# Preliminary Observations on State of Insulin in Human and Bovine Pancreas.* (25632)

HARRY N. ANTONIADES, ALBERT E. RENOLD, YVES M. DAGENAIS† AND JURGEN STEINKE‡ (Introduced by George W. Thorn)
*Protein Foundation Lab., Jamaica Plain, and Baker Clinic Research Lab., Dept. of Medicine, Harvard Medical School, Boston, Mass.*

Previous reports demonstrated that blood insulin-like activity could be adsorbed on cationic exchange resin, in the sodium form, at physiological pH. This activity could then be eluted from the resin with dilute acids(1,2, 3). Since insulin is negatively charged at physiological pH, its adsorption on cationic exchange resin suggested the presence of an insulin complex in blood, insulin being subsequently freed from the complex during elution with acid. Control experiments demonstrated that crystalline insulin was not adsorbed on the same cationic exchange resin at physiological pH, when dissolved in presence of human albumin(1). Insulin-$I^{131}$ added to blood serum similarly was not adsorbed on the same resin. It was further observed that the blood insulin complex eluted from resin with 0.1 N $H_2SO_4$ (pH 3) was biologically inactive when tested for insulin-like activity with the rat diaphragm assay. However, when the eluate was subjected to precipitation at pH 10, insulin-like activity toward rat diaphragm reappeared,§ suggesting removal of a basic protein component of the complex at that pH. Present studies on insulin in human and bovine pancreas indicate that a major fraction of insulin in pancreas, as in blood, is readily adsorbed on cationic exchange resin, in the sodium form, from homogenates prepared in 0.15 M sodium chloride, at pH 6.6 $\pm$ 0.04. The studies presented here suggest that a major fraction of insulin in pancreas, as in blood, may be in a complex form, possibly with basic proteins, the insulin complex being insoluble in saline at physiological pH.

*Materials and methods.* Dowex-50 cationic exchange resin (x8; x2) in the sodium form, has been employed. The resin was prepared as for routine blood collection by washing with distilled water, sodium chloride, sodium hydroxide and disodium phosphate, the final pH of 5 ml 0.01 M sodium sulfate over 1 ml resin beads being 6.6 $\pm$ 0.4. The following procedure was used for extraction of insulin activity from pancreas: Frozen human or bovine pancreas was homogenized and suspended in 5 to 10 volumes of 0.15 M sodium chloride solution with a Waring blendor at 2°C. One volume of Dowex-50-x2 or 2 volumes of Dowex-50-x8 exchange resin (sodium form) were added to the pancreatic suspension, and the mixture stirred for 5 minutes at 2°C. Supernatant fluid was removed from the resin which was repeatedly washed with cold 0.15 M sodium chloride. Elution of insulin-like activity from the resin was carried out at 2°C with 2 to 5 resin volumes of 0.1 N sulfuric acid (5 to 15 min.) followed by elution (5 min.) with one to 3 volumes of 1 N $NH_4OH$ at 2°C. The combined eluates were neutralized, diluted with 10 to 100 volumes of bicarbonate buffer and assayed for insulin-like activity. Biological assays for insulin-like activity were carried out with the rat adipose tissue technic based on the effect of insulin on $C^{14}O_2$ production from glucose-1-$C^{14}$ by this tissue(4). Anti-serum neutralization studies of the insulin-like activity were carried out with anti-insulin serum obtained by immunizing guinea pigs against bovine insulin according to the procedure of Moloney (5).‖ Resin eluates for paper electrophoresis were obtained with 0.1 N $NH_4OH$. The pH of the eluates was adjusted to 3.0 with

* This work was supported by grants from Nat. Foundation, Eli Lilly and Co. and Rear Admiral W. L. Capps Fund at Harvard Med. School.

† Recipient of a Research Fellowship, R. Samuel McLaughlin Fn., Toronto, Ont.

‡ Recipient of Research Fellowship from Am. Diabetes Assn.

§ Gundersen, K., personal communication.

‖ Insulin anti-serum was prepared by Drs. Werner Creutzfeldt and Paul Lacy and was made available to us through their courtesy and generosity.

1 N $H_2SO_4$, and the precipitate removed by centrifugation at 2°C. The supernatant fluid was brought to pH 9.5 with 1 N $NH_4OH$, and the solution stored overnight at 2°C. Precipitate was removed by centrifugation at 2°C, washed with dilute ammonium hydroxide and suspended in the minimum volume of cold distilled water. In the presence of 0.1 N $H_2SO_4$ (pH 3.0) the sulfate salt of the basic protein was water soluble, and samples of this solution were applied to paper for electrophoresis. Pater electrophoresis for detection of pancreatic basic proteins present in resin eluate concentrates was carried out at pH 7.6 (phosphate buffer, ionic strength 0.1) and at pH 4.5 (acetate buffer, ionic strength 0.1) on the Spinco model R, series D, paper electrophoresis cell, at room temperature.

*Results.* Table I summarizes results obtained to date. Although these values are preliminary, comparison with recovery obtained with the classic acid-alcohol extraction procedure suggests that resin-extractable insulin accounts for an important fraction of total pancreatic insulin. Ten ml of wet resin volume (Dowex-50-x2) were sufficient for adsorption of at least 85% of the adsorbable insulin present in 1 g of bovine pancreas (Table II).

The values obtained relate to insulin-*like* activity only, as measured with the rat adipose tissue technic. However, the following observations suggest that the insulin-like activity measured was indeed insulin: (1) Eluates obtained from resin exposed under identical conditions to homogenates of tissues other than the pancreas exhibited no insulin-like activity; (2) insulin-like activity could be demonstrated at dilutions as great as hundredfold in buffer, greatly reducing the likelihood of nonspecific interference; (3) hypoglycemic activity of resin eluates has been established in 24-hour fasted rabbits on 4 separate occasions; (4) anti-insulin serum against bovine insulin neutralized the insulin-like activity extracted from bovine pancreas with the use of Dowex-50-x2 resin (Na+ form) after acid-alcohol treatment of the extracts; (5) insulin-like activity of the eluates was destroyed by incubation with 0.02 M reduced glutathione.

These findings suggest that the portion of pancreatic insulin activity extractable with cationic exchange resin under the conditions described exists in a complex form with other proteins, presumably basic proteins. Adsorption at near-neutral pH, as described here, should not be confused with adsorption of crystalline insulin (or insulin partially purified) on acidic cationic exchange resin in the hydrogen form, which has been observed at pH values below 5.0(6,7). At these low pH values insulin bears a positive charge and can be adsorbed on the cationic resin and subsequently eluted with citric acid(6) or ammonium hydroxide(7). Control experiments have shown that insulin-$I^{131}$, added to pancreatic tissue during its homogenization, is not adsorbed on the cationic exchange resin at physiological pH, indicating that the insulin complex is not the result of non-specific binding of insulin to basic proteins of pancreatic homogenates. Paper electrophoresis of resin

TABLE I. Insulin-like Activity Extracted from Pancreas with Cationic Exchange Resins (Na+ form) at pH 6.6 ± 0.4 (Units/100 g of Pancreas).

| Pancreas | Exchange resin | Insulin-like activity* (units) |
|---|---|---|
| Bovine† | Dowex-50 x8 | 150 |
| | " | 162 |
| | " | 142 |
| | Dowex-50-x2 | 240 |
| | " | 180 |
| | " | 300 |
| Human | Dowex-50-x8 | 63‡ |
| | Dowex-50-x2 | 234 |
| | " | 397 |

* Combined 0.1 N $H_2SO_4$ and 1 N $NH_4OH$ eluates.

† The rather wide range within each species may in part be due to differences in fatty infiltration of pancreatic tissue.

‡ Pancreas from 37-year-old diabetic. This value is not reported to estimate total extractable insulin in a diabetic pancreas, but solely to demonstrate presence of the resin extractable insulin complex in such tissue.

TABLE II. Insulin-like Activity Extracted from 1 g of Bovine Pancreas during Repeated Treatment of Pancreas Homogenate with Dowex-50-x2 Resin, in Sodium Form (Each Treatment Carried Out with 10 ml Wet Volume Resin).

| Resin treatment | Exp. I (units) | Exp. II (units) |
|---|---|---|
| 1 | >1.8 | 1.5 |
| 2 | .12 | .2 |
| 3 | .06 | .05 |
| 4 | < .02 | < .02 |

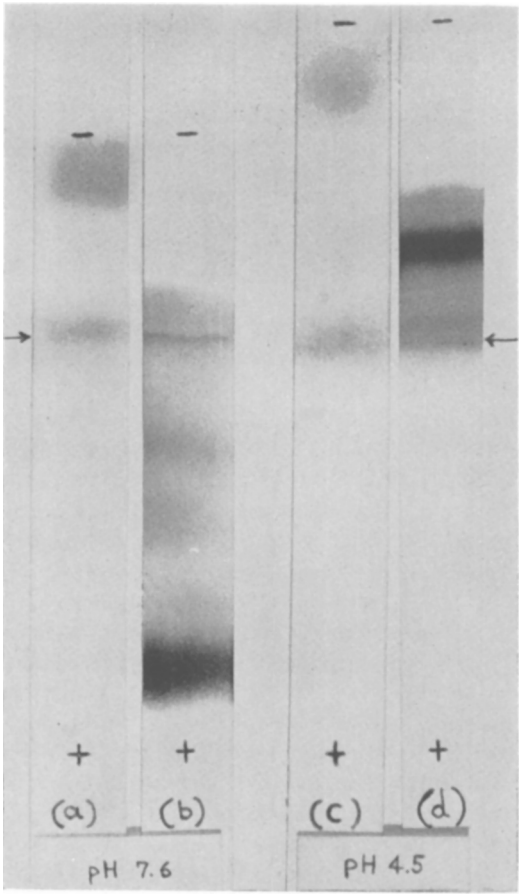


FIG. 1. Paper electrophoresis of pancreatic basic protein concentrate prepared from resin eluates, at pH 7.6 (phosphate buffer, ionic strength 0.1) and at pH 4.5 (acetate buffer, ionic strength 0.1). (a) and (c) basic protein concentrate; (b) and (d) plasma control.

eluates obtained from bovine pancreas indicates the presence of protein(s) which migrate as cations (Fig. 1). At 2 different pH values (pH 7.6 and pH 4.5) only a single basic protein appears on paper electrophoresis, although additional information will be needed before homogeneity of this protein fraction may be assumed.

Consistent with the presence of an insulin complex in pancreas is the observation that insulin-like activity present in resin eluates was not inhibited by addition of anti-insulin serum. Subsequent treatment of the eluate by acid-alcohol precipitation altered insulin-like activity of the supernatant in that neutralization of the activity by addition of anti-serum could now be obtained. This suggests that insulin present in the eluate was bound to other proteins which prevented its reaction with the anti-serum. Upon removal of these proteins by acid-ethanol precipitation, reaction with the anti-serum could proceed.

The physiological significance of these observations is unknown. Lindner(8) has previously reported that insulin immediately extracted from fresh beef pancreas under special conditions is combined with a basic protein of low molecular weight, probably a histone. The preparation, termed "Nativinsulin," is poorly soluble at the pH of blood and tissue and is but slowly adsorbed from subcutaneous injection sites. The major association of insulin with basic proteins in pancreas, postulated here on the basis of observations with cationic exchange resin, may primarily serve to allow for insulin storage at physiological pH.

*Summary.* A major fraction of insulin in human and bovine pancreas is readily adsorbed on cationic exchange resin, in sodium form, from homogenates prepared in 0.15 M sodium chloride at pH 6.6 ± 0.4. These studies suggest that this fraction of insulin in pancreas may be in a complex form, possibly with basic proteins, the insulin complex being adsorbed on the resin under specified conditions.

1. Antoniades, H. N., Beigelman, P. M., Pennell, R. B., Thorn, G. W., Oncley, J. L., *Metabolism Clin. and Exp.,* 1958, v7, 266.

2. Antoniades, H. N., *Science,* 1958, v127, 593.

3. Beigelman, P. M., Antoniades, H. N., Goetz, F. C., Renold, A. E., Oncley, J. L., and Thorn, G. W., *Metabolism,* 1956, v5, 44.

4. Martin, D. B., Dagenais, Y. M., Renold, A. E., *Lancet,* 1958, vii, 76.

5. Mcloney, T. J., Coval, M., *Biochem. J.,* 1955, v59, 179.

6. Boardman, N. K., *Biochem. et Biophys. Acta,* 1955, v18, 290.

7. Samsonov, G. W., Faleev, M. D., *Biochemistry,* 1956, v21, 407. (Eng. Trans.)

8. Lindner, F., *Medizin. u. Chemie,* 1938, v4, 248. Also referred to by Lindner, F. in *Insulin u. Insulintherapie,* Editors: Stich, W., Maske, H., *Urban u. Scharzenberg,* Munich, 1956, p22.

Received January 8, 1960. P.S.E.B.M., 1960, v103.