Effect of Pancreatic Proteins on Insulin Assay Systems

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SUMMARY

The following proteins were analyzed for insulin-like behavior in the rat hemidiaphragm, rat fat pad, and two antibody immunoassay systems: ribonuclease A, deoxyribonuclease I, alpha-chymotrypsin, chymotrypsinogen A, trypsin, and trypsinogen. Chymotrypsin stimulated glucose uptake in the rat hemidiaphragm and behaved like insulin in the immunoassay system when the enzyme concentration was 7×10^{-6} M. No similar activity was found for any of the other proteins. Chymotrypsin was inactive at lower concentrations.

The stimulation of glucose uptake in the rat hemidiaphragm by chymotrypsin was not prevented by the presence of guinea pig anti-insulin serum. This and other evidence suggests that the enhanced uptake of sugar is not due to insulin contamination and is probably a result of partial proteolysis of cell membrane with resulting increase in permeability.

Mixtures of chymotrypsin with guinea pig anti-insulin serum and I-131-labeled insulin under the conditions of the immunoassay were analyzed electrophoretically, and the results indicate partial hydrolysis of the labeled insulin. This hydrolysis probably accounts for the apparent insulin response produced by chymotrypsin in the two antibody immunoassay system. DIABETES *15*:342-45, May, 1966.

Reiser and Reiser[5] have reported that several pancreatic proteins have an insulin-like effect on the intact rat diaphragm. Trypsin was found to be as effective as insulin in stimulating glycogen deposition. Chymotrypsin was more effective than insulin in stimulating the intracellular accumulation of d-xylose.

These observations have important bearing on the detailed mechanism of insulin action in muscle. However, there are three questions which arise with respect to these observations: (1) Could the observed insulin-like activity be due to contamination of the enzymes with insulin? (2) Do the enzymes possess insulin-like activity at lower, more physiological concentrations? and (3) Do the enzymes possess insulin-like activity in tissues other than muscle? The present work was initiated to answer these questions.

MATERIALS AND METHODS

Materials. The proteins studied were obtained from Worthington Biochemical Corporation and had the following designations: Deoxyribonuclease 1, 1 × cryst, pancreatic; ribonuclease A, lyophilized, phosphate free; alphachymotrypsin, 3 × cryst, lyophilized; chymotrypsinogen A, 3 × cryst; trypsin, 3 × cryst; trypsinogen, 1 × cryst. These were used as received. Other solvents and chemicals were the best commercial grades available and were used without further purification. Insulin standards were prepared from Crystalline Insulin generously supplied by Dr. Mary Root and Dr. W. R. Kirtley of the Eli Lilly Company.

Biological assays. The method of Shaw and Shuey[7] was modified by incubating hemidiaphragms with radioactive glucose. The tissue was removed, blotted, weighed, and then digested in KOH. The digest was counted in the scintillation counter and activity was determined by subtracting the counts per minute for hemidiaphragm in buffer alone from the counts for a hemidiaphragm in the test solution. The hemidiaphragms were paired; that is, the base line hemidiaphragm and the test hemidiaphragm for a given solution were taken from the same animal. For hemidiaphragms which differed only 10 to 15 per cent in weight, no difference in activity was found whether the results were expressed as counts per minute per mg. of wet or dry hemidiaphragm or as counts per minute per hemidiaphragm. Adipose tissue activity was determined by the rat fat pad method of Renold et al.[6]

Immunoassay. The methods of Morgan and Lazarow[4] were used.

Preparation of guinea pig anti-insulin sera (GPAIS). Guinea pigs were injected subcutaneously with five to ten units of regular pork insulin in 0.5 ml. of Freund's Adjuvant initially at biweekly intervals for two months and monthly thereafter. The animals were bled every four months by cardiac puncture and antibody titer determined by the method of Morgan and Lazarow.[4]

Electrophoresis. A Savant flat plate system cooled with tap water was used. Barbital buffer of ionic strength 0.075, *p*H 8.6, and Whatman #3 paper were employed under potential gradients of 35 to 40 volts/cm. Runs were made for fifteen minutes, and current density was usually 0.12 to 0.14 MA per cm.[2]

Preparation of assay solutions. The following molecular weights were assumed: deoxyribonuclease, 63,000; ribonuclease, 13,700; chymotrypsin and chymotrypsinogen, 25,000; trypsin and trypsinogen, 24,000. Depending on the experiment, 5 to 30 milligrams of protein were dissolved in 1 ml. of 0.01 M HCl on the day of assay and then made up to 10 ml. with the buffer for the given assay. Desired final concentrations were obtained by the serial dilution of the stock solution with the assay buffer.

RESULTS

Diaphragm activities. Stimulation of glucose uptake in the hemidiaphragm was studied with protein concentrations ranging from 7×10^{-9} to 7×10^{-6} molar. The lowest concentration represents the molar equivalent of 1 milliunit of insulin per milliliter. The highest concentration is twice that at which Reiser and Reiser's work[5] was done. Concentrations intermediate between these extremes were also studied. Activity was found only at high concentration. Results are presented in table 1. Only chymotrypsin showed activity which could be regarded as statistically significant.

Fat pad activities. The ability of proteins listed in table 1 to stimulate C-14-O_2-release from labeled glucose was studied at intervals of a power of 10 over the concentration range 7×10^{-10} to 7×10^{-5}. No activity was found at any concentration for any of the proteins assayed.

Interaction with insulin. Insulin (500 *u*U./ml.) was added to assay solutions of the proteins at various protein concentrations. The mixtures were allowed to stand for one hour at room temperatures, then assayed for activity in the fat pad and the hemidiaphragm. Results are presented in table 2.

TABLE 1
The effect of pancreatic proteins on hemidiaphragm glucose uptake

Solution	Number of observations	Average* per cent above baseline	Ninety-five per cent confidence interval
Insulin standards			
500 *u*U./ml.	11	64.5	±14.7
50 *u*U./ml.	11	21.8	± 9.3
Enzymes (7×10^{-6}M)			
Alpha-chymotrypsin	9	50.1	±32.2
Chymotrypsinogen A	4	9.8	±16.5
Trypsin	4	16.1	±24.4
Trypsinogen	4	5.8	± 9.4
Ribonuclease A	4	7.5	±18.1
Deoxyribonuclease I	3	21.7	±44.3

*Per cent above base line = (Glucose uptake of hemidiaphragm in test solution minus glucose uptake of hemidiaphragm in buffer/glucose uptake of hemidiaphragm in buffer) × 100.

TABLE 2
The effect of pancreatic enzymes on the biological activity of 500 microunits/ml. of crystalline bovine insulin in rat fat pad and rat hemidiaphragm

Protein	Concentration (micromoles/liter) 7 F.P.	7 D.	0.7 F.P.	0.7 D	0.07 F.P.	0.07 D.	0.007 F.P.	0.007 D.
Ribonuclease A	+	+	+	+	+	+	+	+
Deoxyribonuclease	+		+		+		+	
Alpha chymotrypsin	0	+	0	0	0	0	+	+
Chymotrypsinogen A	0	0	+	+	+	+	+	+
Trypsin	0	0	+	0	+	+	+	+
Trypsinogen	+	0	+	0	+	+	+	+

F.P. = Fat pad results (conversion of C-14-labeled glucose to C-14-O_2.
D = Hemidiaphragm results (net uptake of C-14 glucose).
+ = Activity comparable to that of 500 *u*U./ml. insulin standard.
0 = Activity significantly below that of insulin standard. Deoxyribonuclease was not assayed for its effect on insulin hemidiaphragm activity.

Immunoassay. All the proteins listed in table 1 were assayed at a concentration of 7×10^{-6} M. Only chymotrypsin showed significant insulin-like activity. The average activity for six assays was equivalent to 42.5 *u*U. per ml. of insulin (95 per cent confidence limits 36.6 to 48.4 *u*U./ml.).

Assays were also done in the presence of 50 *u*U./ml. of added insulin and enzyme concentration of 7×10^{-6} M. Only in the case of chymotrypsin was the assay value for the mixed solution different from the assay value of the insulin alone. For six determinations, the chymotrypsin-insulin mixture gave an assay value of 73.6 *u*U./ml. (95 per cent confidence limits 67.1 to 80.0).

Electrophoresis. The enzyme solutions were mixed with I-131 insulin and guinea pig anti-insulin serum (GPAIS) under the usual immunoassay conditions (except that no rabbit anti-GPAIS was added), and after incubation, the mixtures were analyzed electrophoretically. Protein concentrations were 7×10^{-6} M. Papers from the electrophoresis were scanned in the strip counter. Results for chymotrypsin are shown in figure 1. In the presence of GPAIS, all of the mixtures showed an electrophoretic pattern similar to that of insulin alone plus GPAIS. There was a slow-moving band near the origin containing the bulk of the labeled insulin and also a band with anode mobility of 0.007 to 0.012 cm.^{2}/volt-min. containing a much smaller fraction. This fast-

moving band was larger for the chymotrypsin-insulin mixture than for any of the other protein-insulin mixtures analyzed.

Effect of GPAIS on chymotrypsin diaphragm activity. Glucose uptake of chymotrypsin was determined in the presence of GPAIS. Results are shown in table 3.

DISCUSSION

Diaphragm activity. Chymotrypsin was the only protein studied which showed stimulation of glucose uptake in the hemidiaphragm. In our hands, the lower limit of sensitivity for the isolated hemidiaphragm is near 50

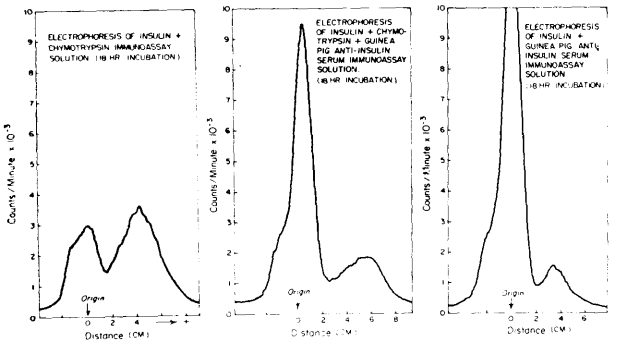

FIG. 1. Ten microliters of solution containing insulin plus enzyme (7 X 10-6 M.), insulin plus enzyme plus GPAIS, and insulin plus GPAIS were applied on a line to 3 X 30 cm. strips on Whatman #3 paper which had been presoaked with barbital buffer (ionic strength 0.075, pH 8.6). Ten-minute runs were made at potential gradients of 35 to 40 volts per cm. and current density of 0.12 to 0.14 milliamps/cm².

*u*U./ml. insulin. The activities listed in table 1 can be regarded as significant only for chymotrypsin. From the diaphragm data alone, it is not possible to determine whether the chymotrypsin effect is due to specific insulin-like action or to a nonspecific increase in membrane permeability due to proteolysis.

However, the work of Hines, Dailey, Wood, and Roe[1] indicates that the results are probably due to proteolysis. These authors found that the uptake of D-xylose by the intact rat diaphragm was enhanced by high concentrations of chymotrypsin and trypsin. At these same enzyme concentrations, they observed complete lysis of fibrin clots. They concluded that the effect on the diaphragm was due to membrane proteolysis. It seems reasonable to attribute the hemidiaphragm effect of chymotrypsin to the same phenomenon. Failure to observe a trypsin effect on the hemidiaphragm comparable to its effect on the intact diaphragm may be due to the lower sensitivity of the hemidiaphragm.

Can the presence of chymotrypsin in fluids derived from biological systems account for any of the reported "insulin" effects of these fluids on diaphragm preparations? A clear answer is available only for normal human serum. Kallos, Arthur, Rizok, and Kahn[2] found that for fifty normal individuals, who were without evidence of pancreatic disease, the amount of serum chymotryptic activity was equivalent to less than 0.1 *u*g. of crystalline chymotrypsin per milliliter of serum. The minimum amount of chymotrypsin required to produce a response in the hemidiaphragm was found in the present work to be greater than 17 *u*g./ml. Thus the amount of chymotrypsin required to produce an enhanced uptake of sugar by the hemidiaphragm is 100-fold greater than the amount present in normal human serum.

Fat pad activity. The lack of response of adipose tissue to any of these proteins might be attributed, in the case of the proteinases and their precursors, to proteolytic damage of the fat cells with impairment of function or cell death. However, even at concentrations as high as 7 × 10⁻⁶ M., neither trypsin nor chymotrypsin substantially reduced the evolution of C-14-O_2 from fat tissue with which they were incubated.

Effect on insulin activity. The nucleases had no effect on insulin activity in either adipose or muscle tissue. High concentrations of trypsin and chymotrypsin reduced insulin activity in both tissues. This interference is probably due to enzyme-catalyzed hydrolysis of the insulin. The loss of insulin activity in the presence of trypsinogen and chymotrypsinogen is probably due to generation of active enzymes from the precursors during the assay.

The response of the two tissues to insulin in the

TABLE 3

The effect of guinea pig anti-insulin serum (GPAIS) on the rat hemidiaphragm glucose uptake stimulated by insulin and by chymotrypsin*

Insulin 100 *u*U./ml.	GPAIS	Chymotrypsin concentration 7 × 10⁻⁶ M	Chymotrypsin concentration 0.7 × 10⁻⁶ M	Number of observations	Per cent uptake above baseline†
X				6	63.4±23.1
	X			12	7.0±12.7
X	X			4	0.0± 0.0
			X	4	4.2± 6.4
		X		6	80.7±29.0
	X	X		4	72.9±47.4

*Substance added is indicated by X in the appropriate column.

†Results are expressed as [(glucose uptake of test solution minus glucose uptake of buffer alone) /glucose uptake of buffer alone] × 100 ± 95 per cent confidence limits.

presence of chymotrypsin and trypsin is similar except for the reappearance of "insulin-like" hemidiaphragm response at high concentrations of chymotrypsin. The hemidiaphragm response to insulin seems to be more sensitive to added trypsin than the fat pad response.

The blocking of diaphragm response to added insulin by chymotrypsin at an enzyme concentration of 7×10^{-7} M makes it unlikely that the diaphragm response to a tenfold increase in chymotrypsin alone is due to insulin contamination of the chymotrypsin. That is, if chymotrypsin can destroy 500 *u*U./ml. of added insulin when the enzyme concentration is 7×10^{-7} M, surely a small amount of contaminating insulin would not survive an enzyme concentration tenfold greater.

Immunoassay. Only chymotrypsin gave a significant response in the immunoassay. This response could be due to the following factors: (1) insulin contamination, (2) cross-reaction of insulin antibodies with chymotrypsin, (3) chymotrypsin interference with the insulin I-131-GPAIS reaction. Insulin contamination seems unlikely in view of the lack of adipose tissue response to the chymotrypsin preparation. A definite conclusion about the possibility of cross-reaction of chymotrypsin with GPAIS cannot be reached on the basis of the evidence presented here. However, it is interesting to note that Yagi, Maier, and Pressman[8] found that insulin B-chain antibodies cross-react. Since insulin is similar to chymotrypsin in several ways (both molecules have their histidine residues located near an amino terminal end, both have a terminal asparagine carboxyl residue, and chymotrypsin has an ileu-val-gly-NH_2 sequence which compares with the insulin A-chain val-ileu-gly-NH_2), a cross-reaction of insulin antibodies and chymotrypsin might occur.

However, the third possibility seems most likely. Should chymotrypsin interfere in any way with the GPAIS-insulin I-131 reaction either by damage to the antibody reactive site or by cleaving insulin I-131 into nonreactive, labeled peptides, the result would be an apparent decrease in the "per cent bound" of I-131 insulin and a spurious assay for insulin. This possibility must always be considered in interpreting the results of the insulin immunoassay. The presence of proteolytic enzymes in tumors[3] might explain the immunoassayable "insulin" in these tumors.

Electrophoretic analysis. Figure 1 shows that chymotrypsin does alter the electrophoretic pattern of I-131 insulin and that this effect is suppressed but not prevented by GPAIS. A large, fast-moving peak is present in the chymotrypsin GPAIS-I-131 insulin solution, suggesting partial cleavage of labeled insulin into smaller labeled peptides during the immunoassay.

The effect of GPAIS on chymotrypsin diaphragm activity. The data in table 3 show that the GPAIS has no direct effect on rat diaphragm and that it has potent insulin-neutralizing capacity. However, the chymotrypsin hemidiaphragm activity is not neutralized by added GPAIS. This is further evidence that the diaphragm activity of chymotrypsin is not due to insulin contamination.

CONCLUSIONS

1. High concentration of chymotrypsin stimulates net glucose uptake in the rat hemidiaphragm, but ribonuclease, deoxyribonuclease, trypsin, trypsinogen, and chymotrypsinogen do not. Since the chymotrypsin activity is observed only at enzyme concentrations in excess of 7×10^{-7} M, such activity probably has little biological significance.

2. Over the concentration range 7×10^{-5} to 7×10^{-10} M, no stimulation of C-14-O_2 release by fat pad is demonstrable for ribonuclease, deoxyribonuclease, chymotrypsin, chymotrypsinogen, trypsin, or trypsinogen.

3. Chymotrypsin gives a definite response in the immunoassay system. Since it is inactive in rat adipose tissue, and since its hemidiaphragm activity is not suppressed by GPAIS, the immunoassay response is not due to contaminating insulin and is probably the result of interference by chymotrypsin with the insulin I-131-GPAIS reaction.

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