Interaction of Pancreas Extracts with Bovine Insulin

1. PURIFICATION AND CHEMICAL PROPERTIES

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(Received 3 September 1963)

This paper is concerned with pancreatic substances that can antagonize insulin. Previous work (Marks & Young, 1939; Thorogood & Zimmermann, 1945; Mirsky, Futterman, Wachman & Perisutti, 1951) has already suggested that insulin antagonists exist in the pancreas. Evidence has been produced for the identity of these antagonists with glucagon, but there is other work suggesting that the pancreas may produce substances that can antagonize insulin in such tissue as muscle (Young, 1962; Antoniades, Renold, Dagenais & Steinke, 1960) when glucagon is without effect (Randle, 1958). Antoniades *et al.* (1960) suggested that insulin might be combined with a basic protein in pancreas. The studies reported below are an attempt to isolate and characterize at least one of the bovine pancreatic substances that is capable of antagonizing insulin in a biological system such as rat diaphragm.

MATERIALS AND METHODS

Materials. DEAE-cellulose and CM-cellulose were obtained from Whatman (W. R. Balston Ltd.). Before use DEAE-cellulose was treated with 2N-NH₃ and 2M-NaCl; CM-cellulose was treated with 2N-acetic acid and 2M-NaCl; both were washed well with distilled water. The bovine insulin (22.2 i.u./mg.) was obtained from Boots Pure Drug Co. Ltd., Nottingham. The proteolytic enzymes trypsin and α -chymotrypsin were recrystallized preparations, obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The pancreatic elastase was prepared by the method of Hall & Czerkawski (1959). Lilley & Vallance-Owen (1961) have shown that dialysis tubing contains substances capable of antagonizing insulin, and that these substances can be removed by boiling the tubing in two changes of distilled water. It was deemed wise to take the same precaution with the 1 in. tubing (Visking) used in the present work. Unless otherwise stated all the reagents used were of AnalaR quality.

Electrophoresis. The purity of the fractions was frequently checked by use of electrophoresis at high voltage on paper and at low voltage on cellulose acetate (Oxoid Division of Oxo Ltd., London, E.C. 4) as supporting medium. The electrophoresis was usually performed in a 75 mM-barbiturate (veronal) buffer solution, pH 8.6, which,

for the paper electrophoresis, was made 0.05M with respect to EDTA (the chelating agent gives a better resolution on paper). The paper strips were stained with bromophenol blue to show protein (Smith, 1960) and with ninhydrin to show peptides. The cellulose acetate strips were usually developed with azocarmine [0.2% in 5% (v/v) acetic acid] and washed with several changes of 5% acetic acid. Good separation could be achieved on paper (Whatman 3MM) at 20V/cm. in 1 hr. With the cellulose acetate, 6V/cm. gave a current of about 1 mA per strip (5 cm. wide) and resulted in acceptable resolution in 1 hr.

Enzyme activity. Chymotrypsin and trypsin activities were determined by the spectrophotometric method of Schwert & Takenaka (1955), by using *N*-acetyltyrosine ethyl ester and toluene-*p*-sulphonylarginine methyl ester respectively as substrate.

Elastase activity was determined by measuring the rate of solution of elastin as described by Hall & Czerkawski (1961).

When a suspension of dry machine-skimmed milk in buffer is incubated with proteolytic enzymes, the turbidity clears rapidly and can be measured photometrically at 560 m μ . The reaction is fast and convenient, and is brought about by very small amounts of enzyme. For instance, trypsin at concentrations of 0.5–1.0 μ g./ml. decreases the extinction by about 50% in 5 min. The reaction is not very accurate and certainly not specific, but is nevertheless very useful, particularly in exploratory purification experiments. The method was as follows: 1.0 ml. of milk suspension (2%, w/v; Cow and Gate Ltd., Guildford, Surrey) in water, prepared at least 1 day before and kept in the refrigerator at 5°, was added to 3.0 ml. of 50 mM-ammediol (2-amino-2-methylpropane-1,3-diol) buffer solution, pH 7.8, in a 25 ml. conical flask. The enzyme (0.1 ml.) was then added and the flask incubated at 37°. The extinction was measured at 1 min. intervals at 560 m μ . The slope of the linear portion of the curve is approximately proportional to the concentration of enzyme.

The proteolytic activity was also measured in a more specific manner by determining the degree of degradation of insulin on a large scale. Borate buffer solution, pH 7.8 and *I* 0.365 (1.0 ml.), was pipetted into centrifuge tubes, and this was followed by 0.5 ml. of bovine insulin (500 μ g./ml. in 3 MN-HCl) and either 0.5 ml. of enzyme solution (4–40 μ g./ml.) or water to serve as control. The tubes were incubated in a water bath at 37° for 30 min., and the reaction was terminated by adding 2.0 ml. of 10% (w/v) trichloroacetic acid. After standing for 15 min., the samples were centrifuged, decanted and allowed to drain by inverting the tubes over filter paper. The precipitate was redissolved in 1.0 ml. of water and the protein concentration was determined by the method of Lowry, Rosebrough,

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Farr & Randall (1951). The activity was obtained by subtracting the spectrophotometer reading of the test samples from that of the control.

Purification of enzyme. The purification procedure finally adopted is shown in Scheme 1. The fresh bovine pancreas (1.0 kg.) was frozen, cut into suitable portions and homogenized with small amounts of 0.2M-ammonium acetate buffer solution, pH 4.7. The thick suspension was extracted twice with 1.0 l. of the same buffer for 24 hr., with intermittent shaking, at 5°. The extracts were isolated by centrifuging at high speed (10000g) at 0°. Most of the fat was removed as a crust from the top of the liquid in the centrifuge vessels and the rest by filtering through a coarse sintered-glass filter; the relatively clear extracts were pooled and dialysed against 5 vol. of distilled water, with daily changes for 5 days at 5°. The copious precipitate of euglobulins, rich in elastase, was removed by centrifuging (at 1800g) at 0°. The clear supernatant ('AES₁ fraction'), which was pink due to haemolysis, was then passed through a composite column (40 cm. × 3 cm.) consisting of DEAE-cellulose (upper 75%) and Hyflo Super-Cel (lower 25%). The water eluate was collected in a refrigerator at 5°. The eluate, which was now colourless, was freeze-dried to give the fraction designated the 'AES₂ fraction' (3.25 g.). This treatment removed all the products of haemolysis and most of the acidic proteins and peptides including insulin.

Further purification was achieved by chromatography on a CM-cellulose column (40 cm. × 3 cm.) in the refrigerator at 5°. A portion (1.0 g.) of the AES₂ fraction was dissolved in 5 ml. of water and applied to the column, which was then washed with water until the eluate gave a negative test for protein. Preliminary experiments indicated (see Fig. 1) that 0.05M-NaCl removes most of material that stains heavily with ninhydrin and contains relatively

little protein. The fractions so obtained had virtually no milk-clearing activity. Most of the activity could be eluted from the column with 0.1–0.2M-NaCl. Thus for preparative purposes it was sufficient to perform the elution in three steps: water, 0.05M-NaCl (750 ml.) and 0.2M-NaCl (750 ml.). The material eluted with 0.2M-NaCl was dialysed in the cold against distilled water to remove NaCl and freeze-dried to give 150 mg. of very active material which has been called 'active pancreatic protein' (APP) (see Scheme 1).

RESULTS

Electrophoresis of the APP fraction indicates (Fig. 2) that it is not homogeneous and that it consists of at least three components, *A*, *B* and *C*. The major component *B* accounts for about 80 % of the protein, and the two minor components *A* and *C* account for about 10 % each.

Chemical and physical properties. The APP fraction gives the typical protein reactions. It is retained during dialysis and has a typical protein ultraviolet-absorption spectrum with an optimum extinction at 283 mμ and a specific extinction, $E_{1\text{cm}}^{1\%}$, at this wavelength of 14.0.

The amino acid composition of the APP fraction was compared with that of trypsin and α-chymotrypsin, by using a modification of the method of Mandelstam & Rogers (1959). The difference is most pronounced in the polar amino acid content and APP fraction has a composition about halfway between chymotrypsin and trypsin; it is

Scheme 1. Diagrammatic representation of the procedure for isolation of the APP fraction. The weights in parentheses give yields and the main route is indicated by the heavy arrows.

probably more closely related to the former. APP fraction migrates in the region of the elastase complex during paper electrophoresis. The major component *B* migrates at a slightly slower rate than α -chymotrypsin and thus has an isoelectric point between pH 7.5 and 8.0.

The protein is readily soluble in water and in dilute solutions of salts. It is stable at low temperatures both in the solid state and in solution. It also exhibits marked stability at high temperatures in the pH range 2–4. Thus, although heating for 5 min. at 100° in the pH range 6–8 results in denaturation as shown by the production of a precipitate and loss of activity (see below), heating under the same conditions at pH 3 has little or no effect.

Enzymic properties. The enzymic properties of the APP fraction were studied mainly in relation to two substrates: milk suspension and bovine insulin. In each case the rate was dependent on temperature and had a broad optimum pH between 7 and 9. There was no activity at pH values lower than 6. The reaction with milk is too complex to allow any insight into its kinetics and mechanism; it will

suffice to say that it is accompanied by liberation of free amino groups, which suggests proteolysis. When insulin is incubated with the APP fraction, it becomes diffusible through a dialysis membrane, becomes soluble in 5% trichloroacetic acid and the reaction results in the cleavage of peptide bonds (increase in ninhydrin colour). The above constitutes sufficient evidence that the insulin molecule is degraded and that this too is due to proteolysis.

The reaction with insulin, measured as described above, permitted kinetic studies to be performed. Some of the results are presented in Fig. 3. The rate of reaction is constant up to 1.0 hr. (Fig. 3*a*), and within limits the rate is proportional to the concentration of enzyme (Fig. 3*b*). The system obeys the Michaelis-Menten equation approximately and the Michaelis constant is about $1 \mu\text{M}$ (assuming that the mol.wt. of insulin is 12000). The low constant indicates the formation of relatively large amounts of the enzyme-substrate complex.

The results described above indicate that the APP fraction contains a proteolytic enzyme with chemical properties similar to those of chymotrypsin. In fact, the thought that we might actually be isolating a form of chymotrypsin was foremost in our minds throughout the investigation. The following results make it unlikely, however, that the active component of the APP fraction is chymotrypsin, although they do not prove it conclusively. The results are summarized in Table 1, in which the findings are expressed as percentages of the activity of the most active enzyme with any particular substrate by using precisely the same weight of each enzyme.

Fig. 1. Fractionation of pancreas extracts on a column of CM-cellulose. A portion (300 mg.) of the AES₂ fraction was dissolved in 5 ml. of water and applied on the column (60 cm. $\times$ 1 cm.). The column was eluted at 2 ml./min. with water and then stepwise with progressively more concentrated NaCl solutions; 5 ml. fractions were collected. The protein in the fractions was determined by the biuret reaction, and the fractions corresponding to each step were pooled. The cut sections were subjected to electrophoresis and the bands developed with ninhydrin. These are shown diagrammatically in (a). The concentration of the corresponding eluting solutions are shown in the column (c). The specific milk-clearing activity of the cuts, expressed as decrease in extinction at $560 \text{ m}\mu/\text{min.}/\text{mg.}$ of protein, is shown as a histogram in column (b).

Fig. 2. Electrophoresis of pancreatic fractions. The strips marked N were developed with ninhydrin and those marked P with the protein stain. The bands on cellulose acetate were stained with azocarmine. Experimental details are given in the text. (a) Paper electrophoresis of crude extract; (b) paper electrophoresis of the APP fraction; (c) electrophoresis on cellulose acetate of the APP fraction. The significance of the three components A, B and C is discussed in the text.

Fig. 3. (a) Reaction of APP with bovine insulin. The reaction mixture contained 125 μg. of insulin/ml. and 5 μg. of the APP fraction/ml. in borate buffer solution, pH 7.8. The percentage solubility in 5% trichloroacetic acid is plotted against time of incubation at 37°. (b) Reaction of APP with bovine insulin. The reaction mixture contained 125 μg. of insulin/ml. and the indicated amounts of the APP fraction. The reaction was terminated by addition of 1 vol. of 10% trichloroacetic acid after incubation at 37° for 20 min. (○) or 40 min. (●).

The fact that the active substance in the APP fraction is not trypsin was demonstrated by comparing its action and that of trypsin on toluene-*p*-sulphonylarginine methyl ester and on insulin (first and last rows in Table 1). It is possible, however, that the fraction is contaminated with trypsin (the more basic of the two minor electrophoretic components). Further, the maximum contamination of the APP fraction with elastase is only 2–3% (third row in Table 1). This low level was already inferred from the solubility of the APP fraction in water (Hall & Czerkawski, 1959), and from the method of its preparation.

The results with respect to α-chymotrypsin are not as clear, but again much information may be gained from examination of Table 1. The APP fraction has only half the specific activity of chymotrypsin with *N*-acetyltyrosine ethyl ester as substrate. If one accepts the electrophoretic results as a true representation of the composition of the pancreatic protein (10% of *A*, 80% of *B* and 10% of *C*), and if one of these components is α-chymotrypsin, then one would expect a value of 10 or 80% for the specific activity and not 50%. There is no reason why two different enzymes should not have similar specificity, and the activity of the APP fraction on *N*-acetyltyrosine ethyl ester is probably an integral property of the enzyme. The chymotrypsin activity of a mixture of equal amounts of chymotrypsin and APP fraction is less than the sum of their separate activities. The lowering is not great (15%), but it occurs consistently. The experiment was done under such conditions that the rate was proportional to enzyme concentration, and such lowering would not occur if the two enzymes were identical.

By far the strongest evidence that the active component of the APP fraction is not chymotrypsin comes from its reaction with insulin (last row in Table 1). Here, the pure well-characterized chymotrypsin has only half the activity of the relatively impure APP. Another important result

Table 1. *Specificity of the active pancreatic protein*

With each substrate the activity of all enzymes was determined simultaneously by using, where possible, sufficient amounts of enzyme to give a measurable value. The specific activity, i.e. activity/ μ g. of enzyme, was then calculated. The activity of each enzyme reacting with any given substrate was then expressed as percentage of the most active one.									
					Relative enzyme activity				
Enzyme	...	...	...	...	Trypsin	α -Chymo- trypsin	Elastase	APP fraction	Reference
Substrate									
Toluene- <i>p</i> -sulphonylarginine methyl ester					100	—	—	15	Schwert & Takenaka (1955)
<i>N</i> -Acetyltyrosine ethyl ester					—	100	—	49	Schwert & Takenaka (1955)
Elastin					2	5	100	3	Hall & Czerkawski (1959)
Milk					80	100	20	80	This paper
Insulin					0	50	50	100	This paper

is the inhibition of the reaction between the APP fraction and insulin by Ca^{2+} ions, in which 40 mM- Ca^{2+} ion gives about 50% inhibition (J. W. Czerkawski, unpublished work). It is well known that Ca^{2+} ions do not inhibit chymotrypsin; rather,

they have been reported to activate it (see Desnuelle, 1960).

Thermal stability. Preliminary experiments in which the APP fraction was heated for 5 min. at 100° in 0.05M-acetate buffer solution in the pH range 1-8, and in which the activity was tested subsequently on milk and insulin, have demonstrated that the preparation is most stable between pH 2 and 4, and that it is completely inactivated at pH 7 under similar conditions. The stability of this protein was investigated further by heating it in solution at pH 3 or at pH 7 for 5 min. at 100° at various concentrations. The stability at pH 3 and denaturation at pH 7 were independent of the concentration of the APP fraction between 50 and 500 $\mu\text{g./ml.}$ It remained to show how the stability of APP was affected at high temperature by the duration of heating.

Two series of solutions of the APP fraction were prepared: 250 $\mu\text{g./ml.}$ in 0.05M-acetate buffer, pH 3.0 and pH 7.3. They were heated at 100° for various times, cooled rapidly, centrifuged and decanted. The protein in the supernatant was determined by the method of Lowry *et al.* (1951) and the remainder of the supernatant was used for the determination of activity with milk and insulin.

The results are shown in Fig. 4. The appearance of the copious white precipitate at pH 7.3 is somewhat misleading, and even after heating for 30 min. not more than 30% of the available protein becomes insoluble (Fig. 4b). The specific enzyme activity of the remaining soluble 70% of the protein is zero under these conditions. No enzyme activity could be demonstrated in the precipitate. At pH 3.0 the extent of precipitation is insignificant, and even heating for 10 min. produces a relatively small loss of activity (Fig. 4a). At this pH the APP fraction loses about 1% of its activity/min. at 100° . α -Chymotrypsin and trypsin also show remarkable thermal stability at pH 3.0 and are labile at neutrality. For instance, at pH 3.0 α -chymotrypsin loses its ability to degrade insulin at about 1.5%/min.

Fig. 4 (a) Thermal stability of APP. The APP fraction was dissolved in 0.05M-acetate buffer solution (250 $\mu\text{g./ml.}$), heated at 100° , cooled rapidly and centrifuged. Part of the supernatant was used for the determination of protein concentration (see Fig. 4b) and part, after adjustment of the pH, was used for the determination of enzyme activity, with milk ($\circ$, $\triangle$) or insulin ($\bullet$, $\blacktriangle$) as substrate. The specific activity (per mg. of protein) was calculated by assuming that the unheated material had 100% activity. ($\bullet$), Heated at pH 3.0, insulin substrate; ($\circ$), heated at pH 3.0, milk substrate; ($\blacktriangle$), heated at pH 7.3, insulin substrate; ($\triangle$), heated at pH 7.3, milk substrate. (b) Heat precipitation of APP. Experimental details are given in the text and the caption for Fig. 4(a).

DISCUSSION

The results presented above show that the substance APP isolated from fresh bovine pancreas is a protein. There is also much evidence that it is a proteolytic enzyme probably related to chymotrypsin and that it has a great specificity for insulin. When this had been established, and when many other proteolytic enzymes from the pancreas had been eliminated on the basis of their specificity (trypsin and elastase) or their physical properties (carboxypeptidase, leucine aminopeptidase, and the 'insulinase' described by Lewis & Thiele, 1957), it became of importance to show that

APP was distinct from chymotrypsin. The evidence that this is so is based partly on physical and chemical properties. It is also based on the consideration of the method of preparation, but the strongest evidence arises from its enzymic behaviour.

Naughton & Sanger (1961) have subjected 'crystalline' elastase to chromatography on CM-cellulose and have shown that the main peak has a shoulder on its leading edge. This had no elastolytic activity and was eluted with 0.15M-sodium chloride solution. When the same preparation of elastase was treated with radioactive di-isopropyl phosphorofluoridate (DFP) and subjected to chromatography the leading edge of elastase peak gave rise to two definite peaks of which only the faster one was radioactive (eluted with 0.15M-sodium chloride). Naughton & Sanger (1961) did not report any additional enzymic properties of these substances, in particular their behaviour towards intact insulin. However, the identity of at least one of these compounds is of importance in connexion with the present work, because the APP fraction is strongly inhibited by di-isopropyl phosphorofluoridate (see Bingle & Czerkawski, 1964). Naughton & Sanger (1961) could not identify the di-isopropyl phosphorofluoridate-sensitive substance with any known enzyme and it is possible that it is the same as the APP fraction.

The investigation of the thermal stability of the APP fraction began as a normal step in its characterization, and led to some confusion. It could be argued that, by having such marked stability at pH 2-4, the active component was more likely to be a purely complex-forming protein than an enzyme. The results obtained show that the stability applies to the enzymic properties, and that this behaviour is not unique, but similar to that of at least two common proteolytic enzymes, namely trypsin and chymotrypsin. F. G. Young has found a similar thermal stability with his pancreatic insulin antagonist (communicated during discussion at a meeting of the British Diabetic Association at Dublin in 1963). The above results are paralleled when the measured parameter is the power to antagonize the insulin-stimulated uptake of glucose by rat diaphragm (see Bingle & Czerkawski, 1964), and so thermal-stability studies provide further evidence that the antagonist activity of the APP fraction is related to its enzymic nature.

SUMMARY

1. A method of purification of a basic pancreatic protein antagonizing insulin is described. It is based on acid extraction of fresh bovine pancreas,

fractional dialysis and fractionation on columns of modified cellulose.

2. The active pancreatic protein fraction is not homogeneous, and is composed of water-soluble proteins. Electrophoretically, it consists of one major (80 %) and two minor (10 % each) components, with isoelectric points in the region pH 7.5-8.0.

3. The major component of the active pancreatic protein fraction is capable of proteolytic activity. It is distinct from most of the common pancreas proteases, but in many respects resembles α -chymotrypsin. Weight for weight this major component attacks insulin faster than other enzymes that have been studied.

4. The major component of the active pancreatic protein fraction is remarkably stable at pH 2-4 even at 100°, but is readily denatured at the pH values near neutrality. Denaturation is accompanied by partial precipitation and complete loss of enzymic activity.

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