

## THE ISOLATION AND CRYSTALLIZATION OF HUMAN INSULIN \*

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The introduction of immunologic procedures for the assay of insulin in the plasma or serum of man (1, 2), and the demonstration of various degrees of species specificity (3), prompted a need for highly purified or crystalline human insulin. Procedures for the isolation and crystallization of insulin from the pancreas of various species on a commercial scale are well-known (4-8). Repeated attempts by us and others (9), however, to apply these procedures, on a laboratory scale, to the human pancreas proved unsuccessful. For example, whereas acid-alcohol extracts of beef or pork pancreas yield a relatively white precipitate on the addition of from 10 to 25% NaCl, similar extracts of human pancreas yield a muddy brown precipitate that does not become any cleaner after repeated reprecipitations. Furthermore, adjusting the pH of a solution of the precipitate to the isoelectric point for insulin does not yield a cleaner product.

The demonstration that mixtures of proteins and peptides can be separated according to relative size by filtration through dextran gels (Sephadex<sup>1</sup>) (10, 11) led to the attempt to isolate human insulin by this technique. After trying various dextrans as well as various buffers, a procedure was developed that permits the crystallization of human insulin with fairly good yields.

### METHODS

The human pancreases utilized in this study were obtained at autopsy at various undetermined intervals after death, frozen as rapidly as possible, and stored in a deep freezer until ready for use. The frozen pancreases were extracted by either the acid-alcohol procedure (4, 5) or the salt-alcohol procedure described by Romans (7, 8). Since preliminary experiments suggested that the latter procedure, with minor modifications, resulted in a greater yield, it was used in most of the studies conducted during the past 2 years.

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<sup>1</sup> Pharmacia Fine Chemicals, Inc., Rochester, Minn.

The frozen pancreas was diced and weighed, and 100-g portions were homogenized for 30 to 60 seconds in a blender with 250 ml of 92% ethyl alcohol containing 20 g sodium chloride and adjusted to pH 4 with concentrated HCl. The homogenate was adjusted rapidly to pH 5.0 to 5.2 with 3 N HCl and maintained there for 10 minutes during which the suspension was stirred continuously. The homogenate was centrifuged at 4° C for 15 to 20 minutes, and the supernatant fluid was removed. The residue was re-extracted twice with 250 ml of 75% ethyl alcohol for 30 minutes during which the pH was maintained at 5.0 to 5.2. After centrifugation, the supernatant fluids were combined and concentrated to  $\frac{1}{3}$  vol in a flash evaporator at 34° C. The concentrate was defatted by the addition of 15% ligrin, shaken vigorously for 5 minutes, and layered by centrifugation for 15 minutes. The lower, clear, alcohol phase was removed, filtered through Whatman 3 paper, and adjusted to pH 3 with N HCl, and 4 g NaCl per 100 ml was added. The resultant solution was concentrated to approximately half its volume, and the precipitate that formed was collected by centrifugation. The precipitate was dissolved in 1 N acetic acid (or 0.01 N HCl) in a volume equivalent to approximately one-third of the defatted extract. The solutions of the precipitates from the equivalent of 400 to 500 g of pancreas were combined, and 10% NaCl, wt/vol, was added. The resultant precipitate was collected by centrifugation, dissolved in approximately 100 ml 1 N acetic acid (or 0.01 N HCl) and filtered, and the filtrate was placed on a 4.4- × 140-cm column of coarse Sephadex G50.

The column was prepared according to the procedure described by Flodin (12) after removal of fines from the dextran gel suspended in 1 N acetic acid (or 0.01 N HCl), which was used as the eluting solution. The sample was washed in with several small portions of the eluent, and the elution was started at a flow rate of from 60 to 80 ml per hour. The effluent was collected in 15- to 20-ml fractions, and its absorbancy at 280 m $\mu$  was monitored with a Gilson absorption meter.

The fractions containing insulin (Figure 1) were pooled, and 15% NaCl, wt/vol, was added. The precipitate was collected by centrifugation and dissolved in 100 ml 0.01 N HCl, the resultant solution was adjusted to pH 5.1 to 5.4 with NaOH and maintained at 4° C for 24 to 48 hours. The isoelectric precipitate was dissolved in a minimal volume of 0.01 N HCl, acetone was added to a final concentration of 16%, and the mixture was adjusted to pH 7.4 with 5 N NaOH. The precipitate that formed was removed by centrifugation and subsequently

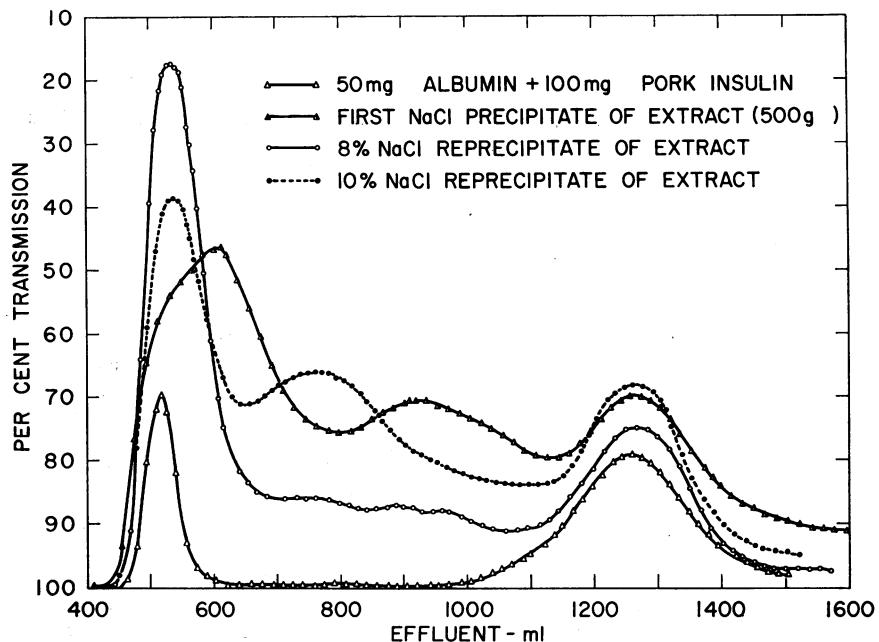


FIG. 1. ELUTION PATTERNS OF EXTRACTS OF HUMAN PANCREAS. Various preparations chromatographed on Sephadex G50C (see text).

reprocessed. The protein concentration of the supernatant fluid was determined, and the solution was subsequently adjusted to a concentration of from 0.5 to 1%. To this was added an equal volume of a citrate buffer containing  $Zn^{++}$  (13) (56 g sodium hydroxide, 100 g citric acid, 400 ml of a 4.4% solution of zinc sulfate, 100 ml acetone, and water to 1,000 ml). The supernatant fluid was adjusted to pH 6.1 to 6.2, stirred for 1 hour at room temperature, and then stored at 4° C. Crystals appeared in from a few minutes to several hours and were harvested at various intervals after the first 72 hours. The crystals were removed by slow speed centrifugation, repeatedly suspended in distilled water and centrifuged, and finally suspended in absolute ethanol and permitted to dry *in vacuo* at room temperature.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (14) using both crystalline bovine albumin and crystalline pork insulin<sup>2</sup> as standards. The hypoglycemic response of rabbits to the injection of various quantities of the effluents was utilized as a qualitative index of the presence of insulin. Quantitative assays were performed with the immunoassay described by Yalow and Berson (1) and the adipose tissue assay described by Leonards, Landau, and Bartsch (15). Blood glucose was determined by the ferricyanide autoanalyzer procedure (16).

<sup>2</sup> We are indebted to Dr. W. R. Kirtley of the Eli Lilly Company, Indianapolis, Ind., for generous supplies of crystalline pork insulin.

#### RESULTS AND DISCUSSION

The pancreatic extracts were prepared by homogenization rather than by the usual extraction of minces for approximately 6 hours because no appreciable differences in the yields of insulin obtained by the two procedures were noted. Accordingly, it became possible to complete the preparation of an extract from 400 to 500 g of pancreas and to start its separation on a column in from 6 to 8 hours.

The data depicted in Figure 1 illustrate the reproducibility of the effluent patterns obtained with gel filtration of various preparations through a 4.4- × 140-cm column of coarse Sephadex G50. Thus, a mixture of 50 mg bovine serum albumin and 100 mg crystalline pork insulin yielded two distinct peaks, the first appearing at an effluent volume of from 425 to 600 ml and the second at from 1,100 to 1,400 ml; 98 mg insulin crystals was recovered from the second peak. The first salt precipitate obtained by concentrating the defatted extract of 500 g pancreas yielded three peaks of protein, the third occurring in the same volume as the insulin. Although some hypoglycemic activity was detected occasionally in the effluent constituting the fractions from 900 to 1,000 ml, the

major hypoglycemic activity was found in the third fraction. Gel filtration of the solution of a second precipitation with 10% NaCl (see Methods) resulted in a sharper separation between the second and third peaks. When 8% NaCl was used for the second precipitation, most of the protein responsible for the second peak was removed. Since the latter treatment appeared to reduce the quantity of insulin present in the third peak, 10% NaCl was used routinely to precipitate the solution of the first salt precipitate. The results obtained when 0.01 N HCl was used as the eluent were essentially the same as those obtained with 1 N acetic acid. The latter, however, was found to be preferable because the dextran gel did not have to be cleaned so frequently as was necessary when HCl was used.

Although crystals of human insulin can be obtained with the ammonium acetate buffer that many use for the crystallization of insulin from other species, the citrate buffer procedure (6) as modified by Schlichtkrull (13) is preferable because it yields insulin that is relatively free of glucagon (17). In accord is our observation that whereas acrylamide disc electrophoretograms (18) of crystalline human insulin prepared with ammonium acetate buffer revealed the presence of glucagon, crystals prepared as described here were free of glucagon.

The crystals of human insulin vary in size and resemble those of other species in that they are rhombohedral with perfectly sharp edges. As can be anticipated from the way the pancreases were obtained, the yield of crystals from more than 200 preparations varied markedly, from 0 to 75 mg per kg with a mean of  $28.2 \pm 2.4$  SE mg per kg pancreas. On the basis of  $\text{C}^{14}\text{O}_2$  production and  $\text{C}^{14}$  incorporation into glycogen from glucose-1- $\text{C}^{14}$  by rat epididymal adipose tissue (15), the potency (mean  $\pm$  SE) of the crystals was  $27 \pm 0.5$  U per mg and  $23.7 \pm 0.7$  U per mg, respectively. Immunoassays revealed the crystalline human insulin to have the same potency as crystalline porcine insulin.

The procedure reported here provides a relatively simple method for the isolation and crystallization of insulin from human pancreas. That this may be accomplished by other procedures is quite evident since two pharmaceutical companies have made crystalline human insulin available for

investigative purposes. The procedures and yields obtained by the two groups, however, have not been published. More recently, crystalline human insulin has been prepared by a modification of earlier processes by Jamieson and Fisher (19). The gel filtration procedure reported here, however, appears to be much simpler to apply than other methods that have been reported. Since the gel bed is ready to be used as soon as the slowest solute has been washed out by the eluate, it is possible to use the sample column for many fractionations. Flodin's recent introduction of an automatic semicontinuous procedure (20) suggests the applicability of the gel filtration procedure to fairly large scale isolations of insulin from pancreatic extracts.

During the past 2 years, in addition to the above, the dextran gel filtration procedure described here has been used successfully for the isolation and crystallization of insulin from monkey, beef, pig, dog, rat, and chicken pancreases. The use of gel filtration in the isolation of insulin from cat pancreas has been reported recently by Davorin (21). After this report was submitted for publication, our attention was drawn to the recent report on the use of a gel filtration procedure by Epstein and Anfinsen (22) for the isolation of insulin from small quantities of bovine pancreatic tissue.

#### SUMMARY

A relatively rapid, simple procedure for the isolation and crystallization of human insulin is described. Yields of approximately 30 mg of crystals per kg of pancreas were obtained.

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#### REFERENCES

1. Yalow, R. S., and S. A. Berson. Immunoassay of endogenous plasma insulin in man. *J. clin. Invest.* 1960, **39**, 1157.

2. Arquilla, E. R. Immunological studies of insulin. *Ciba Foundation Colloquium Endocr.* 1962, **14**, 146.
3. Berson, S. A., and R. S. Yalow. Species-specificity of human antipeef, pork insulin serum. *J. clin. Invest.* 1959, **38**, 2017.
4. Scott, D. A., and A. M. Fisher. Crystalline insulin. *Biochem. J.* 1935, **29**, 1048.
5. Romans, R. G., D. A. Scott, and A. M. Fisher. Preparation of crystalline insulin. *Industr. eng. Chem.* 1940, **32**, 908.
6. Petersen, K. Method of producing crystalline insulin. U. S. Patent Office no. 2,626,228, 1953.
7. Romans, R. G. The preparation and chemistry of crystalline insulin. *Recent Progr. Hormone Res.* 1954, **10**, 241.
8. Romans, R. G. Insulin process. U. S. Patent Office no. 2,897,117, 1959.
9. Field, J. B., F. Tietze, and D. Stetten, Jr. Further characterization of an insulin antagonist in the serum of patients in diabetic acidosis. *J. clin. Invest.* 1957, **36**, 1588.
10. Porath, J., and P. Flodin. Gel filtration: a method for desalting and group separation. *Nature (Lond.)* 1959, **183**, 1657.
11. Porath, J. Gel filtration of proteins, peptides and amino acids. *Biochim. biophys. Acta (Amst.)* 1960, **39**, 193.
12. Flodin, P. Methodological aspects of gel filtration with special reference to desalting operations. *J. Chromatography* 1961, **5**, 103.
13. Schlichtkrull, J. Insulin crystals: II. Shape of rhombohedral zinc-insulin crystals in relation to species and crystallization media. *Acta chem. scand.* 1956, **10**, 1459.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951, **193**, 265.
15. Leonards, J. R., B. R. Landau, and G. Bartsch. Assay of insulin and insulin-like activity with rat epididymal fat pad. *J. Lab. clin. Med.* 1962, **60**, 552.
16. Grady, H. J., and M. A. Lamar. Glucose determination by automatic chemical analysis. *Clin. Chem.* 1959, **5**, 542.
17. De Duve, C., and H. G. Hers. La pretendue action glycogenolytique de l'insuline. *C. R. Soc. Biol. (Paris)* 1947, **141**, 1147.
18. Ornstein, L., and B. J. Davis. Disc Electrophoresis. Rochester, N. Y., Distillation Products Industries, 1962.
19. Fisher, A. M. Personal communication, 1963.
20. Flodin, P. Dextran Gels and Their Application to Gel Filtration. Uppsala, Sweden, Pharmacia, 1962.
21. Davoren, P. R. The isolation of insulin from a single cat pancreas. *Biochim. biophys. Acta (Amst.)* 1962, **63**, 150.
22. Epstein, C. J., and C. B. Anfinsen. The use of gel filtration in the isolation and purification of beef insulin. *Biochemistry* 1963, **2**, 461.