

# The Biological and Immunological Properties of Pork and Beef Insulin, Proinsulin, and Connecting Peptides

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**ABSTRACT** The recently discovered hormone precursors, pork and beef proinsulins, their respective connecting peptides, and beef proinsulin intermediates have been compared to insulin in their ability to stimulate the conversion of glucose- $U-^{14}C$  to  $^{14}CO_2$  and lipids in isolated fat cells. The concentrations of beef and pork proinsulins required to achieve the same biological effect were, respectively, 15 and 10 times that of insulin. Beef proinsulin intermediates required only 2.6 times the concentration of insulin for the same effect. Pork and beef connecting peptides in high or low concentrations alone or in combination with proinsulin, insulin, or proinsulin intermediates showed no biological effect on the isolated fat cell system. The insulin-like activity of beef and pork proinsulins on the isolated fat cell system was not abolished with pancreatic trypsin or kallikrein inhibitors. Pork insulin antiserum inhibited the biological activity of pork insulin and proinsulin as well as that of beef insulin or proinsulin. Pork proinsulin antiserum also inhibited the insulin-like activity of both pork insulin and proinsulin. By the radioimmunoassay method, pork insulin antiserum bound only  $\frac{1}{4}$  to  $\frac{1}{3}$  as much proinsulin as insulin. Beef proinsulin intermediates, on the other hand, were found to react with the pork insulin antiserum to an extent nearly equal to that of insulin. These data suggest that (a) proinsulin exhibits its effect on the isolated fat cells independent of its conversion to insulin, (b) connecting peptides have no biological effect under present experimental conditions, and (c) in comparison to insulin, immunological reactivity of proinsulin is greater than its biological activity using our pork insulin antiserum; thus, the

comparison of antibody specificity with the fat cell receptor specificity suggests that the biological site of action is different from the immunologic site.

## INTRODUCTION

Recent investigations have established the presence and identity of a single chain insulin precursor in the pancreas designated as proinsulin<sup>1</sup> (2, 3). The structure of both pork and beef proinsulins has been elucidated in various laboratories (4, 5). Although purified pork proinsulin has been shown to have insulin-like activity in the epididymal adipose tissue and the isolated diaphragm of the normal rat, this effect was reported to be blocked by pancreatic trypsin inhibitor (6). The conversion of proinsulin to insulin by the action of trypsin in these tissues has been suggested as the mechanism of action of proinsulin upon these tissues. Whether or not proinsulin exhibits any direct biological effect has not been established. Furthermore, recent work of Clark, Cho, Rubenstein, and Steiner (7) indicates that the peptide released when proinsulin is converted to insulin is not degraded within the islet cell, but is liberated within the circulation. It is therefore possible that connecting peptide or proinsulin intermediates may have some biological effect on the peripheral tissue. This communication presents data indicating that proinsulin and the

<sup>1</sup> The following nomenclature is used for the compounds utilized in these studies. Beef connecting peptide—a polypeptide liberated by proteolysis from beef proinsulin containing residue Nos. 33 through 58. Beef intermediates—mixture containing (a) beef proinsulin lacking residues Nos. 31 and 32, and (b) beef proinsulin lacking residues Nos. 58 and 59. The ratio of (a):(b) in the mixture is 40:60. Pork connecting peptide—31 residue glutamyl peptide (B 33 through 63) liberated from intact pork proinsulin by limited tryptic digestion. C-peptide refers to that form of connecting peptide from which all terminal basic residues have been removed.

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proinsulin intermediates, but not the connecting peptide, act directly on isolated fat cells, which suggests that the mechanism is probably independent of conversion of proinsulin to insulin. Furthermore, these data suggest a greater specificity of insulin interaction with the fat cell "receptor" than with its homologous antiserum.

## METHODS

*Animals.* Male Holtzman rats weighing 90–140 g were used in these studies. The rats were on Purina Laboratory Chow and had access to food and water up to the time of sacrifice.

*Chemicals.* Bovine plasma albumin (fraction V, lot E30308) was obtained from Armour Pharmaceutical Co.,

TABLE I  
*Effect of Inhibitors of Kallikrein (Trasylol) and Trypsin (KPTI) on the Biological Activities of Insulin and Proinsulin Preparations\**

Preparation and concentration	Inhibitor		No. of expts.	Amount of glucose- <sup>14</sup> C carbon converted to	
	Prep	Quantity		CO <sub>2</sub>	Lipids
		μg/ml		nanoatoms	
Baseline	—		10	36.6	76.0
	Trasylol	50	6	39.3	88.5
		200	7	46.3	78.9
	KPTI	50	4	24.8	93.3
		200	5	37.3	73.0
Porcine insulin 5 μU/ml (2.5 × 10 <sup>-4</sup> μg/ml)	—		5	152.3	298.0
	Trasylol	200	5	208.8	355.9
Porcine insulin 100 μU/ml (5 × 10 <sup>-3</sup> μg/ml)	KPTI	200	3	157.5	280.6
	—		10	242.2	438.2
	Trasylol	50	5	291.2	479.2
		200	7	263.0	451.2
	KPTI	50	3	210.6	481.4
		200	5	241.2	397.7
Porcine proinsulin (2.1 × 10 <sup>-3</sup> μg/ml)	—		7	86.9	183.9
	Trasylol	200	4	95.7	173.0
Porcine proinsulin (4.2 × 10 <sup>-2</sup> μg/ml)	KPTI	200	3	87.2	183.1
	—		9	233.3	423.8
	Trasylol	50	5	256.7	479.0
		200	6	243.4	413.0
	KPTI	50	4	214.1	387.6
		200	5	245.8	416.4
Beef proinsulin (4.3 × 10 <sup>-3</sup> μg/ml)	—		7	138.6	267.5
	Trasylol	200	4	148.5	268.1
Beef proinsulin (8.6 × 10 <sup>-2</sup> μg/ml)	KPTI	200	3	150.6	253.9
	—		9	236.5	419.9
	Trasylol	50	5	263.0	453.9
		200	6	259.4	435.9
	KPTI	50	4	217.9	399.4
		200	5	254.4	430.8

\* Incubation mixture: each incubation vessel contained 70–100 × 10<sup>3</sup> fat cells, 180 × 10<sup>3</sup> cpm as glucose-U-<sup>14</sup>C with 0.55 mM glucose in total volume of 2 ml containing Krebs-Ringer bicarbonate, pH 7.4 with 4% bovine serum albumin. Incubation was carried out under 5% CO<sub>2</sub> 95% O<sub>2</sub> for 2 hr. Results are reported as nanoatoms of glucose-<sup>14</sup>C carbon converted into <sup>14</sup>CO<sub>2</sub> and lipids per 100,000 cells in 2 hr.

Chicago, Ill. Crude bacterial collagenase (lots CLS9AA and CLS), Kunitz pancreatic trypsin inhibitors (KPTI, lot PISF8CA), and trypsin (lot TRL81B with specific activity of 200 U/mg) were purchased from Worthington Biochemical Corp., Freehold, N. J. Uniformly labeled glucose- $^{14}\text{C}$  was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Kallikrein inhibitor (Trasylol) with specific activity of 4900 kIU/mg was the gift of Dr. Gert Haberland of A. G. Bayer Company of Germany. Glucagon-free pork insulin (lot PJ5589), pork proinsulin (lot 615-1039B-45-C), and connecting peptide consisting of 31 residue glutamyl peptide (lot 615-984B81-F-2) were the gifts of Dr. Ronald Chance; human insulin and pork proinsulin antiserum were from Dr. Mary Root, and beef insulin was supplied by Dr. Walter Shaw, all of the Eli Lilly Co., Indianapolis, Ind. Beef proinsulin (lots BCM1aA and NNCMIAC), beef connecting peptide (C-peptide B), and intermediates (NBCD-F) were kindly supplied by Dr. Donald Steiner of the University of Chicago. Insulin- $^{125}\text{I}$  was purchased from Cambridge Nuclear Corporation, Cambridge, Mass., and rabbit antiserum globulin to guinea pig serum was purchased from Cappell Laboratories, West Chester, Pa.

**Preparation of adipose tissue.** Isolated fat cells were prepared by the method of Rodbell (8), as modified by Lavis, Kitabchi, and Williams (9). Epididymal fat pads from three or four rats were pooled and divided into two 700-1000 mg portions. Each portion was placed in a plastic vial containing 2 ml of Krebs-Ringer bicarbonate, pH 7.4, with bovine plasma albumin and 5 mg of collagenase. Each flask was incubated for 45 min in a Dubnoff metabolic shaker at  $37^\circ\text{C}$ . At the end of the incubation period the contents of each vial were centrifuged, and the fat cells were washed three to four times with additional buffer and resuspended in the same medium. The cells from two vials were then pooled, filtered through one layer of clean white silk organza, and diluted so that a 1 ml aliquot of the pooled isolated fat cell had between 70,000 and 100,000 fat cells. The fat cells were counted by the method of Gliemann

(10). The mean count of at least four independent dilutions was used. Aliquots were withdrawn and distributed in a random manner. The content of each incubation vessel is described in the legend of Table I.

**Method of fat cell bioassay.** The incorporation of radioactive carbon from glucose U- $^{14}\text{C}$  into  $^{14}\text{CO}_2$  and lipids was determined by previously published methods (9). Counting was performed in a Packard Liquid Scintillation Spectrometer. Results are reported as nanoatoms of glucose-carbon incorporated into  $\text{CO}_2$  or lipids/ $10^5$  fat cells per 2 hr of incubation.

**Method of immunoassay.** The double antibody immunoassay method of Morgan and Lazarow (11), as modified by Samols and Bilkus (12) was used. The first antibody was prepared by repeated injection of proinsulin-free insulin with Freund adjuvant into guinea pigs. The final dilution of insulin antiserum used for immunoassay was 1:300,000. The second antibody consisted of gamma globulin obtained from rabbits immunized with normal guinea pig serum globulin.

## RESULTS

### *Insulin-like activity of beef and pork proinsulins.*

Fig. 1 depicts the dose-response curve of pork insulin, beef proinsulin, and pork proinsulin as well as pork connecting peptide on the oxidation of glucose- $^{14}\text{C}$  to  $^{14}\text{CO}_2$ . Essentially similar results were obtained for incorporation of glucose-carbon into lipids. As shown in Fig. 1, a sigmoid curve was obtained for the logarithmic concentration-response curve of insulin and proinsulin. The concentration of beef proinsulin required to obtain half the maximal response was 15 times that of insulin. The corresponding concentration for pork proinsulin was 10 times that of insulin.

Pork connecting peptide had no significant insulin-like activity. Fig. 2 compares the effect of beef insulin, pro-

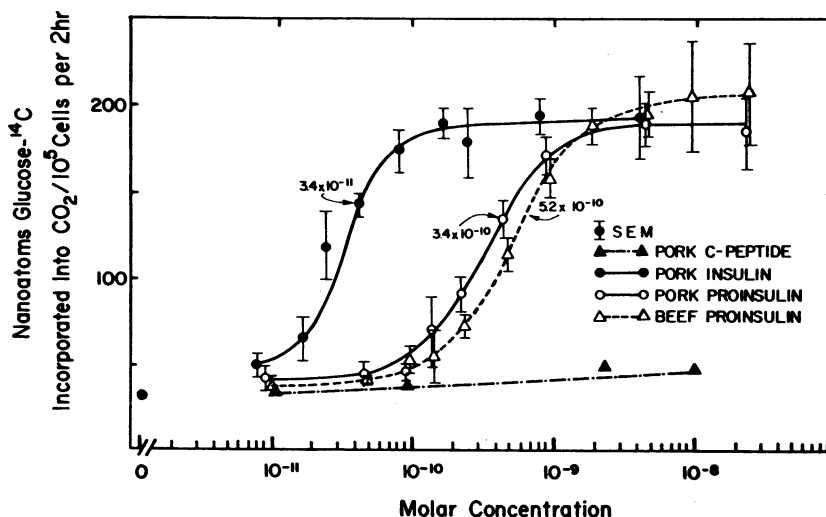


FIGURE 1 Dose response curves for pork insulin, pork proinsulin, beef proinsulin, and pork C-peptide on isolated fat cells. Conditions of the experiment are similar to those of Table I with various concentrations of the above components. Arrows point to the concentration of each compound at half-maximum stimulation. Each point represents an average of four experiments.

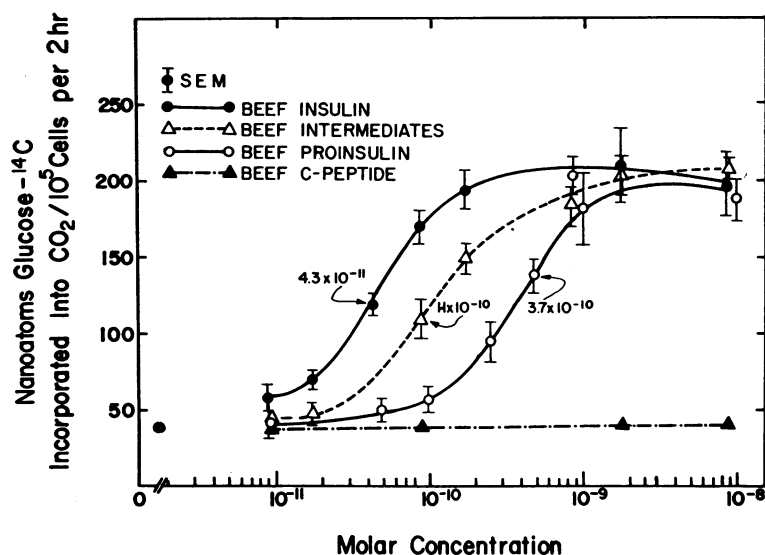


FIGURE 2 Dose response curve of beef insulin, proinsulin, intermediates, and C-peptides on the isolated fat cell system. Arrows point to the concentration of each compound at half-maximum stimulation. Each point represents an average of three experiments.

insulin, and intermediates as well as connecting peptide. In this experiment, the same maximal responses were obtained with quantities of beef proinsulin and beef proinsulin intermediates. The values for half-maximum responses of beef proinsulin and intermediates were, respectively, 9 times and 2.6 times that of insulin. Again, lack of activity of beef connecting peptide is noted at all concentrations tested.

**Kinetic studies on the insulin-like activity of insulin and proinsulin.** Kinetic studies showed that the formation of  $^{14}\text{CO}_2$  at 5, 10, 15, 30, 60, and 120 min of incubation was linear, without a lag period in the presence of either insulin or proinsulin (Fig. 3). This suggests that proinsulin was not converted to insulin during the incubation period.

**Effect of trypsin and kallikrein inhibitors on insulin-like activity of proinsulin.** The results recorded in Table I show the effect of insulin and proinsulin on the oxidation of glucose to  $\text{CO}_2$  and the formation of lipid in the presence and absence of KPTI and Trasylol. Neither of these preparations blocked the action of proinsulin or insulin on isolated fat cells. The results also show that the effects of insulin or proinsulin at lower concentrations were not inhibited by the highest concentrations of KPTI or Trasylol tested. The insulin-like activity of the beef proinsulin intermediates was also unaltered with KPTI or Trasylol (data not shown). Since our results with isolated fat cells in the presence of KPTI were opposite to those reported by Shaw and Chance who used intact fat pads and diaphragm (6), additional experiments were done to insure that a colla-

genase contaminant with proteolytic activity was not present in the isolated fat cell preparations. Crude collagenase is known to contain an acid protease (13). Residual collagenase, if present, would not be sensitive to the effect of Trasylol or KPTI, and the possibility existed that it might convert proinsulin to insulin. For this reason, the above experiments were repeated on fat cell preparations which were washed an additional three to five times in Krebs-Ringer buffer in an attempt to remove all residual collagenase. Although these fat cells showed decreased sensitivity to insulin and proinsulin, no effect of KPTI was noted on the biological response to proinsulin (data not shown).

To demonstrate that the effect of proinsulin is not due to small amounts of contaminating insulin in pork proinsulin, the following experiment was done. 100- $\mu\text{g}$  samples of pork proinsulin were subjected to polyacrylamide-gel electrophoresis with and without the addition of 3  $\mu\text{g}$  of porcine insulin. As shown in Fig. 4, only proinsulin was detected in the proinsulin preparations, whereas a distinct visible band of insulin was apparent in the preparation containing 3  $\mu\text{g}$  of insulin. These experiments demonstrated that the pork proinsulin contained less than 3% of free insulin, which could not account for the observed degree of biological activity of proinsulin at the minute concentration tested.

**Assay of pancreatic and kallikrein inhibitors.** To demonstrate that trypsin, KPTI, and Trasylol are active, the inhibitory effects of the latter two compounds were tested on the conversion of proinsulin to insulin with trypsin by incubating 40  $\mu\text{g}$  of pork proinsulin with 0.4

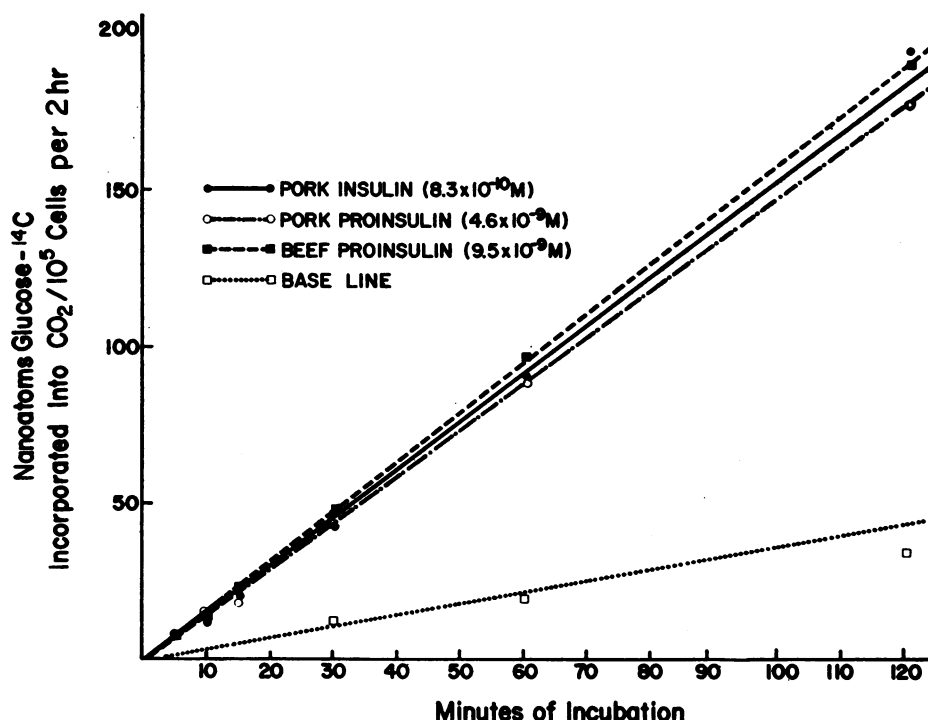


FIGURE 3 Time course of glucose-U- $^{14}\text{C}$  oxidation by isolated fat cells in the absence of insulin ( $\square$ ) and in the presence of pork insulin ( $\bullet$ ), beef proinsulin ( $\blacksquare$ ) and pork proinsulin ( $\circ$ ) at various times. Incubation mixture is similar to that in Table I.

$\mu\text{g}$  of trypsin in the presence and absence of 1.6  $\mu\text{g}$  of KPTI or Trasylol by the method of Chance, Ellis, and Bromer (4). The product of proinsulin digestion was then identified by disc electrophoresis. As shown in Fig.

5, both inhibitors prevented conversion of proinsulin to insulin. In the absence of inhibitors, conversion of proinsulin to insulin was complete in 30 min.

*Additive effect of beef proinsulin, connecting peptide, and proinsulin intermediates.* Since proinsulin exhibited

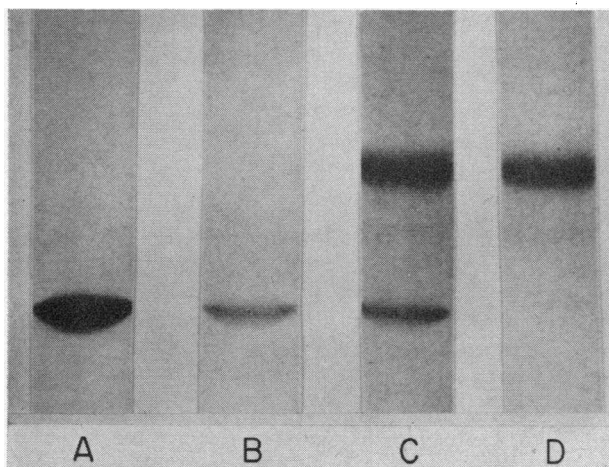


FIGURE 4 Polyacrylamide-gel electrophoretic patterns of 100  $\mu\text{g}$  of pork insulin (A); 3  $\mu\text{g}$  of porcine insulin (B); 100  $\mu\text{g}$  pork proinsulin plus 3  $\mu\text{g}$  of porcine insulin (C); and 100  $\mu\text{g}$  of pork proinsulin (D). The method of polyacrylamide-gel electrophoresis was identical with that of Steiner and Oyer (3).

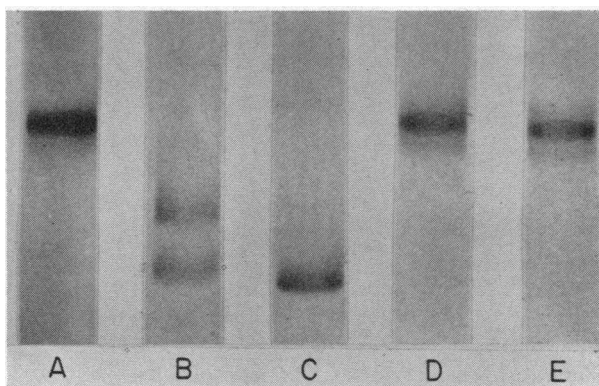


FIGURE 5 Polyacrylamide-gel electrophoretic pattern of 40  $\mu\text{g}$  of pork proinsulin (A); 40  $\mu\text{g}$  of pork proinsulin plus 0.4  $\mu\text{g}$  of trypsin after 20 sec of incubation (B); 40  $\mu\text{g}$  of pork proinsulin plus 0.4  $\mu\text{g}$  trypsin after 30 min of incubation (C); the latter mixtures plus 1.6  $\mu\text{g}$  of KPTI (D); and the latter mixtures plus 1.6  $\mu\text{g}$  of Trasylol (E). The method of incubation and condition of assay were similar to that of Chance et al. (4).

TABLE II  
*Effect of Beef Insulin, C-Peptide, Intermediate and Proinsulin on Glucose Oxidation and Lipogenesis in the Isolated Fat Cells of Rat\**

Preparation(s) and concentration				Amount of glucose- <sup>14</sup> C-carbon converted to	
Insulin 8.3 × 10 <sup>-10</sup> M	C-peptide 8.3 × 10 <sup>-10</sup> M	Intermediates 8.3 × 10 <sup>-10</sup> M	Proinsulin 4.75 × 10 <sup>-9</sup> M	CO <sub>2</sub>	Lipids
				<i>nanoatoms</i>	
+				253.3	495.5
	+			39.3	87.3
		+		223.1	490.2
			+	240.5	461.0
+	+			242.1	460.6
+		+		272.3	478.3
+			+	255.1	498.2
	+	+		224.0	400.1
	+		+	223.7	362.3
		+	+	261.4	473.0
+	+		+	267.1	483.9
+	+	+		263.8	487.0
+		+	+	260.3	492.3
4.7 × 10 <sup>-11</sup> M	1.41 × 10 <sup>-10</sup> M	1.41 × 10 <sup>-10</sup> M	4.5 × 10 <sup>-10</sup> M		
+				174.0	355.0
	+			38.8	80.2
		+		180.4	369.3
			+	177.7	363.5
+	+			165.3	325.0
+		+		213.9	423.4
+			+	226.0	428.8
	+	+		200.7	366.7
	+		+	164.0	309.1
		+	+	209.5	389.7

+ Signifies addition of the preparation to the incubation mixture. Base line values for CO<sub>2</sub> and lipids are 42.5 and 83.3 nanoatoms respectively.

\* The condition of the incubation mixture is identical with that of Table I. Each value is an average of three experiments.

small but significant biological activity on isolated fat cells, it was of interest to study the biological effect of beef proinsulin and its various breakdown products on insulin. For these studies, the effect of insulin, C-peptide, proinsulin, and proinsulin intermediates was tested separately or in combination with each other on the isolated fat cells at concentrations which produced maximum and intermediate responses. Results of these experiments (Table II) suggest that (a) connecting peptide has no influence on the insulin-like activity of the other compounds, (b) proinsulin and its intermediates show some combined effect on insulin when insulin is added at intermediate concentrations, and (c) insulin, proinsulin, and proinsulin intermediates at higher concentrations exert no inhibitory or stimulating effect on each other.

*Effect of insulin and proinsulin antisera on insulin-like activity.* Because of the similarity of the insulin and proinsulin molecules in their immunologic reac-

tivity, it was of interest to know the effect of pork proinsulin antiserum on the biological properties of various preparations. For this purpose, the studies summarized in Fig. 6 were conducted using pork and beef insulins and proinsulins and beef proinsulin intermediates in the presence of pork proinsulin antiserum or pork insulin antiserum. The antisera were used at a final dilution of 1:400. The concentrations of insulin, proinsulin, and proinsulin intermediates selected were such as to produce half their maximum activity. Under these experimental conditions, both antisera significantly inhibited the biological activity of all three types of preparations—insulin, proinsulin, and intermediates. Thus, suppression of biological activity at the concentration of antisera used was not specific.

*Antibody reactivity of connecting peptides, insulin, proinsulin, and intermediates.* Fig. 7 shows the results of radioimmunoassay of insulin, proinsulin, connecting

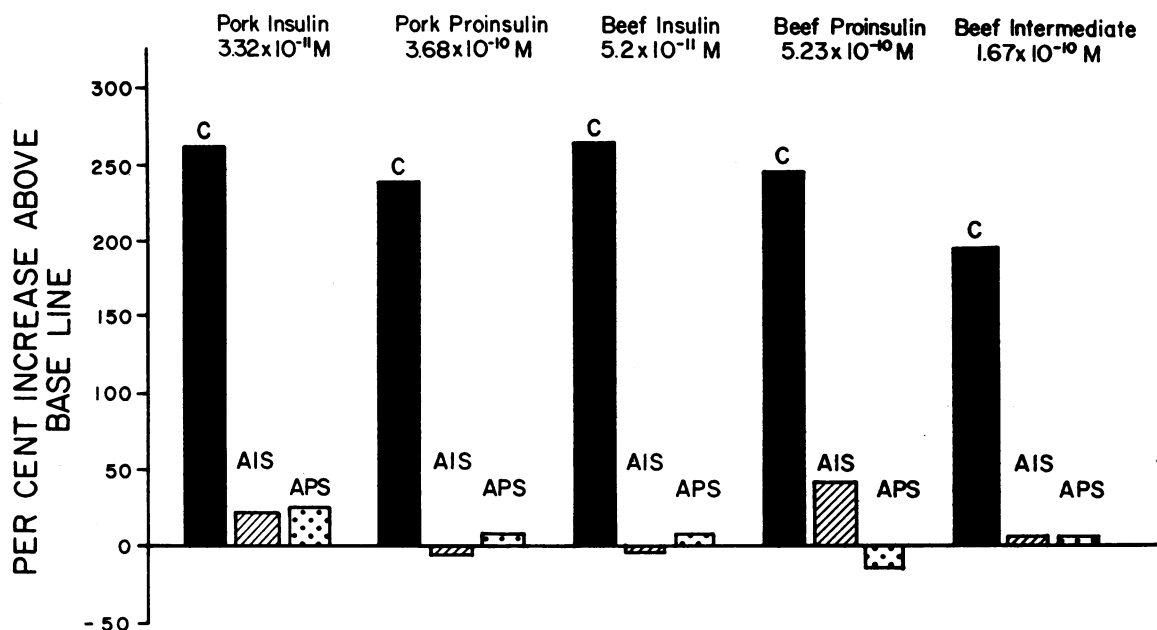


FIGURE 6 Effect of pork proinsulin and insulin antisera on the insulin-like activity of pork insulin and proinsulin and beef insulin, proinsulin, and intermediates. For the condition of assay see text. C = control; AIS = pork insulin antiserum; APS = pork proinsulin antiserum.

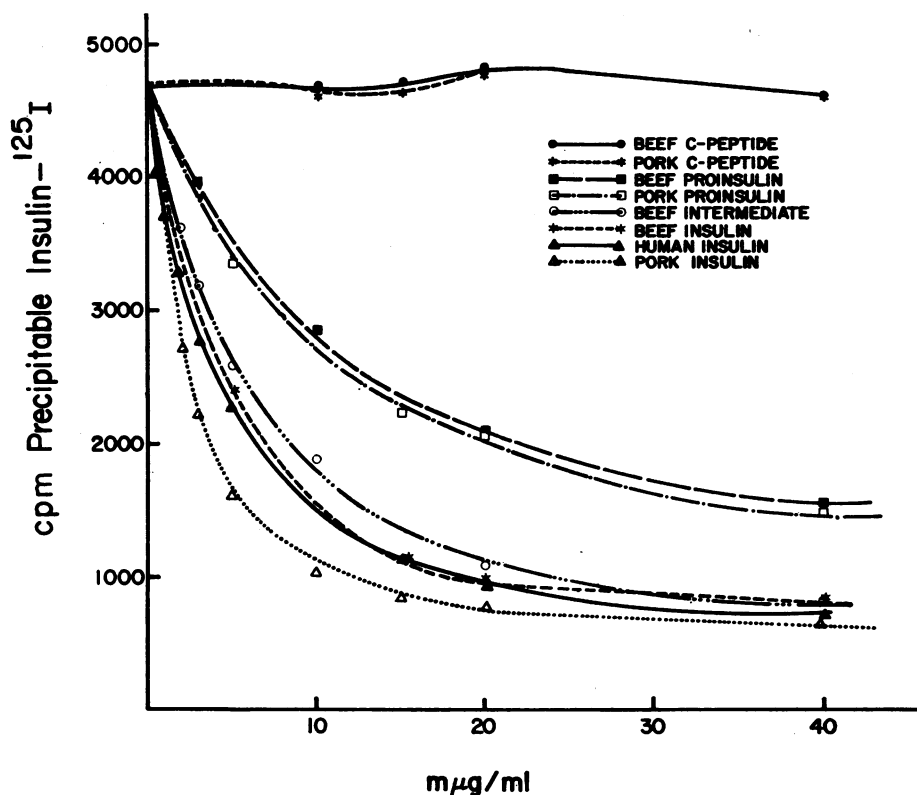


FIGURE 7 Immunoprecipitability of various preparations of insulin, proinsulin, and C-peptide and beef intermediates by the insulin double antibody immunoassay. The final dilution of insulin antiserum for 50% immunoprecipitability of labeled insulin is 1:300,000.

peptides, and beef proinsulin intermediates using pork insulin antiserum. Both pork and beef proinsulins exhibit a similar degree of reactivity, indicating that proinsulin reactivity with the insulin antibody is  $\frac{1}{4}$  to  $\frac{1}{3}$  that of insulin. The reactivity of beef proinsulin intermediates, on the other hand, appears approximately equal to that of insulin. Connecting peptides of beef and pork show no significant immunoreactivity.

## DISCUSSION

The biological effect of beef proinsulin on isolated fat cells has been demonstrated in other laboratories by Gliemann and Moody as quoted by Steiner (2). The amount of this substance required to produce an equivalent response is 20–100 times that of insulin (14). The amount of proinsulin required in the whole animal to produce an equivalent response is only 5–7 times that of insulin. Although fat and muscle tissues appear to contain a large amount of insulin-degrading enzymes (15), it is not known if these enzymes are able to convert proinsulin to insulin. Preliminary results in our laboratories<sup>2</sup> suggest that an apparent insulin-specific protease in rat muscle is not capable of attacking proinsulin or converting it to insulin. Furthermore, Steiner has reported that <sup>125</sup>I-labeled beef proinsulin was not apparently transformed into an insulin-like compound upon injection into rats (14). It is doubtful if isolated fat cells have any appreciable proinsulin-converting enzymes. Our studies which demonstrated the inability of trypsin inhibitors to block the effect of proinsulin activity support such a conclusion. With the addition of up to 200  $\mu$ g of KPTI and Trasylol per ml of incubation, we have not been able to block the insulin-like activity of proinsulin in the isolated fat cell system. The data from Table II on combining effects of proinsulin, C-peptide, and proinsulin intermediates on insulin preclude the possibility that any of the former compounds exert a significant antagonistic effect on insulin activity either in the intermediate or the maximal dose concentrations. Furthermore, additional experiments (data not shown) were performed in which insulin or proinsulin were first preincubated with the fat cells and then followed by the addition of the opposite material. The results were identical with those in Table II where insulin and proinsulin were added simultaneously. These studies also demonstrate lack of an insulin antagonism to proinsulin action as well as an apparent lack of preemptive binding by proinsulin or insulin to the fat cell receptor site.

Immunological studies of insulins and proinsulins from beef or pork suggest that insulin and proinsulin

possess common antigenic determinants. It is interesting that the connecting peptide showed very little immunoreactivity with insulin antiserum in view of the observation that it may interfere in the specific immunoassay designed for proinsulin where insulin antibodies have been removed (16, 17). The C-peptide which apparently is liberated in the circulation (7) under present experimental conditions has no demonstrable biologic properties.

Studies cited (2, 6) as well as the data from present studies suggests that relative to insulin the biologic effect of proinsulin is less on the fat cell than on the intact rat diaphragm. The concentration ratio of proinsulin and insulin required to produce 50% of the maximal response in isolated fat cells is at least 10, as shown by our data, whereas the ratio in muscle is approximately 4 (6). It should be noted, however, that approximately 80 and 20 times as much proinsulin and insulin, respectively, are required to produce the same effect in intact diaphragm (6) as in isolated fat cells. If proinsulin produces its insulin-like effects by combining with the same receptor site as does insulin, then it is evident that the presence of the connecting segment considerably weakens the binding of proinsulin in comparison with insulin. Whether this is merely due to the blockage of the respective terminal groups of the B and A chains or to other factors such as steric hindrance cannot be unequivocally decided from these data. The fact that the bovine intermediate fraction still retains the bulk of the connecting segment but exhibits much higher biologic activity than intact proinsulin suggests that the deblocking of at least one of the termini of the insulin chains may be an important factor for the increased activity.

The present studies demonstrate a definite disparity between immunoassayable and bioassayable properties of proinsulin. This discrepancy makes an accurate direct quantitative assessment of proinsulin in the circulation by the immunoreactive insulin (IRI) method difficult, especially in those instances (18) where proinsulin might constitute considerable portion of IRI in the circulation.

## ADDENDUM

Recent studies in our laboratory (19) on biological properties of beef and pork proinsulin and beef intermediates have extended our findings in regard to antilipolytic properties of these compounds on ACTH-induced lipolysis under isolated fat cells. The half-maximal response doses calculated for the above compounds give the following figures expressed as molar concentration for antilipolytic properties of pork insulin and proinsulin, as well as beef insulin, proinsulin, and intermediates, respectively:  $1.4 \times 10^{-11}$ ,  $1.3 \times 10^{-10}$ ,  $1.7 \times 10^{-11}$ ,  $2.7 \times 10^{-10}$ , and  $3.5 \times 10^{-11}$ . The antilipolytic effect of proinsulin or intermediates was not blocked by Trasylol or KPTI. The C peptides of beef or pork showed no anti-

<sup>2</sup> Brush, J. S., and A. E. Kitabchi. Unpublished observations.



lipolytic activity alone or combination with insulin, proinsulin, or intermediates.

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