Interaction of Pancreas Extracts with Bovine Insulin

2. REACTION IN A BIOLOGICAL SYSTEM

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Crude pancreatic extracts described as trypsin (Dudley, 1923; Shonle & Waldo, 1923; Witzemann & Livshis, 1923) were found to interfere with the biological activity of insulin. Epstein & Rosenthal (1924) showed that, whereas a mixture of pancreatic extracts and insulin was biologically inactive when injected into animals immediately after mixing, little or no proteolytic destruction of the insulin by the pancreatic protein could have occurred. Later workers, using purified enzymes, have shown that trypsin acts slowly on insulin, breaking off a heptapeptide and alanine, and that the residuum possesses little or no biological activity (Nicol, 1960; Carpenter & Baum, 1962). Young & Carpenter (1961) found that the biological inactivation of insulin is complete when as little as 20% of the insulin has been split by trypsin, and they also noted the formation of a precipitate when trypsin and insulin were mixed at a neutral pH and suggested, like earlier workers (Scott, 1925), that an enzyme-substrate complex had been formed.

Chymotrypsin, the other major pancreatic enzyme, attacks insulin more rapidly than does trypsin, and does so at several different points (Butler, Dodds, Phillips & Stephen, 1948, 1949). Butler *et al.* (1948, 1949) have also suggested that the biological activity of insulin is not lost under the action of chymotrypsin until 50% of the insulin is in the form of non-protein nitrogen. Young (1962) mentioned that an extract of pancreas of alloxan-diabetic rats injected into rabbits could result in hyperglycaemia. He considered that in the conditions used it was unlikely that these effects were due to glucagon.

There is thus evidence that there are present in the pancreas substances that can both degrade insulin and impair its biological activity. There is also a suggestion that biological inhibition may be more extensive than the degree of chemical degradation, possibly as a result of the formation of an inactive enzyme-substrate complex.

Antoniades, Renold, Dagenais & Steinke (1960) extracted pancreas with a cation-exchange resin and showed that a proportion of the insulin-like

activity of the pancreas became attached to the resin. Later work by Antoniades (1961) on blood suggests that the reason for the linking of insulin on to a cation-exchange resin is that insulin forms complexes in blood and also in tissue with a basic protein. The complex with basic protein does not show insulin activity on muscle unless an adipose-tissue extract is present.

A method of extraction and fractionation of bovine pancreas has been detailed by Czerkowski & Bingle (1964). In the prepared extract was present an active pancreatic protein (APP) capable of interacting with and degrading bovine insulin.

The present paper is concerned with the effect of APP on the biological activity of bovine insulin, as shown by the rat-diaphragm assay of insulin. Investigations into the mechanism of this interaction, utilizing the solubility of ¹³¹I-labelled insulin and working in the concentration range normally used in the rat-diaphragm assay, are also reported. Finally, a comparison of the action of APP on bovine insulin is made with that of APP on porcine insulin.

MATERIALS AND METHODS

Materials. All chemicals used were of AnalaR grade. Incubations were carried out in Krebs-Ringer bicarbonate buffer solution (Krebs & Henseleit, 1932). The buffer solution was prepared at the beginning of each experiment and gassed for 1 hr. with O₂ plus CO₂ (95:5).

Recrystallized bovine insulin (22.2 i.u./mg.) was obtained from Boots Pure Drug Co., Nottingham, and porcine insulin (24.0 i.u./mg.) from Novo Pharmaceuticals, Denmark. The comparative potencies of the two insulins stated by the manufacturers were checked by rat-diaphragm assays. The insulins were made up in stock solutions of 20 i.u./ml. in 3.3 M-hydrochloric acid and stored at -20°. Adsorption of diluted solutions of insulin on glass was prevented by the addition of bovine serum albumin (0.5 mg./ml.) to all solutions containing insulin.

Pancreatic extracts and their fractions were prepared according to the method of Czerkowski & Bingle (1964) and kept in a solid state at -20° until required. Glassware used in the diaphragm assay was kept separate, and before use cleaned with conc. sulphuric acid followed by numerous washings in tap water and then distilled water. Male Wistar-strain rats weighing 100-140 g. were used in the assay. They were kept in a constant-temperature room for at least 24 hr. before used and starved for 18-24 hr.

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Table 1. *Design of experiment to determine the effect of active pancreatic protein on insulin, by using the rat-diaphragm assay to measure impairment of biological activity of insulin*

The incubations were for 90 min. at 37° in 2.0 ml. of Krebs-Ringer bicarbonate buffer, with the additions indicated.

Diaphragm nos.	Preincubation	Additions to incubation medium
1-6	None	None (basal medium)
7-12	None	2×10^{-3} i.u. of bovine insulin/ml.
13-18	None	4 μ g. of bovine APP fraction/ml.
19-24	Insulin + APP incubated at 37° for 15 min. before addition of diaphragm	2×10^{-3} i.u. of bovine insulin/ml. + 4 μ g. of bovine APP fraction/ml.
25-30	Insulin + APP incubated at 37° for 15 min. before addition of diaphragm	2×10^{-3} i.u. of bovine insulin/ml. + 4 μ g. of bovine APP fraction/ml. (heated at 100° at pH 7 for 5 min.)

[131 I]Iodide of high specific activity was obtained from The Radiochemical Centre, Amersham, Bucks. Insulin was iodinated according to the method of Hales & Randle (1963*a, b*) based on iodination at pH 1 (Springell, 1961) with iodine monochloride (McFarlane, 1958). The specific activity of the 131 I-labelled insulin was 14–29 μ C/ μ g. as measured in an ionization chamber. The 131 I-labelled insulin was added in proportions of 1:10 to 1:50 to the unlabelled insulin used.

Soya-bean trypsin inhibitor was supplied by L. Light and Co., Colnbrook, Bucks. DFP* was obtained from Boots Pure Drug Co. as a 0.02M solution in propylene glycol.

Assay of insulin and insulin antagonists. The rat-diaphragm assay, which is almost specific for insulin (Randle, 1957), has been used by several workers for the detection of insulin antagonists (Vallance-Owen & Lukens, 1957; Field, Tietze & Stetten, 1957; Randle, Taylor & Vargas, 1960).

In the present investigation, the rat-diaphragm assay was carried out according to the methods of Randle (1954) and Vallance-Owen & Hurlock (1954). The activity of 2×10^{-3} i.u. (0.1 μ g.) of bovine insulin/ml. in buffer solution was compared with the activity of a similar concentration of insulin after incubation with the pancreatic extracts and fractions described by Czerkawski & Bingle (1964). In other experiments the actions of the APP fraction on similar concentrations of bovine and porcine insulins were compared.

Care was taken to ensure that any comparison between groups of flasks was made under similar conditions in the same temporal experiment, apart from the one variable that was being studied. The proportions of pancreatic protein to insulin used in these experiments were approximately that present in pancreas. Allowance was made for the difference in uptake between right and left hemidiaphragms (Bingle, 1963).

A typical experiment with 30 hemidiaphragms is shown in Table 1. The glucose concentration in the incubation fluid was measured before and after incubation, by using a Technicon autoanalyser and the ferricyanide-reduction method of Hoffman (1937). Groups of diaphragms were compared by using Student's *t* test, *P* < 0.05 being accepted as significant with regard to the presence or inhibition of an insulin effect.

* The abbreviation used is: DFP, di-isopropyl phosphorofluoridate.

The glucose uptakes, calculated as means $\pm$ S.E.M., were expressed as mg. of glucose/ml. of incubation solution/g. dry wt. of diaphragm/hr. This unit of glucose uptake is used throughout the present paper.

The basal glucose uptake of the diaphragm was not affected by APP. In one such experiment the basal glucose uptake was 5.04 ± 0.43 and the glucose uptake in the presence of 4 μ g. of the APP fraction/ml. was 5.56 ± 0.36 , the difference not being significant (*P* > 0.5).

Degradation of insulin. The action of proteolytic enzymes on insulin has been measured on a macro-scale by using the solubility of insulin in 8% (w/v) trichloroacetic acid as a measure of the average molecular size of the products of proteolysis (Hiller & Van Slyke, 1922). It is realized that absolute values of the degree of solubility of a substance in 8% trichloroacetic acid are dependent on other factors apart from molecular size (Linderström-Lang, 1949), and the method was used for comparison only.

When 131 I-labelled insulin is used as a tracer, the solubility-in-trichloroacetic acid method can be applied to concentrations of insulin normally used in the rat-diaphragm assay (Tomizawa, Nutley, Narahara & Williams, 1955).

In all experiments the effect of the pancreatic protein on bovine insulin was compared with controls in which water was substituted for the pancreatic protein. 131 I-labelled insulin in the concentration necessary for the experiment was made up in a 0.066M-phosphate buffer solution, pH 7.4, or with the Krebs-Ringer buffer solution used in the rat-diaphragm assay. No difference in results could be attributed to the type of buffer used. The pancreatic protein fractions were made up in water to the required concentration.

Incubation was carried out in centrifuge tubes treated with a silicone solution to prevent surface adsorption of small quantities of protein. Agitation in a Dubnoff shaker at 60 cyc./min. was continued throughout the incubation at 37°. The reaction was terminated by the addition of 2% (w/v) bovine serum albumin followed 30 sec. later by 20% (w/v) trichloroacetic acid. At this stage each incubation tube contained 1.0 ml. of 131 I-labelled insulin solution, 1.0 ml. of a solution of pancreatic protein or water, 1.0 ml. of 2% bovine serum albumin and 2.0 ml. of 20% trichloroacetic acid. The incubation tubes were centrifuged at 5000 rev./min. (2500*g*) for 20 min. after being allowed to stand for 30 min. The supernatant (insulin soluble in 8% trichloroacetic acid) was then decanted and the precipitate

(insulin insoluble in 8% trichloroacetic acid) washed with 8% (w/v) trichloroacetic acid, the washings being discarded. The precipitate was then dissolved over 12 hr. in 5 ml. of aq. 30% (w/v) potassium hydroxide.

Portions (4 ml.) of the supernatant and of the dissolved precipitate were compared with similar portions from the control incubations. The radioactivity was measured in a well-type thallium-activated sodium iodide-scintillation counter. At least 10 000 counts were collected for each sample and correction was made for background radioactivity.

The radioactivity of the insulin soluble in 8% trichloroacetic acid was expressed as a percentage of the insulin initially insoluble in 8% trichloroacetic acid.

Preparation of inhibitors of proteolytic enzymes. There are a number of naturally occurring and synthetic substances that can inhibit proteolytic enzyme activity. This reaction is useful in confirming the presence of a proteolytic enzyme. In this investigation two inhibitors were used: the naturally occurring soya-bean trypsin inhibitor (Laskowski & Laskowski, 1954), and the synthetic and very potent DFP (Balls & Jansen, 1952).

The soya-bean trypsin inhibitor was mixed with the pancreatic protein before incubation with insulin. DFP is poisonous to numerous enzyme systems but the stable compound with proteolytic enzymes is not. DFP reacts with serine that is part of a definite sequence of amino acids in certain proteolytic enzymes (Hartley, Naughton & Sanger, 1959). The DFP compound with the pancreatic protein fraction used was prepared according to the method of Hartley *et al.* (1959) for elastase by using a 0.02M solution of DFP in propylene glycol. Free DFP was removed from the DFP compound with the enzyme by dialysis, and this compound was freeze-dried and stored at -20° until required.

RESULTS

In the first group of experiments, the crude pancreatic extract (AES₁ fraction) and the APP fraction obtained by separation on a CM-cellulose column (Czerkawski & Bingle, 1964) were tested for their activity against bovine insulin by using the rat-diaphragm assay. The pancreatic protein extract or its APP fraction was incubated with bovine insulin for 15 min. at 37° before the addition of the rat hemidiaphragm. The results are shown in Table 2.

These results show that the crude pancreatic extract and the fractions of it (c-f) eluted from a CM-cellulose column with 0.1-0.2M-sodium chloride (APP fraction) were capable of inhibiting the insulin-stimulated uptake of glucose by the rat hemidiaphragm. This active fraction (Czerkawski & Bingle, 1964) has been shown to consist of three protein bands on electrophoresis. Initial experiments suggest that the insulin-antagonistic activity is associated with the major component (APP). Separate experiments have shown that the APP fraction was inactivated with regard to its inhibitory action against the insulin-stimulated uptake of glucose by the diaphragm by previously

Table 2. Action of crude pancreatic protein extract and fractions obtained from a carboxymethylcellulose column on bovine insulin

Preparation or fraction	Eluted from CM-cellulose column with	Concn. of pancreatic protein (μg./ml.)	Uptake of glucose (mg./g. dry wt. of diaphragm/ml./60 min.)				Significance of inhibition (P)
			In basal medium	In basal medium + 2 × 10 ⁻³ i.u. of bovine insulin/ml.	In basal medium + 2 × 10 ⁻³ i.u. of bovine insulin/ml. + pancreatic protein	Inhibition (%)	
Crude pancreatic protein	—	50	3.52 ± 0.28	6.91 ± 0.45	5.77 ± 0.31	33	< 0.05
Crude pancreatic protein	—	100	5.94 ± 0.55	5.94 ± 0.55	3.52 ± 0.31	73	< 0.01
Fraction (a)	Water	8	4.26 ± 0.30	6.84 ± 0.53	7.84 ± 0.57	0	—
Fraction (b)	0.04M-NaCl	4	5.55 ± 0.38	7.11 ± 0.37	7.44 ± 0.57	0	—
Fraction (c)	0.1M-NaCl	8	4.26 ± 0.30	6.84 ± 0.53	4.70 ± 0.27	83	< 0.01
Fraction (d)	0.2M-NaCl	8	3.80 ± 0.23	7.04 ± 0.33	4.13 ± 0.43	73	< 0.001
Fraction (e)	0.2M-NaCl	4	5.06 ± 0.69	7.71 ± 0.37	5.23 ± 0.40	94	< 0.001
Fraction (f)	0.2M-NaCl	8	4.17 ± 0.53	7.74 ± 0.67	4.03 ± 0.50	100	< 0.001

The pancreatic protein and insulin were preincubated for 15 min. at 37° and the inhibition was measured by using the rat-diaphragm assay. Experimental details are given in the text. Fractions (d), (e) and (f) were obtained from three different preparations of crude pancreatic protein. The results for glucose uptake are given as means ± s.e.m.

Table 3. *Effect of heat at pH 2 and at pH 7 on the inhibitory activity of active pancreatic protein against bovine insulin*

Experimental details are given in the text. The results for glucose uptake are given as means $\pm$ s.e.m. N.S., Not significant.

Concn. of APP fraction ($\mu\text{g./ml.}$)	Procedure	Uptake of glucose (mg./g. dry wt. of diaphragm/ml./60 min.)			Significance of inhibition (<i>P</i>)
		In basal medium	In basal medium + 2×10^{-3} i.u. of bovine insulin/ml.	In basal medium + 2×10^{-3} i.u. of bovine insulin/ml. + bovine APP fraction	
8	Heated at pH 7	4.10 $\pm$ 0.63	7.00 $\pm$ 0.53	7.27 $\pm$ 0.70	N.S.
8	Heated at pH 7	4.50 $\pm$ 0.73	6.52 $\pm$ 0.57	7.24 $\pm$ 0.47	N.S.
8	Heated at pH 2	3.27 $\pm$ 0.17	6.30 $\pm$ 0.37	3.74 $\pm$ 0.33	< 0.001

heating it at 100° for 5 min. at a pH of 7. No inactivation occurred if the APP fraction was heated at pH 2 (Table 3).

Antoniades *et al.* (1960) suggested that the basic protein that combined with insulin rendered it biologically inactive on the rat diaphragm unless a rat-adipose-tissue extract was present. It was therefore decided to investigate the activity of such extracts on the reaction between the pancreatic protein fraction (APP) capable of inhibiting the action of bovine insulin on the rat diaphragm. The glucose uptake in the presence of 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. was 5.87 ± 0.30 , with 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. plus 4 $\mu\text{g.}$ of the APP fraction/ml. it was 4.06 ± 0.30 , and with 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. plus 4 $\mu\text{g.}$ of the APP fraction (preincubated with rat-adipose-tissue extract)/ml. it was 4.30 ± 0.27 . These results show that APP causes significant inhibition of insulin ($P < 0.01$) in both the presence and absence of adipose-tissue extract. The latter does not affect the degree of inhibition.

Mechanism of the inhibition of bovine insulin by the active pancreatic protein fraction

The active fraction APP, which inhibits the insulin-stimulated uptake of glucose by the rat diaphragm, could do so either by combining with insulin or the diaphragm and preventing the action of the former, or by degrading insulin. If APP combined with insulin and did not alter its molecule, the reaction would presumably take place rapidly and APP would not affect the solubility of insulin in 8% trichloroacetic acid. If, however, APP was a proteolytic enzyme it would take time to degrade and inactivate insulin, it would increase the solubility of insulin in 8% trichloroacetic acid and its action might be prevented by inhibitors of proteolytic enzymes. These possibilities were investigated.

Effect of preincubation of active pancreatic protein with insulin. It has been shown above that incubation of the APP fraction with insulin for 15 min. at 37° before the addition of the rat hemidiaphragm

results in significant inhibition of the insulin-stimulated uptake of glucose by the diaphragm. This procedure has been compared with the inhibitory effect produced by mixing the APP fraction and insulin immediately before the addition of the diaphragm. The glucose uptake of the diaphragm with 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. was 6.64 ± 0.33 , with 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. preincubated with 4 $\mu\text{g.}$ of the APP fraction/ml. for 15 min. at 37° it was 4.70 ± 0.53 , and with 2×10^{-3} i.u. (0.1 $\mu\text{g.}$) of bovine insulin/ml. plus 4 $\mu\text{g.}$ of the APP fraction/ml. and no preincubation it was 6.40 ± 0.37 . These results show a significant inhibition ($P < 0.01$) when the APP fraction is preincubated with insulin, but no significant inhibition when there is no preincubation period. The difference in glucose uptake with and without preincubation is also significant ($P < 0.05$). A short period of incubation of the APP fraction with insulin is required to produce inhibition.

Effect of active pancreatic protein on the solubility of bovine insulin in trichloroacetic acid. In this experiment 8 $\mu\text{g.}$ of the APP fraction/ml. was incubated with 4×10^{-3} i.u. (0.2 $\mu\text{g.}$) of bovine insulin/ml. for various times. The solubility of insulin in 8% trichloroacetic acid was determined for each incubation period by using ^{125}I -labelled insulin as a tracer. Fig. 1 shows the variation of the solubility of insulin in 8% trichloroacetic acid with time when incubated with the APP fraction. The results suggest that this protein is capable of degrading insulin, presumably by breaking the molecule into smaller peptides or amino acids.

Effect of proteolytic enzyme inhibitors on the reaction between active pancreatic protein and insulin. The above experiments and those described by Czerkawski & Bingle (1964) suggest that APP may be a proteolytic enzyme, and this view would be supported if its action were inhibited by known protease inhibitors.

(a) Action of soya-bean trypsin inhibitor. Commercial soya-bean trypsin inhibitor was mixed in a 2:1 weight ratio with the APP fraction in a solution

in water. The activity of this mixture was compared with that of the APP fraction to determine the relative inhibitory activity against bovine insulin, as shown by both the rat-diaphragm assay and the method for estimating the solubility of insulin in 8% trichloroacetic acid. The uptake of glucose by the diaphragm with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. was 8.13 ± 0.33 , with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. plus $8 \mu\text{g.}$ of the APP fraction/ml. it was 5.46 ± 0.33 , and with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. plus $8 \mu\text{g.}$ of the APP fraction/ml. plus $16 \mu\text{g.}$ of soya-bean trypsin inhibitor/ml. it was 7.16 ± 0.67 . These results show that soya-bean trypsin inhibitor partly but significantly ($P < 0.05$) prevents the inhibition of bovine insulin by APP, as shown by the rat-hemidiaphragm assay.

The effect of soya-bean trypsin inhibitor on the reaction between APP and bovine insulin, as shown by the solubility of the insulin in 8% trichloroacetic acid after incubation for 90 min. at 37° , was also studied. In the same experiment the effect on trypsin was also investigated. It was found that $4 \mu\text{g.}$ of trypsin/ml. rendered 3.0% of 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. soluble in 8% trichloroacetic acid after 90 min., whereas in the presence of $8 \mu\text{g.}$ of soya-bean trypsin inhibitor/ml. this action of trypsin on insulin was completely prevented. In comparison $4 \mu\text{g.}$ of the APP fraction/ml. rendered 27.9% of 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. soluble in 8% trichloroacetic acid after 90 min.; the addition of $8 \mu\text{g.}$ of soya-

bean trypsin inhibitor/ml. to the incubation mixture diminished the action of $4 \mu\text{g.}$ of the APP fraction/ml. so that only 5.4% of 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. became soluble in 8% trichloroacetic acid in 90 min.

Thus there is partial inhibition of the action of APP in insulin by the soya-bean trypsin inhibitor compared with complete inhibition of the action of trypsin. Previous work (Laskowski & Laskowski, 1954) has shown that soya-bean trypsin inhibitor causes partial inhibition of other pancreatic enzymes such as chymotrypsin.

(b) *Effect of the DFP compound with APP on bovine insulin.* The reaction between APP and bovine insulin was compared with the reaction between the DFP compound with APP by using both the rat-diaphragm assay and the effect on the solubility of the insulin in 8% trichloroacetic acid. The uptake of glucose by the diaphragm with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. was 7.16 ± 0.33 , with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. plus $8 \mu\text{g.}$ of the APP fraction/ml. was 4.74 ± 0.13 , and with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine insulin/ml. and $8 \mu\text{g.}$ of the DFP compound with APP/ml. it was 6.84 ± 0.23 . These results show that the DFP compound with APP is almost inactive against bovine insulin on the rat diaphragm and differs very significantly ($P < 0.001$) from the action of the intact APP fraction.

In a second experiment the effect of APP on the solubility of bovine insulin in 8% trichloroacetic acid was compared with the effects of its DFP compound. Thus, whereas $8 \mu\text{g.}$ of the APP fraction/ml. incubated with 4×10^{-3} i.u. ($0.2 \mu\text{g.}$) of bovine insulin/ml. rendered 26% soluble in 8% trichloroacetic acid after 90 min., a similar concentration of the DFP compound with APP was completely inactive and did not affect the solubility of bovine insulin in 8% trichloroacetic acid.

The results in both these experiments show that DFP is capable of inhibiting the reaction between APP and bovine insulin. This constitutes further evidence that APP is a proteolytic enzyme.

Species variation

Comparison of the action of bovine active pancreatic protein on bovine and porcine insulins by using the rat-diaphragm assay. In these experiments a standard technique was used. Either 4 or $8 \mu\text{g.}$ of the APP fraction/ml. was preincubated with 2×10^{-3} i.u. ($0.1 \mu\text{g.}$) of bovine or porcine insulin/ml. for 15 min. at 37° before the addition of the rat hemidiaphragm. The uptakes of glucose by the diaphragm in the presence of each species of insulin were compared with each other and with the uptakes in the presence of each insulin incubated with the inhibitor. In all experiments there was no significant difference between the action of

Fig. 1. Action of $8 \mu\text{g.}$ of APP fraction/ml. on 4×10^{-3} i.u. ($0.2 \mu\text{g.}$) of bovine insulin/ml. The solubility of insulin in 8% trichloroacetic acid was measured as described in the text after various periods of incubation at 37° .

2×10^{-3} i.u. (0.1 μ g.) of bovine insulin/ml. and that of 2×10^{-3} i.u. (0.1 μ g.) of porcine insulin/ml. on the rat diaphragm. The results for the three separate preparations of the APP fraction used are shown in Table 4 (first three rows). The three samples of APP fraction were prepared individually, in each case from a different supply of fresh bovine pancreas.

These results show that the inhibition of bovine insulin by APP was always greater than that of porcine insulin, and that the difference was on each occasion statistically significant. In a further experiment (fourth row in Table 4), when 8 μ g. of the APP fraction/ml. was preincubated with 2×10^{-3} i.u. (0.1 μ g.) of insulin/ml. complete inhibition of both bovine and porcine insulins occurred, so that a difference in activity could not be shown. Chymotrypsin (fifth row in Table 4) used under similar conditions but at three times the concentration caused a similar degree of inhibition to 4 μ g. of the APP fraction/ml., but no significant difference between the inhibition of the two species insulins was detected. These results suggest that bovine APP reacts more readily with homologous bovine insulin than with heterologous porcine insulin.

Comparison of the action of bovine active pancreatic protein on bovine and porcine insulins: measurement of solubility of insulin in trichloroacetic acid. Bovine APP increases the solubility of bovine insulin in 8% trichloroacetic acid (Czerkawski & Bingle, 1964). Three sets of experiments were performed in which the action of APP on the solubilities of bovine and porcine insulins in 8% trichloroacetic acid were compared under similar conditions.

(a) Bovine APP fraction at a concentration of 8 μ g./ml. was incubated with 4×10^{-3} i.u. (0.2 μ g.) of either bovine or porcine insulin/ml. The variation of the solubilities of the two insulins in 8% trichloroacetic acid with time was measured by using ^{131}I -labelled insulin as a tracer (Fig. 2).

(b) Bovine APP fraction at a concentration of 4 μ g./ml. was incubated with bovine or porcine insulin at various concentrations between 10^{-2} and 1.0 i.u. (0.5 and 50 μ g.)/ml. The solubilities of the two insulins in 8% trichloroacetic acid after incubation for 30 min. at 37° were compared with the concentration of the substrate insulin used (Fig. 3).

(c) Bovine APP fraction at concentrations of 1–100 μ g./ml. was incubated for 30 min. at 37° with 4×10^{-3} i.u. (0.2 μ g.) of either bovine or porcine insulin/ml. The solubilities of the two insulins in 8% trichloroacetic acid were compared with the concentration of APP used (Fig. 4). In each of these experiments bovine APP increased the solubility of the homologous bovine insulin in 8% trichloroacetic acid to a greater extent and at a greater rate than that of the heterologous porcine

Table 4. *Action of three separate preparations of active pancreatic protein and chymotrypsin on bovine and porcine insulins*

Bovine APP fraction or chymotrypsin was preincubated with insulin for 15 min. at 37° . The inhibitory activities of the pancreatic proteins on the insulin-stimulated uptake of glucose by the rat diaphragm were measured. Experimental details are given in the text. The results for glucose uptake are given as means $\pm$ s.e.m. The inhibition of bovine insulin was compared with that of porcine insulin and the statistical significance of any difference calculated. N.S., Not significant.

Pancreatic protein	Uptake of glucose (mg./g. dry wt. of diaphragm/ml./60 min.)				Inhibition of insulin by pancreatic protein (%)	Significance of difference between action of pancreatic protein on bovine and porcine insulins (<i>P</i>)		
	In basal medium	In basal medium + 2×10^{-3} i.u. of bovine insulin/ml.	In basal medium + 2×10^{-3} i.u. of porcine insulin/ml. + pancreatic protein	In basal medium + 2×10^{-3} i.u. of porcine insulin/ml. + pancreatic protein			Bovine insulin	Porcine insulin
APP fraction 1 (4 μ g./ml.)	3.84 $\pm$ 0.53	6.26 $\pm$ 0.33	5.07 $\pm$ 0.27	6.64 $\pm$ 0.23	38	0	< 0.01	
APP fraction 2 (4 μ g./ml.)	2.60 $\pm$ 0.33	7.00 $\pm$ 0.37	5.83 $\pm$ 0.43	7.40 $\pm$ 0.30	28	0	< 0.001	
APP fraction 3 (4 μ g./ml.)	5.06 $\pm$ 0.67	7.69 $\pm$ 0.37	5.24 $\pm$ 0.40	6.76 $\pm$ 0.40	94	55	< 0.01	
APP fraction 3 (4 μ g./ml.)	4.17 $\pm$ 0.53	7.73 $\pm$ 0.66	4.03 $\pm$ 0.50	3.44 $\pm$ 0.33	100	100	N.S.	
APP fraction 3 (8 μ g./ml.)	3.74 $\pm$ 0.27	10.07 $\pm$ 1.06	6.13 $\pm$ 0.50	6.48 $\pm$ 0.43	62	55	< 0.7	
Chymotrypsin

Fig. 2. Action of APP on bovine and porcine insulins. The samples contained 8 μ g. of APP fraction/ml. and 4×10^{-3} i.u. (0.2 μ g.) of insulin/ml. ● and ▲, Two separate experiments with bovine insulin; ○ and △, two separate experiments with porcine insulin. The solubility in 8% trichloroacetic acid was measured as described in the text after various periods of incubation at 37°.

insulin, confirming the results obtained with the rat-diaphragm assay. In an experiment with α -chymotrypsin incubated with bovine or porcine insulin, the variations of the solubilities of the two insulins in 8% trichloroacetic acid with time did not differ from each other.

DISCUSSION

The work described above attempts to account for the inhibitory activity of pancreatic extracts on the action of insulin in a biological system of the rat diaphragm. Previous work suggested that pancreatic extracts can inhibit insulin in muscle but the mechanism for this inhibition is far from clear.

Our experiments have shown that an extract prepared from fresh bovine pancreas and freed from insulin is capable of interfering with the action of bovine insulin on the rat diaphragm. Further investigation into the mechanism of this inhibition suggests that the active inhibitor is a protein with the property of a proteolytic enzyme. Evidence has been put forward that this enzyme possesses certain similarities to chymotrypsin but also a number of properties different from it (Czerkawski & Bingle, 1964). Ryle, Sanger, Smith

Fig. 3. Effect of concentration of insulin on the extent of reaction with APP. The concentration of the APP fraction was kept constant at 4 μ g./ml. and the samples were incubated for 30 min. at 37°. The percentage of total insulin rendered soluble in 8% trichloroacetic acid was measured as described in the text and plotted against the initial concentration of insulin. ● and ▲, Two separate experiments with bovine insulin; ○, porcine insulin.

Fig. 4. Action of APP on bovine (●) and porcine (○) insulins. The curves show the variation of solubility of 4×10^{-3} i.u. (0.2 μ g.) of insulin in 8% trichloroacetic acid, measured as described in the text, after incubation for 30 min. at 37° with various concentrations of the APP fraction.

& Kitai (1955) found that certain crude pancreatic extracts attacked insulin more rapidly and completely than could be accounted for by their content of α -chymotrypsin and trypsin.

The present results suggest that the degree of biological inactivation of insulin is in excess of what would be expected from the degree of chemical degradation of the insulin molecule. However, it is unwise to make such deductions based on the absolute value of the solubility of the protein in trichloroacetic acid (Linderstrøm-Lang, 1949).

It has not been shown that APP has any physiological significance *in vivo* as an insulin antagonist. Insulin antagonists isolated from the serum have, in general, been poorly characterized and their site of origin is unknown. Such antagonists are thought to form complexes with insulin or to interfere with the attachment of insulin at its site of action. They could still be enzymes with low Michaelis-Menten constants, suggesting the formation of fairly stable enzyme-substrate complexes that might well be biologically inactive and only later break down with the degradation of insulin.

Bovine APP can attack its own species insulin more readily than the insulin of another species (pig). In this respect also APP differs from bovine chymotrypsin. It is possible that this species difference in enzymic action could depend, as does the immunological specificity, on the differences in the amino acid sequence in the A chain of insulin.

The relative amounts of enzyme and insulin used in the above experiments are different from those usually encountered in enzyme studies. The rat diaphragm is sensitive to amounts of insulin of the order of 10^{-3} i.u., and it was therefore decided to investigate the effects of antagonists at this concentration of insulin which is similar to that occurring *in vivo*. Initial experiments with crude pancreatic extracts suggested that inactivation of insulin occurred in a short time when amounts of extract measurable in micrograms were used. The proportion of APP to insulin in the pancreas was about 20:1. It was therefore in this ratio of APP to insulin that studies on the rat diaphragm and later measurements of the solubility of insulin in 8% trichloroacetic acid were made. Czerkawski & Bingle (1964) have shown that APP will also attack insulin when more orthodox enzyme:substrate ratios are used. In those experiments substrate concentrations were about 1000-fold greater than in the experiments with rat diaphragm. At low concentrations of insulin the rate of reaction is approximately proportional to substrate concentration, and so the reaction of amounts of insulin of the order of 10^{-3} i.u. with similar or smaller amounts of APP would be very slow and probably not detectable with the biological assay. It is also for this practical reason that, having decided on a biological concentration of insulin, we had to use such abnormal enzyme:substrate ratios.

The experiments presented above show that the enzyme system occurring in pancreas, by degrading insulin, could also account for most of the 'antagonistic action' against insulin detected in pancreatic extracts. It is likely that this enzyme is closely related to chymotrypsin, but definite differences between APP and chymotrypsin, including the species specificity, have been shown. APP is probably produced by the exocrine tissue of

the pancreas and may not have any physiological significance. The pancreatic exocrine enzymes are found in blood but their function is unknown.

SUMMARY

1. Fresh bovine pancreas extracted with a buffer solution, pH 4.6, and further treated on a DEAE-cellulose column to remove insulin yields a mixture containing a protein that can inhibit the action of bovine insulin in a biological system.

2. Fractionation of this mixture on a CM-cellulose column shows that the inhibitory activity against insulin resides in a fraction of the mixture which contains one major and two minor components.

3. The active pancreatic protein fraction can degrade bovine insulin as shown by its ability to increase the solubility of insulin in 8% trichloroacetic acid.

4. There is evidence that the active pancreatic protein is a proteolytic enzyme.

5. The action of the active pancreatic protein in decreasing the insulin-stimulated uptake of glucose by the rat diaphragm is probably due to its ability to degrade insulin. A second possibility is that an enzyme-substrate complex between the active pancreatic protein and insulin makes the latter biologically inactive.

6. Bovine active pancreatic protein inhibits the porcine-insulin-stimulated uptake of glucose by the rat hemidiaphragm, but the inhibition is significantly less than its action on bovine insulin.

7. Bovine active pancreatic protein degrades bovine insulin to a greater extent and at a greater rate than porcine insulin, as indicated by the solubilities of the degraded insulins in 8% trichloroacetic acid.

8. Bovine active pancreatic protein differs from α -chymotrypsin which does not show any species difference with regard to its action on insulin.

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Purification of Pig-Kidney Diamine Oxidase and its Identity with Histaminase

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Best (1929) described an enzyme, histaminase (EC 1.4.3.6, diamine-oxygen oxidoreductase), that catalyses the oxidation of histamine and which is distributed in various animal tissues (Best & McHenry, 1930). Mann (1961) obtained a highly purified preparation of pea-seedling diamine oxidase and showed that it is a metalloenzyme containing cupric copper (Hill & Mann, 1962). Yamada & Yasunobu (1962*a, b*) suggested that crystalline plasma amine oxidase can be represented as protein-(Cu²⁺)₄-(pyridoxal phosphate)₂. The loss in activity of animal diamine oxidase after treatment with carbonyl reagents suggests that this enzyme contains a functional aldehyde or ketone group (Zeller, 1938*a*; Davison, 1956; Goryachenkova, 1956, 1959; Zeller, Stern & Wenk, 1940; Swedin, 1943). Kapeller-Adler (1949) and Kapeller-Adler & MacFarlane (1963) provided evidence that both FAD and pyridoxal phosphate are coenzymes of animal diamine oxidase. Werle & Hartung (1956) have shown that plant and animal diamine oxidases

are inhibited by chelating agents, and Mondovi, Rotilio & Costa (1963) and Goryachenkova & Ershova (1963) have provided evidence that pig-kidney diamine oxidase contains copper.

The purification of pig kidney diamine oxidase has been described by several authors (Stephenson, 1943; Laskowsky, 1945; Tabor, 1951; Swedin, 1958), and highly purified preparations have been obtained by Uspenskaia & Goryachenkova (1958), Mondovi (1961), Goryachenkova & Ershova (1963), Mondovi *et al.* (1963) and Kapeller-Adler & MacFarlane (1963).

Despite these numerous investigations, the identity of pig-kidney diamine oxidase with histaminase has not yet been established. Zeller (1951) and Blaschko, Friedman, Hawes & Nilsson (1959) concluded that the oxidation of histamine, and of such simple diamines as cadaverine and putrescine, was catalysed by a single enzyme that Zeller (1951) named 'diamine oxidase'. Goryachenkova (1958) arrived at similar conclusions, showing that it was