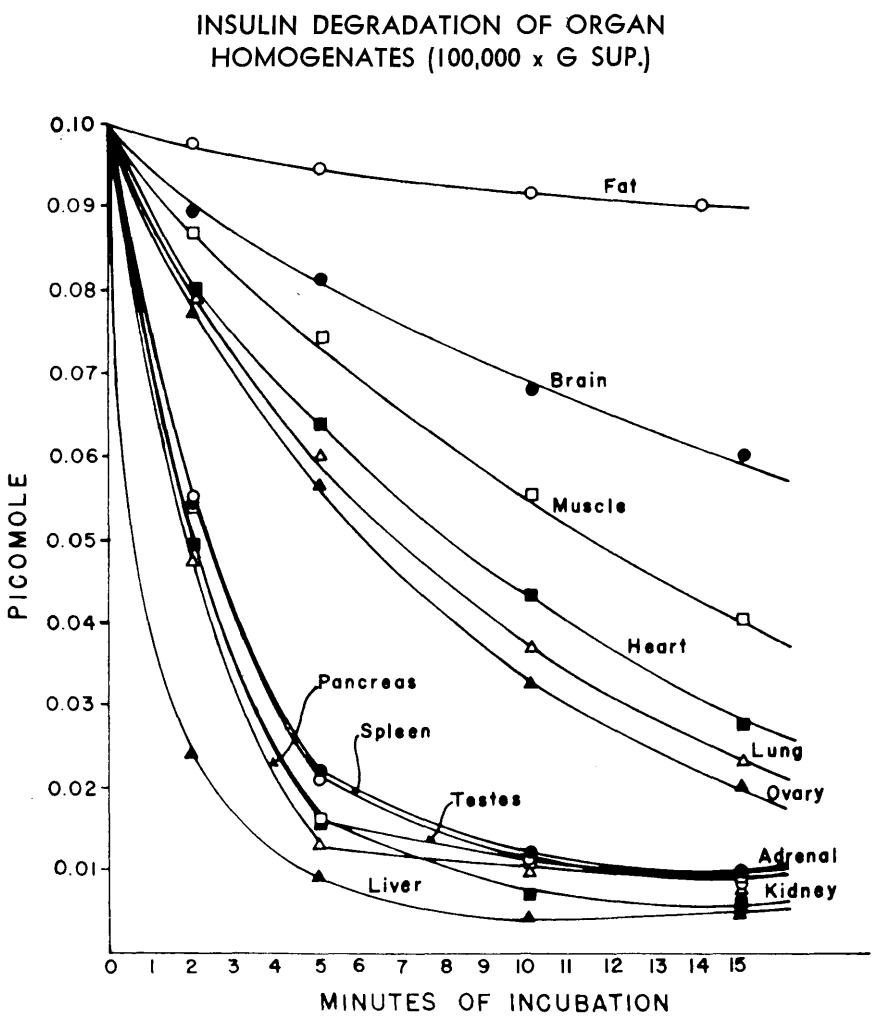

FIGURE 3

Rates of insulin degradation by equivalent wet tissue weights of the various organ homogenates (100,000 x g supernatant). The condition of incubation is similar to that in figure 2. The tissue extract is from 0.625 mg. of tissue for each organ.

been shown to be specific for insulin since other disulfide containing compounds may also be degraded by GIT.[7,19] Furthermore, the extent to which GIT is present in other tissues beside liver has not been fully elucidated. Aside from its lack of specificity, the exact localization of this enzyme in various tissue homogenates has not been fully investigated, since most of the studies on GIT have utilized solvent fractions as a step for purification. This is known to stimulate GIT activity.[20]

Studies by Burghen et al.[12] on liver-degrading enzyme have established that without prior alteration of tissue with lipid solvent fractionation, approximately 95 per cent of the insulin-degrading enzyme in liver homogenate is located in the supernatant which does not contain glutathione, does not require glutathione for degradation of insulin, and the degradation is not by reductive cleavage but by hydrolysis of peptide bonds. Furthermore, the K_m of the enzyme is 0.1 μM,[12] which is more physiological than the 43 μM reported for K_m of GIT.[19]

Present studies on various organ homogenates clearly show that degradative activity for insulin and proinsulin is located in the supernatant fraction and is inactivated by NEM. Furthermore, studies on purified kidney enzyme indicate K_m to be similar to other insulin proteases from muscle and liver.[11,12] The specificity of kidney protease for insulin is further substantiated by the fact that removal of two dibasic amino acids of the connecting peptide from the amino end of the A chain (intermediate I) makes this molecule more susceptible to degradation than removal of a basic dipeptide of the connecting peptide from the carboxy end of the B chain (intermediate II); thus, in agreement with the role of the A chain in determining the tertiary structure of insulin, the deblocking of the A chain may be critical

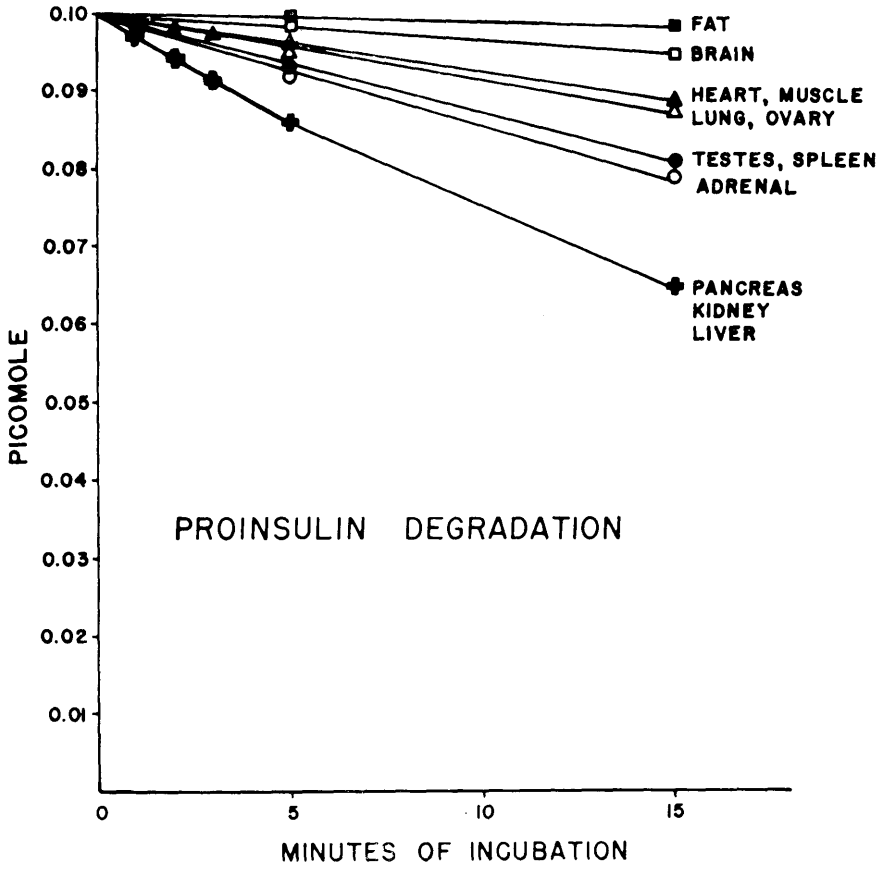

FIGURE 4

Rates of proinsulin degradation using 0.1 ml. of 1 nM proinsulin; the condition of the experiment is similar to that in figure 2 except that additional, earlier, timed studies are included for the first five minutes to demonstrate the lack of increased immunoreactivity in the early part of incubation.

for insulin-like activity of the proinsulin molecule.[10] Slower degradation of proinsulin in most major organs may explain the longer half-life of this prohormone.[21]

Our studies on insulin degradation in various tissues were also undertaken to measure the extent of total degradative activity of insulin and proinsulin in both supernatant and precipitate fraction. In earlier studies of insulin degradation, labeled insulin was used in the crude homogenates of various organs. We have specifically selected immunoreactive hormone in preference to labeled hormone to circumvent difficulties associated with variation of biological activity of labeled insulin,[4] as well as difficulties associated with inaccuracy of assessment of insulin degradation when measured by TCA precipitability.[22] Our studies on insulin degradation, although using the immunoassay method and a completely soluble enzyme system, essentially confirm the earlier work in this area, although giving a more accurate measurement of insulin degradation.

From the available evidence in the literature, it would appear that the major site of proinsulin conversion to insulin is in the pancreas.[23,24] Studies on the peripheral degradation of proinsulin, however, are limited. Labeled proinsulin injected into rats can be recovered unchanged as long as three hours after injection.[25] In support of peripheral conversion of proinsulin to insulin, Mashiter and King[8] reported that incubation of proinsulin with tissue homogenates of liver, kidney and spleen resulted in a change in immunoreactivity, suggestive of a conversion to insulin followed by a degra-

TABLE 2

Purification of rat kidney insulin-degrading enzyme*

Fraction	Specific† activity	Yield (%)	Fold purification
Crude homogenate	0.03	100	1.0
100,000 x g supernatant	0.11	70	3.6
$Ca_3(PO_4)_2$ adsorption elution	0.50	51	16.7

* Conditions of the experiment are similar to those in table 1.

† Picomole substrate destroyed/min./mg. protein.

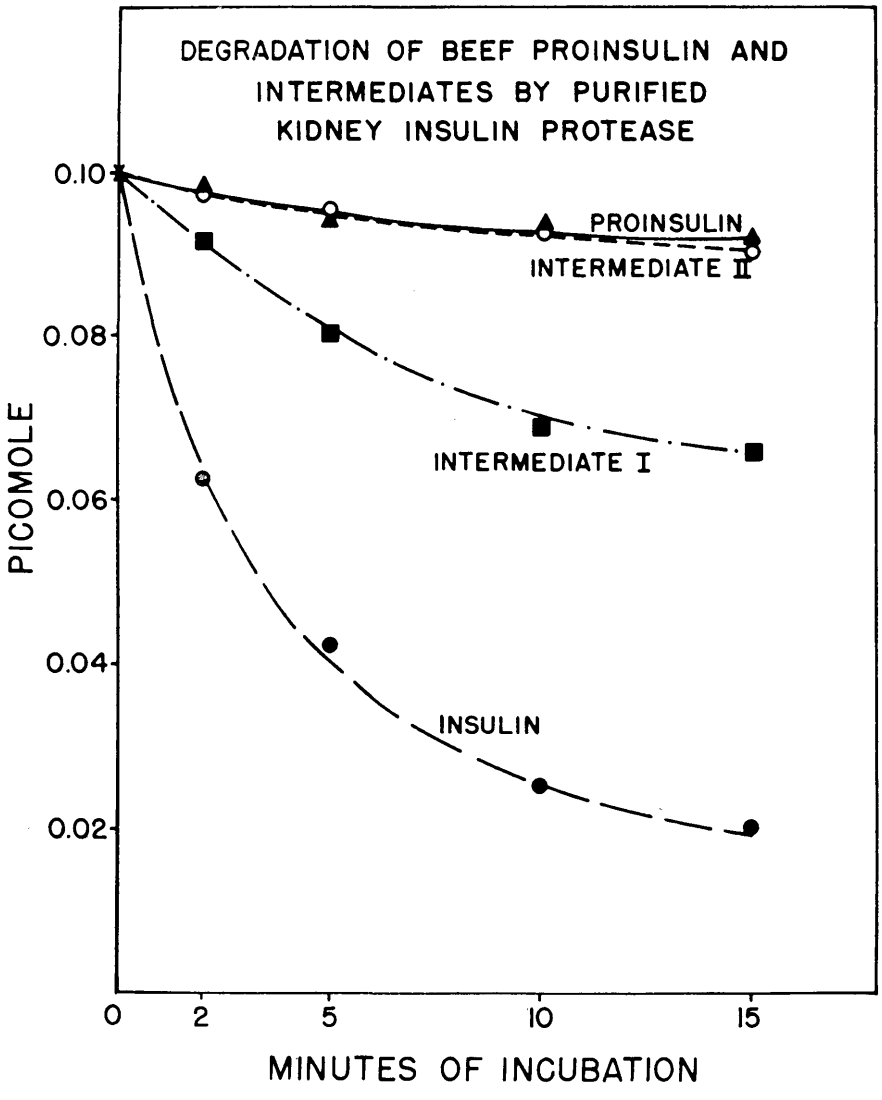

FIGURE 5

Degradation of beef insulin, proinsulin and proinsulin intermediates by kidney insulin protease. The conditions of the experiments are similar to those in table 3.

dation of insulin. The presence of nonspecific hydrolases released from lysosomes in these studies could not be ruled out, since homogenization of the organs was carried out in distilled water. Furthermore, isolation and characterization of the conversion products were not reported.

Challoner,[9] studying insulin and proinsulin degradation in fat, found that labeled insulin and proinsulin degradation to TCA soluble material was similar in fat cells after two hours of incubation. Homogenates of fat pieces, however, showed less degradative activity for proinsulin than for insulin. The presence of residual crude collagenase (which is used in the preparation of fat cells), and the liberation of nonspecific hydrolytic enzymes from lysosomes of these cells by the method of sonication used in disruption of these cells, could not be ruled out as responsible factors for nonspecific protease activities observed in these preparations. From the kinetic studies depicted in figure 4, incubation of proinsulin with various tissue extracts does not appear to lead to a higher immunoreactive material, which would be suggestive of insulin; these findings do not support the work of Mashiter and King.

The kidney and the pancreas have higher ratios of proinsulin/insulin degradation than the other tissues examined in our studies. Much of the proinsulin degradation by the pancreas may be due to the large content of nonspecific proteolytic enzymes in this tissue. Recently, a proinsulin converting enzyme system has been described in the islet[23] and the pancreatic extract.[24]

As to the biological significance of an intracellular enzyme which degrades insulin, we postulate that since

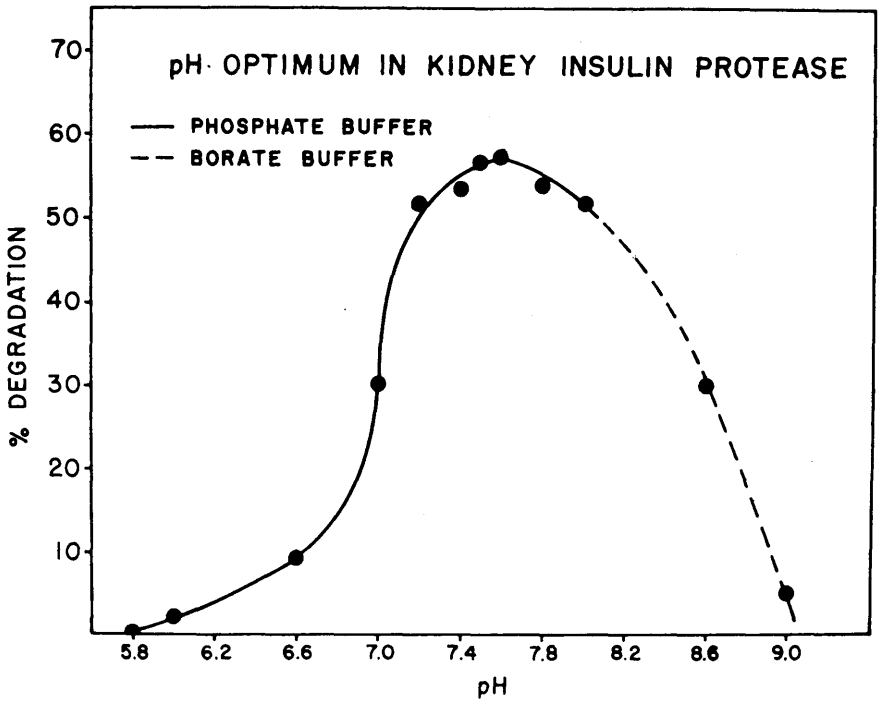

FIGURE 6

Kinetic study of the enzymatic degradation of insulin. Incubations were carried out utilizing the indicated concentrations of insulin. Velocity is expressed as 10^{-13} M degraded per minute. Substrate concentration is expressed at 10^{-8} M. The Lineweaver-Burke plot shows the K_m to be 2 x 10^{-8} M.

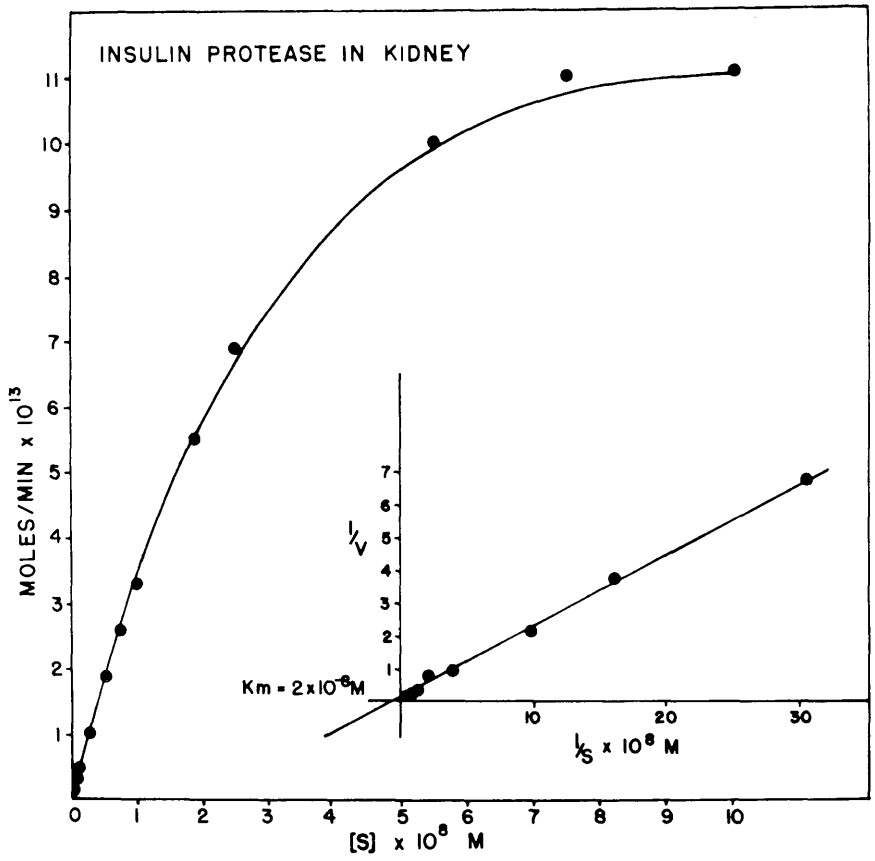

FIGURE 7

Variation of enzyme activity wth pH. Enzymatic activity at each pH was determined using 0.01 M phosphate (0.5 per cent BSA) buffer from pH 5.8 to 8.0 and 0.13 M borate buffer (0.5 per cent BSA) from pH 8.0 to 9.0.

TABLE 3

Insulin (IRI) and proinsulin (IRP) degradation in purified insulin protease of liver, muscle, and kidney of rats*

Enzyme source	Picomole substrate destroyed/min./mg. protein		K_m (μM)
	IRI	IRP	
Liver	0.68	0.072	0.10
Kidney	0.50	0.064	0.02
Muscle	0.54	0.080	0.04

* Incubation system: Enzyme (25μg. protein), borate buffer 0.13 M, pH 7.5, containing 3 mg./ml. bovine serum albumin and 0.1 ml. of 10^{-9} M substrate. Incubation for five minutes at 37° in air in total volume of 225 μl. At the end of incubation, 25 μl. of 0.01 M NEM is added to the tube and the mixture is immunoassayed.

the active site of insulin is on the membrane,[26,27] the degradation of the hormone may be accomplished elsewhere. Preliminary studies from other laboratories support different sites between binding and degradation of insulin.[28] Furthermore, the possibility of an insulin-degrading enzyme bound loosely to the membrane can not be ruled out from the present studies.

As to the presence of less active proinsulin-degrading enzymes, it is tempting to postulate that because of its lesser biological activity,[29,30] and lack of its increase in the postabsorptive state,[31] its immediate destruction may not be as important physiologically as that of insulin.

In conclusion, it would appear that the degradative activity for both insulin and proinsulin appears to be in the soluble portion with the particulate fraction having less than 10 per cent of the total degradative activity, and that the degradative activity for proinsulin in each organ is considerably less than for insulin. A comparison of properties of the partially purified insulin-degrading enzymes from liver, muscle and kidney suggests the presence of similar biochemical properties among the enzymes of these three organs with high specificity for insulin. Recent studies[32] from our laboratory have shown that insulin specific protease from rat skeletal muscle can be purified 1,000-fold by affinity chromatography and appears to be a single enzyme by polyacrylamide gel electrophoresis.

TABLE 4

Effect of various agents on insulin degradation of insulin protease of kidney*

Addition	Concentration	Picomole substrate destroyed/min.
None	—	0.0124
Trasylol	45 μg./ml.	0.0120
Glutathione	0.001 M	0.0124
P-hydroxymercuribenzoate	0.001 M	0
N-ethylmaleimide	0.001 M	0
Heating (60° for 5 min.)	—	0
Dialyzed (overnight)	—	0.0124
Proinsulin†	1×10^{-10} M	0.0124
Proinsulin†	5×10^{-9} M	0.0124
Proinsulin†	1×10^{-7} M	0.0100

* Method of assay is similar to that in table 3.

† In these experiments, degradation of 125-I insulin (10^{-10} M) was studied in the absence and presence of various concentrations of unlabeled proinsulin and the results adjusted for comparison.

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