Separation of Human Insulin, Glucagon and Other Pancreatic Proteins

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SUMMARY

Purified human insulin and beef-pork glucagon were subjected to electrophoresis on cellulose acetate strips. Insulin was found to migrate rapidly towards the anode as a single band, while glucagon migrated slowly towards the anode in two distinct fractions. Virtually complete separation of the two hormones was achieved.

Electrophoresis of acid alcohol extracts of human pancreas revealed eight positively migrating bands. By means of densitometric, radioisotopic, and radioimmunochemical technics, fraction 2 was identified as the insulin-bearing fraction, and the area of fractions 7 and 8 as the glucagon-bearing region. By gel filtration substantial quantities of the unidentified extraneous proteins, which make up the bulk of the pancreatic extract, were removed, thereby providing purer samples for electrophoresis.

The technic makes possible the rapid preparation of relatively pure samples of glucagon-free insulin and insulin-free glucagon from the pancreas of man and other species in quantities sufficient for laboratory studies. In addition, the characterization of the electrophoretic mobility of these hormones provides a new means by which they may be identified in unknown samples.

A simple method for the preparation of relatively pure samples of human insulin and glucagon for laboratory use would be useful in a variety of experimental pursuits. Although a limited supply of human insulin in highly purified form has recently been made available,* human glucagon has not yet been isolated; furthermore, insulin and glucagon from other nonungulate species are not generally available.

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Portions of this study will appear in a Ph.D. thesis by J. de V. Lochner submitted at the University of Stellenbosch, Stellenbosch, South Africa.

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The following report describes a relatively simple, rapid, and inexpensive technic by which human insulin and glucagon can be separated from each other and, in part, from other pancreatic proteins and microgram quantities of each recovered in partially purified form.

MATERIALS AND METHODS

Crude human pancreatic extracts, prepared by the Kenny modification[1] of the acid-alcohol method of Best et al.,[2] were dissolved to a concentration of 0.5 mg. per ml. in a Tris buffer (*p*H 9.0),[3] containing 8M urea. After centrifugation, 5 µl. of the clear supernatant was applied along a line near the cathodal end of a 5 x 12 cm. cellulose acetate strip, placed horizontally in a Shandon Universal electrophoresis apparatus which holds four strips of this size. Electrophoresis was carried out at 4° C. in a Tris buffer solution of *p*H 9 (ionic strength 0.04) at a constant current of 0.4 mA/cm. of paper-width for 150 minutes. The strips were then dried for fifteen minutes at 80° C., strained for ten minutes with a saturated solution of Amido black in a methanol-water-glacial acetic acid mixture (4:5:1), and rinsed in the latter mixture. Permanent recordings of electrophoretograms were obtained by densitometric scanning with a self-registering Spinco Analytrol.

In certain experiments eluates of the various electrophoretically separated pancreatic fractions were prepared. Immediately after electrophoresis each strip was bisected longitudinally. One half was stained as indicated previously and was employed as a guide for division of the unstained strip-half into segments, each containing a separate fraction. Each strip segment was eluted individually by swirling for thirty minutes in a test tube with from 2 to 10 ml. of a 0.2M glycine solution containing 2 per cent human albumin (*p*H 9). The efficiency with which insulin and glucagon can be eluted from the strips by the foregoing method was tested by determining the recovery of insulin-I-131 and glucagon-I-131 after electrophoresis.

In five such experiments, the recovery of glucagon-I-131 averaged 92 per cent (85-97 per cent) and that of insulin-I-131 91 per cent (84-94 per cent).

In other experiments, small amounts of I-131-labeled beef-pork glucagon or pork insulin* of high specific activity were mixed with extract prior to electrophoresis for marking purposes. Following electrophoresis each fraction was eluted, treated with cold 10 per cent trichloracetic acid (TCA). The TCA-precipitable radioactivity was determined in a Tracerlab scintillation counter.

In a third group of experiments, eluates of each fraction were assayed for insulin by the immunochemical method of Yalow and Berson[4] and for glucagon by the method of Unger, Eisentraut, McCall, and Madison.[5]

Evaluation of a gel-filtration technic to enhance the purity of crude extracts prior to electrophoresis was studied. Sephadex† columns (G-25, 100-250 mesh, water regain: 2.4 gm. per gm. of dry weight) were prepared by the Manipol and Spitzy adaptation[6] of the method of Porath and co-workers,[7,8] using Tris-buffer as eluent. The protein content of the eluates was determined spectrophotometrically at 278 mu., the absorption peak of glucagon.[9] The samples to be investigated further were desalted on another Sephadex column, prepared with diluted Tris-buffer (1:64) as described by Porath and Flodin,[7] and the protein material recovered from the desalted product by alcohol-ether precipitation at *p*H 7.5.[1]

RESULTS

Electrophoretic behavior of purified pancreatic hormones

1. *Densitometry.* The premixture of insulin and glucagon prior to electrophoresis did not alter the location or form of the densitometric patterns of either hormone: Purified human insulin‡ was found to migrate rapidly towards the anode in a single band (figure 1). Crystalline beef-pork glucagon§ migrated less rapidly towards the anode and, as first shown by Staub et al.,[9] became separated into two distinct fractions. Densitometric scanning of the strip revealed that the faster-moving, minor component of glucagon comprised approximately 9 per cent of the glucagon applied (figure 1). These findings indicate the feasibility of electrophoresis as a means of separating glucagon and insulin.

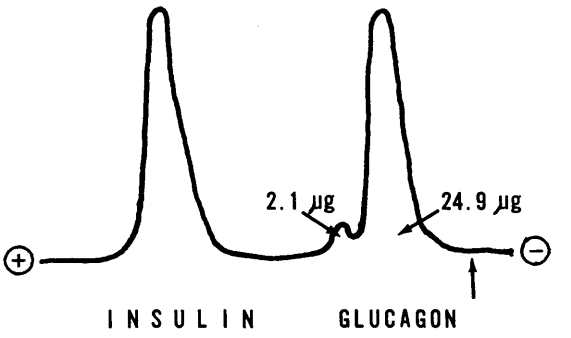

FIG. 1. *Electrophoretic separation of a mixture of purified human insulin and beef-pork glucagon. Glucagon and insulin were quantitated immunochemically. The application point is indicated by the vertical arrow.*

To determine if urea influenced the electrophoretic behavior of insulin or of glucagon, electrophoresis was also performed on mixtures of the hormones dissolved in buffer with and without urea. No differences in the patterns could be demonstrated.

2. *Immunochemical assay of hormones.* To evaluate by more sensitive technics the completeness of electrophoretic separation, an insulin-glucagon mixture, demonstrated immunochemically to contain 57.8 µg of insulin and 29.1 µg of glucagon, was subjected to electrophoresis, and eluates of the insulin- and glucagon-bearing segments of the strip were immunoassayed for insulin and glucagon. In the case of insulin, 43.5 µg, or 75 per cent of the 57.8 µg applied was recovered in the eluate of the insulin-bearing segment, and 27 µg of glucagon, or 93 per cent of the 29 µg applied was recovered from the eluate of the glucagon-bearing sections (figure 1). Of the glucagon recovered, 24.9 µg was present in the major fraction, and 2.1 µg, or approximately 8 per cent of the total recovered, was present in the minor component. This similarity of the immunochemically and densitometrically determined ratios of major to minor glucagon fractions suggests that the two moieties do not differ immunologically.

Electrophoresis of crude extracts of human pancreas

Densitometric scans of electrophoretograms of crude human pancreatic extracts revealed the presence of eight protein fractions which moved towards the an-

*Abbott Laboratories, Oak Ridge, Tennessee, kindly supplied I-131-labeled beef-pork glucagon and pork insulin.

†Pharmacia, Uppsala, Sweden.

‡Kindly furnished by Dr. Mary Root, Eli Lilly, Inc., Indianapolis, Ind. Lot No. 493-10GP-181 had a biologic activity of 25.1 U./µg.

§Kindly supplied by Dr. W. R. Kirtley, Eli Lilly, Inc., Indianapolis, Ind. Lot No. 258-234B-167-1.

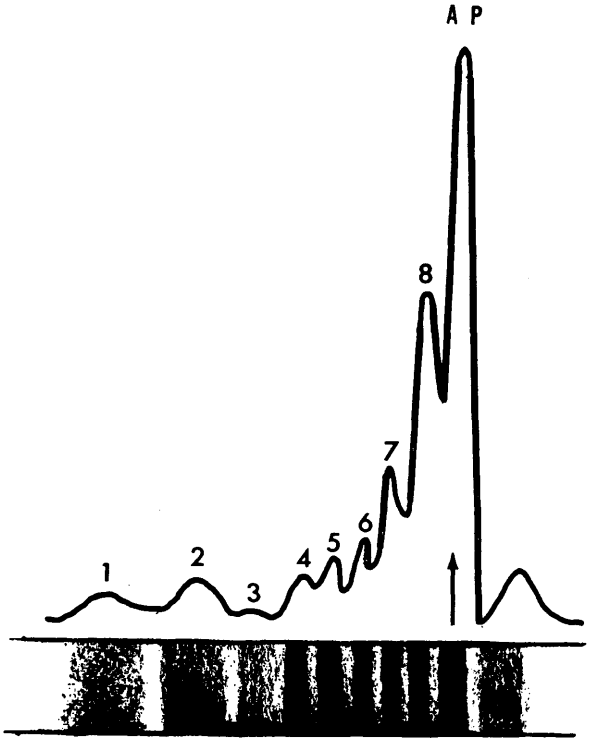

FIG. 2. The electrophoretic separation of a crude, human pancreatic extract: Electrophoretogram (retouched) and densitometric scan pattern show eight components (1-8) migrating towards anode (left), one immobile (AP), and one negatively migrating fraction.

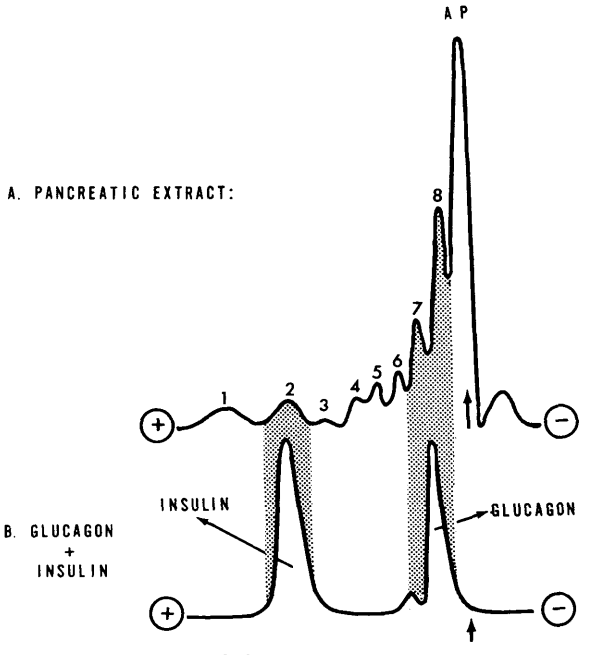

FIG. 3. A comparison of the densitometric scan patterns of a crude, human pancreatic extract (A) and a mixture of human insulin and beef-pork glucagon (B). The application points (vertical arrows) are aligned and insulin corresponds to fraction 2, while the glucagon area spans fractions 7 and 8.

ode, of one immobile component, and of one fraction which migrated towards the cathode (figure 2). The positively moving fractions were assigned the numbers 1 through 8, 1 being the most rapidly moving and 8 the most slowly migrating. The densitometric pattern is shown in figure 2 and provides a semi-quantitative indication of the pattern of protein distribution.

Identification of insulin- and glucagon-bearing fractions of human pancreatic extracts

Comparison of the densitometric scan pattern of an electrophoretogram of a human pancreatic extract (figure 3A) with that of a mixture of purified human insulin and beef-pork glucagon (figure 3B) suggests that insulin corresponds to pancreatic fraction 2, while the glucagon area spans fractions 7 and 8.

Further identification of the hormone-bearing fractions was made by the following technics: Forty μg of purified human insulin was premixed with crude extract prior to electrophoresis and the densitometric scan patterns with and without the added insulin were compared. The addition of insulin to the extract resulted in a 472 per cent increase in the density of fraction 2, without any alteration in the density of other fractions, as judged by the heights of the peaks (figure 4). Similarly, when 10 μg of beef-pork glucagon was premixed with the extract prior to electrophoresis, an 88 per cent increase in fraction 7 and a less imposing rise in fraction 8 were observed (figure 5).

Because of the limited sensitivity of densitometry as a means of hormone localization, I-131-labeled hormones were also employed. Trace quantities of radiohormone were premixed with extract, the mixture subjected to electrophoresis, and eluates of each fraction assayed for TCA-precipitable radioactivity. After the premixture of 0.190 mμg of pork insulin-I-131, 60 per cent of TCA-precipitable radioactivity was present in the eluate of fraction 2, save for minor degrees of trailing, thus providing further identification of fraction 2 as the insulin-bearing fraction. The presence of other pancreatic proteins did not alter the distribution of insulin-I-131 appreciably, since the same pattern was found when labeled insulin was electrophoresed independently.

When 5 mμg beef-pork glucagon-I-131 was similarly premixed with crude extract and electrophoresed, 53 per cent of the TCA-precipitable radioactivity was present in eluates of fractions 7 and 8. However, wide-

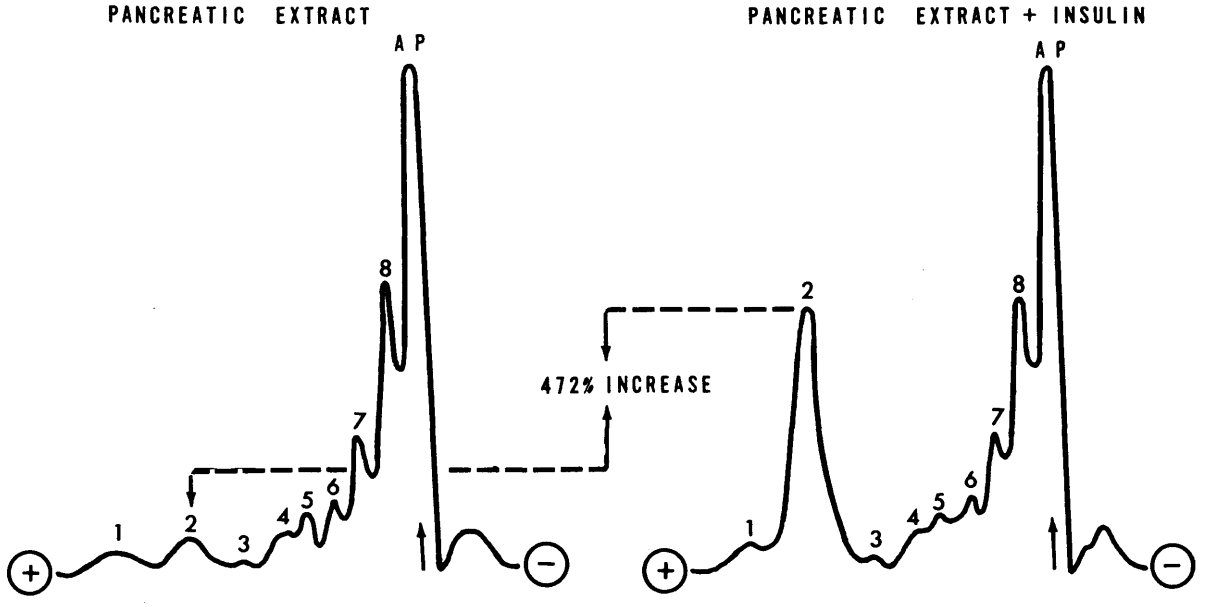

FIG. 4. The localization of the insulin-bearing component in a crude, human pancreatic extract: A comparison of the densitometric patterns of the extract alone (left) and one premixed with 40 μg of human insulin (right). The added insulin caused a 472 per cent increase in fraction 2.

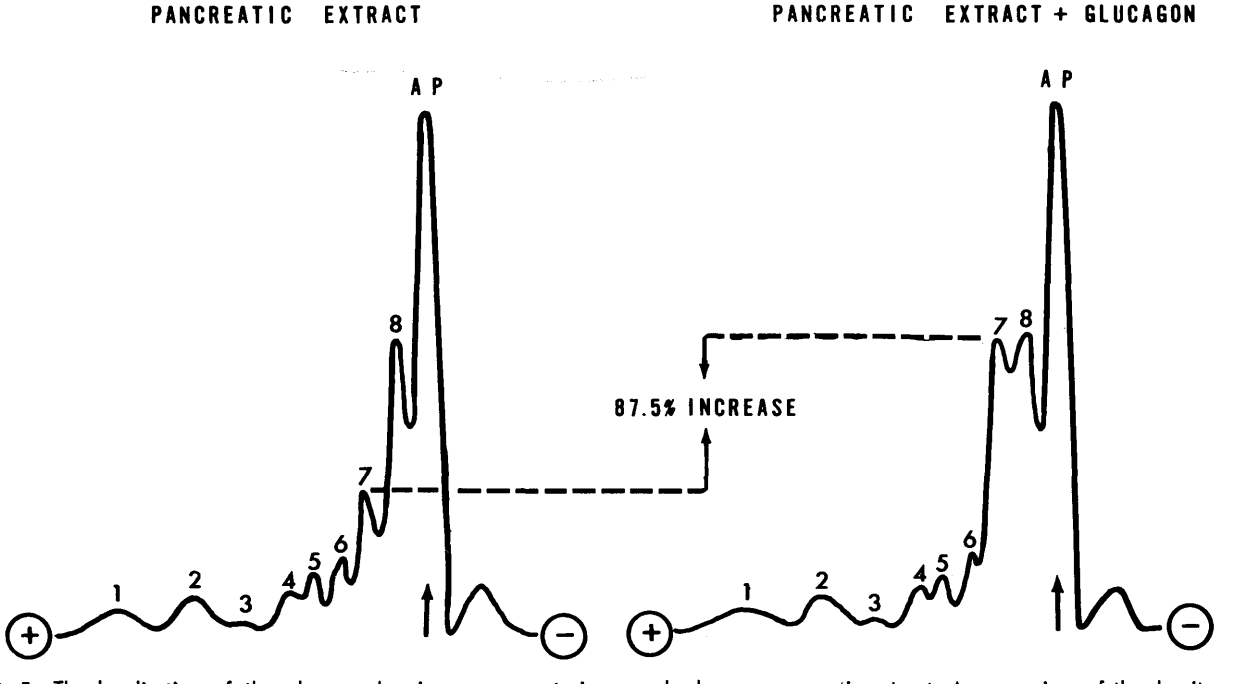

FIG. 5. The localization of the glucagon-bearing components in a crude, human pancreatic extract: A comparison of the densitometric patterns of the extract alone (left) and one premixed with 10 μg of beef-pork glucagon (right). An 87.5 per cent increase in fraction 7 and a modest increase in fraction 8 occurred on addition of glucagon.

spread distribution of TCA-precipitable radioactivity in other fractions was observed. To determine if the presence of high concentrations of pancreatic proteins might have influenced the electrophoretic behavior of trace quantities of glucagon-I-131, the migration of radioglucagon with and without extract was compared. An increase in radioactivity in the faster moving fractions at the expense of fractions 7 and 8 was noted

after exposure of glucagon-I-131 to pancreatic proteins; this may represent damage and may explain, at least in part, the apparent discrepancy in the electrophoretic distribution of labeled and unlabeled glucagon.

As a final means of hormone localization, the insulin and glucagon present in the eluate of each fraction were measured directly by radioimmunochemical technics. In the experiment illustrated in figure 6, 79

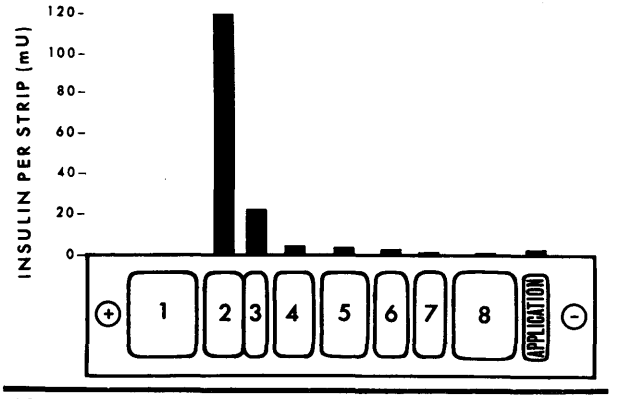

FIG. 6. Immunochemical identification of the insulin-bearing fraction of pancreatic extract: Insulin content of eluates of electrophoretically separated fractions of a crude, human pancreatic extract as determined by immunoassay.

per cent of the insulin (.12 U. per strip) was present in the eluate of fraction 2, and a total of 21 per cent (0.032 U. per strip) was recovered in a dwindling trail which spanned fractions 3 through 8.

To determine if the immunochemically measureable insulin was biologically active, the eluate of fraction 2 was assayed by the rat diaphragm method* and compared in table 1 with the eluate of fraction 7, employed as a control. A 1:100 dilution of the fraction 2 eluate contained 884 μU./ml. of insulin-like activity by bioassay as compared to 604 μU./ml. calculated to be present by the immunoassay. The eluate of fraction 7 was devoid of insulin-like activity and contained no immunochemically assayable insulin.

Immunoassay of the eluate of each fraction for glucagon revealed 77 per cent of the glucagon to be present in fractions 7 and 8 (160 mμg. equiv. per strip) while 21 per cent remained at the origin (figure 7).

Preliminary purification by gel-filtration

In the hope of removing the large quantities of extraneous protein present in the pancreatic extract, particularly in fractions 7 and 8, a gel-filtration method

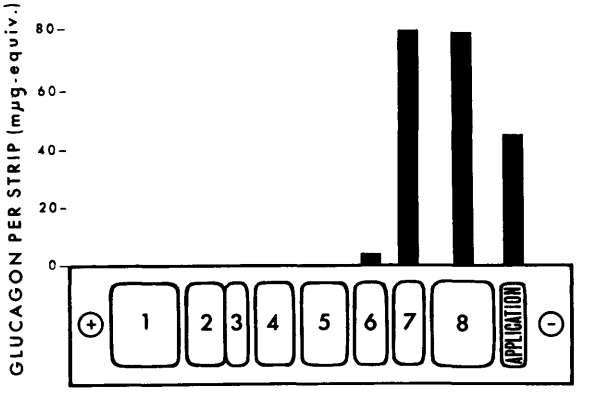

FIG. 7. Immunochemical identification of the glucagon-bearing fractions of pancreatic extract: Glucagon content of eluates of electrophoretically separated fractions of a crude, human pancreatic extract as determined by immunoassay.

was evaluated in respect to its ability to separate the hormones from the protein prior to electrophoresis. The results, shown in figure 8, indicate that a considerable degree of separation can be obtained. Only 30.7 per cent of the insulin, and 6.4 per cent of the glucagon were present in the recovery fractions which contained 85.3 per cent of the protein, whereas 58.5 per cent of the glucagon and 56.4 per cent of the insulin were recovered in a later recovery fraction in which only 14.7 per cent of the protein was present. It seems likely that with additional modifications further removal of extraneous protein from the hormone-containing fraction would be feasible.

TABLE 1

Assay of electrophoretic fractions by rat hemidiaphragm and immunoassay technics

Fraction	Dilution	Rat diaphragm assay (μU./ml.)	Immuno-assay (μU./ml.)
2	1:10	>1,000	6,040
	1:100	884	604*
7	1:10	0	0

*Not assayed; calculated from assay of 1:10 dilution.

*Kindly performed by Dr. Holbrooke Seltzer, University of Texas, Southwestern Medical School and V.A. Hospital, Dallas, Texas.

DISCUSSION

The present investigations have defined the electrophoretic behavior on cellulose acetate paper strips of purified human insulin and beef-pork glucagon, and of acid-alcohol extracts of human pancreas.

Insulin was shown to migrate as a single component, confirming earlier studies of diverse insulin preparations in which paper electrophoresis,[10,11] moving boundary (Tiselius) electrophoresis,[12] chromatography on filter paper,[13-19] and kieselgühr columns[20] were em-

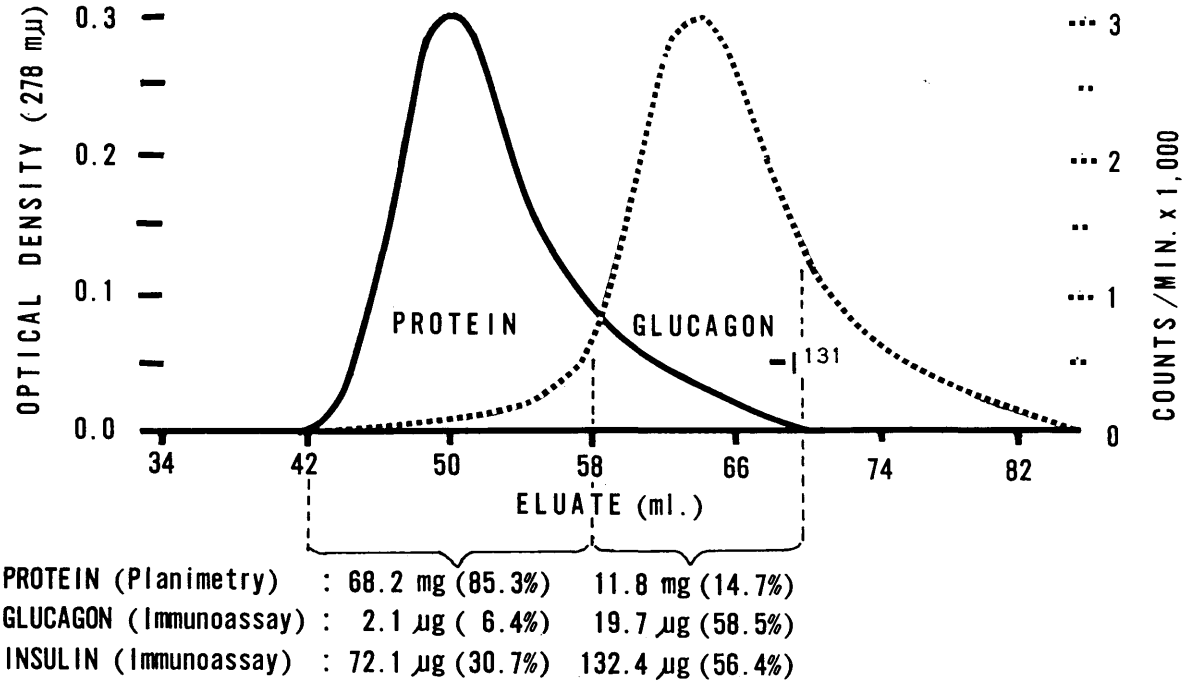

FIG. 8. Gel filtration of a crude, human pancreatic extract on a Sephadex G-25 (110 X 1.2 cm.) with Tris-buffer. Partial purification of glucagon and insulin is achieved with removal of 85.3 per cent of the extraneous proteins.

ployed. However, in several other studies of the physicochemical homogeneity of insulin, two or more components have been demonstrated by means of technics such as partition column chromatography,[21-26] countercurrent distribution,[27,28] paper chromatography,[29] and electrophoresis.[30-32] Although the explanation for the apparent discrepancy is not clear, it is possible that decomposition products of insulin, resulting from exposure to certain of the solvents employed or to prolonged storage,[33] may have contributed to the latter findings. In the present investigation, a second insulin component was noted to appear on storage of the insulin solutions and seemed to increase in quantity with time of storage while the original fraction diminished proportionately. However, variation between insulin lots may possibly account for varying results.

Glucagon, by contrast, was noted to migrate in two distinct fractions. The faster-migrating moiety comprised approximately 9 per cent of the glucagon applied, as determined both by densitometric and by immunochemical measurements. These findings confirm in general the studies of Cole,[34] performed by ion exchange chromatography and those of Staub, Sinn, and Behrens[9] and Barrett et al.[32] using starch gel electrophoresis. The minor glucagon fraction, which reappears upon re-electrophoresis of the major component,[9] has been shown to be qualitatively identical in amino acid composition, but to have only 50 per cent of the biological activity of the major fraction.[9] In the present study the quantitative similarity of the densitometric and the immunochemical measurements suggests that the two components are immunologically indistinguishable.

Virtually complete separation of mixtures of purified insulin and glucagon, demonstrated by sensitive immunochemical technics, was achieved by the cellulose acetate method, in contrast to the partial separation obtained by the column chromatography method of Cole,[34] and the resin-exchange paper chromatographic procedure of Wiesel et al.[35]

Electrophoretograms of crude extracts of human pancreas revealed eight positively migrating fractions, one immobile component and a single negatively migrating fraction. By means of densitometric, radioisotopic, and immunochemical methods, fraction 2 was identified as the insulin-bearing fraction, and 7-8 as the principle glucagon-bearing region. Fraction 2 appears to be composed primarily of insulin itself, since after incubation with cysteine the peak is replaced by a diffuse smear.[36] However, glucagon is not sufficiently abundant to be visible after staining, and the intense staining of fractions 7 and 8 in the glucagon-bearing region must be

the result of unidentified proteins. At least a prominent portion of fractions 7 and 8 are of acinar cellular origin, since they are markedly diminished in extracts of duct-ligated pancreases with atrophy of acinar tissue.[36]

The apparent discrepancy between the distribution of glucagon-I-131 and that of unlabeled glucagon, as determined densitometrically (figure 5), and immunochemically (figure 7) may be explained in two ways: First, the difference may be more apparent than real, a consequence of the fact that, in these experiments, the sensitivity of radioactivity measurements extended to less than 100 $\mu\mu$g./ml. of glucagon-I-131, well below the sensitivity of either the immunochemical or densitometric procedures as employed here. Trace quantities of unlabeled glucagon in the faster-moving fractions might, therefore, have been overlooked by the two latter technics. However, at least some of the faster-moving TCA-precipitable radioactivity may represent damaged rather than intact glucagon-I-131, altered either by the iodination procedure or by contact with other pancreatic proteins, as suggested by figure 8, or by both.

Insulin and glucagon were recovered from the Sephadex column with an important reduction in the unidentified protein components of the crude extract. With additional improvement, this method should provide a simple preliminary step by which the purity of the hormone preparations can be further enhanced.

The foregoing technics provide a means by which microgram quantities of relatively pure preparations of insulin and glucagon from any species can readily be prepared in the laboratory. Thus far, extracts of canine and guinea pig pancreas have been shown to have electrophoretic characteristics essentially similar to those of human pancreas and there is no reason to doubt that insulin and glucagon from these and other species can easily be isolated. In addition, the method provides a new physicochemical means for identifying an unknown material as glucagon and insulin.

SUMMARIO IN INTERLINGUA

Separation, in le Humano, de Insulina, Glucagon, e Altere Proteinas Pancreatic

Purificate insulina human e glucagon bovi-porcin esseva subjicite a electrophorese in bandas de acetato de cellulosa. Esseva trovate que insulina migra rapidemente verso le anodo in un unic banda, durante que glucagon migra lentemente verso le anodo in duo distincte fractiones. Un virtualmente complete separation del duo hormones esseva effectuate.

Electrophorese de extractos a acido-alcohol ex le pancreas human revelava octo positivemente migrante bandas. Per medio de technicas densitometric, radioisotopic, e radioimmunochimic, fraction duo esseva identificate como le fraction que porta le insulina, e le area del fractiones septe e octo esseva identificate como portator de glucagon. Per medio de filtration a gel, quantitates considerabile del non-identificate proteinas extranee que representa le massa del extracto pancreatic pote esser eliminate, con le resultato que on obtene plus pur specimens pro le studio electrophoretic.

Le technica permitte le rapide preparation de relativemente pur specimens de insulina libere de glucagon e de glucagon libere de insulina ab le pancreas de humanos e altere species, in quantitates sufficiente pro studios laboratorial. In plus, le characterisation del mobilitate electrophoretic de iste hormones provide un nove methodo pro identificar los in anonyme specimens.

ACKNOWLEDGMENT

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