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An Assay of Insulin by Fibril Formation from Small Samples of Pancreas

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(Received 26 June 1957)

A paper-chromatographic technique for the isolation and assay of insulin from 1 g. samples of foetal ox pancreas has been described in an earlier report (Grotsky & Tarver, 1956). It was effective at levels of insulin above 100 $\mu\text{g./g.}$ of tissue, but attempts to increase the sensitivity so as to make it applicable to studies of insulin metabolism, where the hormone level would be very much lower than this, have been consistently marred by the presence of relatively large amounts of other protein material which moved from the origin at the same rate as the insulin itself. For this reason it has been thought worth while to investigate an alternative procedure.

Waugh (1946), following the pioneer observation of Du Vigneaux, Geiling & Eddy (1928), observed that insulin in acid solution separated as fibrils when the temperature was raised. A preparation of these insoluble fibrils would act as a seed in crude dilute solutions of insulin, and when this was carried out at the right pH the insulin was quantitatively precipitated.

This method of assay has been criticized by Foster, MacDonald & Smart (1951) and by Light & Simpson (1956), who have reported that recoveries

may be erratic in the presence of a crude mixture of tissue proteins. There is, indeed, evidence that globin (Foster *et al.* 1951), muscle protein, serum albumin and fibrin (Waugh, Wilhelmson, Commerford & Sackler, 1953) can form insoluble aggregates under the prescribed conditions, and Sutherland, Cori, Haynes & Olsen (1949) found that glucagon could be precipitated as part of the fibril mat.

By suitable modification of the Waugh (1946) procedure it has been found possible to assay the insulin in 1 g. of adult animal pancreas without any apparent interference from glucagon or other extraneous proteins. It has been applied under limited conditions to the pancreas of several animal species and the efficiencies of seed fibrils from pig, sheep and ox insulins have been compared.

EXPERIMENTAL

Materials. Crystalline ox insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham (batch no. 9011G). The sterile solution of glucagon was prepared by Eli Lilly and Co., Indianapolis, Ind., U.S.A. (batch no. 208-158B 197). I am grateful to Doctors F. Sanger, D. S. H. W. Nickols and L. F. Smith for samples of esterified insulin, of insulin that had been treated with carboxypeptidase so as to remove the terminal alanine and asparagine residues, and of the A and B fractions of oxidized insulin. The bovine serum albumin was obtained from Armour and Co. Chicago, Ill., U.S.A.

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Assay of insulin by paper chromatography. The procedure of Grodsky & Tarver (1956), with minor changes dictated partly by the atmospheric conditions of the laboratory and the filter-paper sheet available, was first explored. If the insulin band, after staining with bromophenol blue, was extracted with $0.1\text{ M-K}_2\text{HPO}_4$ instead of with acidic ethanol and the total volume of eluting solution was reduced from 25 to 6 ml., an eightfold increase in sensitivity of the colorimetric determination was thereby obtained. Nevertheless, when this modified technique was applied to extracts from liver tissue, which contain an appropriate mixture of proteins other than insulin, a small amount of foreign material, approx. $20\text{--}25\text{ }\mu\text{g./g.}$ of tissue, travelled at the same rate as insulin itself. Other products which interfere in the same way were glucagon (Grodsky & Tarver, 1956), esterified insulin, insulin modified with carboxypeptidase and insulins from sheep and pig, all of which move at the same rate as ox insulin. The procedure was clearly not applicable at the level of insulin dealt with in this paper and was accordingly abandoned.

Assay of insulin by precipitation of fibrils. The pancreatic tissue was treated by the technique of Grodsky & Tarver (1956) until the stage when the crude insulin was precipitated with alcohol-ether. When this precipitate was extracted with 0.1 N-HCl (0.5 ml.) containing 0.1% NH_4Cl , the major part of the protein material passed into solution. The extract was filtered through a fluted Whatman no. 2 filter paper into a flat-bottomed vial of the type described below. A second extract was prepared and used to wash the filter paper. The filtrate, which should be clear and at approx. pH 1.5, was adjusted to 1 ml. by adding water. Standard solutions of insulin of the same pH were prepared by dissolving the crystalline material in 1 ml. of 0.05 N-HCl containing 0.1% NH_4Cl .

After preparation of the clear solution, $20\text{ }\mu\text{g.}$ of freshly prepared insulin seeding fibrils (Waugh, Thompson & Weimer, 1950) were added to each vial, the magnetic-stirrer rods ('fleas', see below) were inserted and the vials firmly sealed with close-fitting rubber stoppers. Fig. 1 shows the apparatus used for stirring the contents of the vials at a uniform rate. A glass-walled constant-temper-

ature water bath with stirrer was placed on two identical magnetic stirrers (Towers Co. Ltd., Manchester). The radius of sweep of the electromagnet in these instruments is 4.5 cm. Nine holes (outside diam. 1.2 cm.) each large enough to accommodate loosely a vial were drilled in a circle 4.5 cm. from the centre of each of two stainless-steel disks. The centre of each disk was drilled and tapped to fit a stainless-steel rod which acted as a handle. Alongside each hole was fastened a small strip of spring metal which held each vial firmly in place. The acid-resistant nickel-silver rods ('fleas') which rotate freely in the moving electromagnetic field, were approx. 7 mm. long and of 2 mm. diam. By placing the holders over the centre of the magnetic stirrer, the 'fleas' in the vials lying on the path of the electromagnet could be agitated. By adjusting the speed of the stirrer, this agitation could be transformed into a rotary motion which caused a rapid stirring of the contents of the vials with little or no surface foaming.

The seeded solutions were stirred at 45° for 16–18 hr. The contents of the vials were then transferred to 12 ml. centrifuge tubes. Each vial was washed with 0.05 N-HCl (1 ml.) containing 0.1% NH_4Cl , and any fibrils adhering to the glass surface of the vial were removed with a glass rod. The fibril suspension and wash were combined, the fibrils centrifuged down and the supernatant discarded. The fibrils were washed once in the acid solution and twice in 1.5 ml. of methanol.

Two methods for the quantitative determination of the insulin were used. After the last methanol wash, the fibrils were re-suspended in 1.5 ml. of the acid solution. The extinction of this suspension at $275\text{ m}\mu$ was compared with those obtained from similarly treated standard solutions of insulin. Alternatively, the fibrils were washed once more with the acid solution, dissolved by boiling in 1 ml. of NaOH for 15 min., the solution was centrifuged to remove insoluble particles, the volume adjusted with water and the tyrosine content of the solution compared with similarly treated solutions of insulin, the extinction being measured at $293\text{ m}\mu$ (Beaven & Holiday, 1951).

Comments. Waugh *et al.* (1950) were able to produce insulin fibrils from commercial 'still concentrates' dissolved in H_2SO_4 . In the present investigation the crude mixture of proteins from the alcoholic extracts of pancreas was not readily soluble in $0.1\text{ N-H}_2\text{SO}_4$. Filtrates were cloudy and during fibril formation a large amount of non-specific material separated. The addition of 1% Na_2SO_4 increased the amount of extraneous precipitation. The protein mixture, however, was readily soluble in 0.1 N-HCl containing 0.1% NH_4Cl . The final washing of the separated fibrils with methanol removed a large amount of extraneous material, and good recoveries of added insulin could not be obtained if this step were omitted. Although the stirring technique resulted in a minimum of splashing, the fibrils massed in a ring at the surface of the liquid on the sides of the glass vials. This partial separation of the fibrils, however, did not affect the assay.

The two methods used for estimating the insulin fibrils gave closely agreeing results. That based on

Fig. 1. Apparatus for stirring the vials used for preparing insulin fibrils.

a direct reading was preferred, as it called for less manipulation. The standard error obtained by repeated assays of the same crude insulin preparation was $\pm 5\%$.

Specificity of the method. Waugh *et al.* (1953) reported that many proteins form fibrils and, of those investigated, albumin is normally present in pancreas and could be expected to interfere with the assay. In the present procedure, however, the major portion of albumin was precipitated during the alkaline-alcohol treatment for preparation of the crude insulin. Glucagon could also be a possible contaminant (Sutherland *et al.* 1949). Neither albumin nor glucagon forms fibrils under the conditions of the present procedure, nor do they interfere with the fibril formation of insulin whether in pure solution or in extracts of pancreas.

RESULTS

Fig. 2 demonstrates the recovery of insulin which had been added to the protein precipitated from acid alcoholic extracts of rat liver and ox pancreas. Recoveries of increasing amounts of added insulin (including that added as seeding fibrils) are linear and 95% quantitative.

To investigate the possibility of seed specificity, as described by Waugh *et al.* (1950), standard solutions of ox, pig, sheep and calf insulins, carboxypeptidase-modified insulin, esterified insulin and Sanger's *A* and *B* fractions of oxidized insulin were seeded with fibrils from ox, pig and sheep respectively. Although more than 250

observations were made, the recoveries did not demonstrate any consistent species difference in the fibrils used (Table 1). Fibril formation occurs in the absence of seed, but generally in low yield (Table 1).

Values for the insulin content of pancreas obtained from samples of various animal species are given in Table 2. The figures for standard error refer to the individual values and because the number of animals available for study was limited,

Fig. 2. Recovery of insulin added to the alcohol-ether precipitate obtained from the acid-ethanol extracts of: ●, ox pancreas; ○, rat liver.

Table 1. Recovery of insulin from pure solution by the use of fibrils from various species as seed

Solutions contained 200 μg. of modified or of unmodified insulin; recoveries are expressed as percentages. Standard error, 4.9%.

Seed fibrils	Species of insulin				Oxidized insulin		Esterified insulin	Decarboxylase-modified insulin
	Ox	Pig	Sheep	Calf	A fraction	B fraction		
Ox	103.6	103.1	100.8	99.2	0.0	100.1	91.0	110.3
Pig	106.0	97.3	99.9	98.0	—	—	—	—
Sheep	108.7	93.0	97.1	101.5	—	—	—	—
None	80.5	93.3	86.6	97.1	—	43.7	—	—

Table 2. Yield of insulin from pancreas of various species

Results are expressed as μg./g. of pancreas; standard error, 5.2%. Figures in parentheses refer to the number of pancreases pooled.

Seed fibrils	Pancreas				
	Ox (5)	Pig (1)	Calf (2)	Cat (3)	Rat (48)
Ox	140	143	485	56	41
Pig	—	119	400	—	41
Sheep	—	125	460	56	31
None	—	151	436	—	19
Quoted by Haist (1944)	150–170	100–150	500	35–125	30–65

no attempt has been made to determine the statistical average of insulin content for each species. No consistent differences were observed when seed from different species was used.

DISCUSSION

The observation that the use of sulphuric acid in the original fibril method described by Waugh (1946) caused considerable precipitation of foreign protein could explain the lack of specificity reported by Foster *et al.* (1951) and Sutherland *et al.* (1949). Most of this interference was prevented by using hydrochloric acid and rapid stirring. Glucagon did not form fibrils. Small amounts which might come down with the insulin (Sutherland *et al.* 1949), although possibly of high biological activity, were negligible as protein impurities.

Contrary to Waugh *et al.* (1950) and Foster *et al.* (1951), who found pig fibrils to be the most efficient seed for recovery of insulin, no consistent species difference was observed. It may be suggested that the long period of rapid stirring used in the present procedure carried the fibril formation to completion so that individual differences in rate were unobservable.

The formation of fibrils by modified insulins agrees with the earlier observations of Waugh *et al.* (1950), of Koltun, Waugh & Bear (1954) and of Mommaerts & Neurath (1950). Evidence that the B fraction of oxidized insulin also forms fibrils suggests that the disulphide bonds are not involved and supports the view of Koltun *et al.* (1954) that the linkage may be due to the interaction of small non-polar side-chain groups.

Values for the insulin content of pancreas obtained from samples of various animal species (Table 2) are in general agreement with those quoted in the review by Haist (1944).

The present procedure, though perhaps not specific for the unmodified insulin molecule, appears to give satisfactory assays at the insulin level normally present in adult-animal pancreas. It thus seems to be a suitable one for metabolic studies which may be concerned with the level of insulin during the incubation of pancreatic slices, one of the purposes for which it was designed.

SUMMARY

1. A chemical method for the assay of insulin in 1 g. samples of pancreas tissue, utilizing the phenomenon of fibril formation, is described.

2. Recoveries are linear and near quantitative. Although some modified insulins can form fibrils, a general protein mixture from liver, as well as albumin and glucagon, does not interfere with the assay.

3. The levels of insulin found in the pancreas of ox, pig, sheep, calf, cat and rat were comparable with those obtained by biological-assay methods.

4. No differences in insulin yield were observed when seeding fibrils from ox, pig, and sheep insulins were used.

5. An apparatus is described for the simultaneous determination of 18 samples.

I should like to thank Dr A. C. Chibnall for suggestions. The continued interest, advice, and helpful discussions of Mr Maurice Rees are gratefully acknowledged.

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