Studies on the Guinea Pig Pancreas

FRACTIONATION AND PARTIAL CHARACTERIZATION OF EXOCRINE PROTEINS

(Received for publication, March 15, 1974)

ALAN TARTAKOFF,* LEWIS J. GREENE,† and GEORGE E. PALADE§
From the Rockefeller University, New York, New York and Brookhaven National Laboratory, Upton, New York 11973

SUMMARY

The protein mixture discharged by guinea pig pancreatic lobules incubated in vitro has been analyzed by two complementary techniques: isoelectric focusing (which separates proteins according to charge), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (which separates proteins according to size).

Isoelectric focusing columns spanning a set of pH ranges were used to fractionate secretory proteins biosynthetically labeled with 3H- or 14C-amino acids. Effluent fractions were used to determine radioactivity distribution and to locate (by specific enzyme assays) ribonuclease, amylase, chymotrypsin, trypsinogen, procarboxypeptidase A and procarboxypeptidase B either directly or after trypsin activation. There are single species of the first four proteins all of which are isoelectric above pH 9.2, and two forms of each procarboxypeptidase isolectric near pH 5 (procarboxypeptidase A) and pH 7 (procarboxypeptidase B). The average isoelectric point of secretion mass-labeled with a mixture of 15 14C-amino acids is at pH 9.2. The most acidic species isoelectric near pH 3.4 can be labeled with both 35S042- and amino acids. Its charge complementarity with the bulk of the secretory proteins is discussed with reference to the concentration and packaging of secretory proteins in the condensing vacuoles of the acinar cells.

Sodium dodecyl sulfate-polyacrylamide gels resolve eight major proteins from the secretion. Six of these have been identified by cross-referencing aliquots from the isoelectric focusing columns onto the gels. No enzyme activities or potential activities have been assigned to two of the major proteins: the first has a molecular weight of 68,000 and the second appears to be slightly smaller than chymotrypsinogen.

The results indicate that the guinea pig pancreas produces essentially the same complement of enzymes and zymogens as found in other species; they also demonstrate that the zymogen granules of the acinar cells contain the same proteins as the secretion discharged by pancreatic lobules upon carbamylcholine stimulation.

* Present address, Département de Pathologie, Faculté de Medicine, Université de Genève, 40 Boul. de la Cluse, 1211 Genève 4, Switzerland.
† Supported by the United States Atomic Energy Commission.
§ Present address, Section of Cell Biology, Yale University School of Medicine, 333 Cedar Street New Haven, Conn. 06510.

The procedures described can be used to analyze on a protein-specific basis a variety of events in the secretory process.
the available information concerning intracellular events has
drawn from studies on the pancreatic tissue of this species;
and to provide a method for investigating the various phases
of the secretory process on a protein-specific basis.

MATERIALS AND METHODS

Materials

Materials were purchased as follows: Acrylamide, N,N'-methyl-
enebisacrylamide, and N, N', N'-tetramethylethylenediamine,
from Eastman; ampholytes, from LKB (Batch 39 for pH 3 to 6,
batch 30 for pH 6 to 8, Batch 54 for pH 3 to 10, and experimental
Batch 4/72 for pH 9 to 11); benzamidine HCl hydrate, from Ald-
Mann; Coomassie brilliant blue, iPr2 P-F, ovalbumin (twice
crystallized), hippuryl-Arg, and Tos-Arg-OMe, from Schwarz-
Mann; hippuril-Phe, from Fox Chemical Co.; Sephadex
from Pharmacia Fine Chemicals; grade VI yeast RNA and sodium
dodecyl sulfate, from Sigma; soluble starch, from Mallinckrodt;
concanavalin A (twice crystallized) and goat γ-G, from Miles;
eel thymus DNA, bovine trypsinogen (PSP 9DA, 98 units per mg),
porcine α-amylase, soybean trypsin inhibitor (crystallized),
concanavalin A (twice crystallized) and goat γG-II, from Miles;
arginase, from calf thymus DNA, bovine thyroglobulin (Part, 9DA,
98 units per mg), porcine α-amylase, soybean trypsin inhibitor
(crystallized), porcine lipase, pepsin, bovine DNase I, bovine chymotrypsinogen
and bovine trypsinogen, from Worthington; 2,5 diphenyloxa-
zone (PPO) and butyl PBD, from ICN; Aquasol, from New Eng-
land Nuclear. All other chemicals were of reagent grade.

Animals

Male albino guinea pigs (Rockefeller University colony),
weighing 450 to 500 g were fasted overnight, with water ad libitum,
before each experiment. Bovine pancreatic juice was obtained from
pancreatic lobules system used has been studied in detail by Scheele and
Palade.

Incubation Medium and Technique—The lobules were incubated
in KRB with a full amino acid supplement by procedures used for
pancreatic slices (17, 18). When radioactive amino acids or sul-
fate were incorporated by the tissue, the corresponding nonradio-
active components were omitted from the medium. Discharge of
secretory proteins was promoted by optimal doses1 of carboxyl-
choline (10−5 M). In all experiments in which discharge was
studied, 20 μg per ml of bovine plasma albumin were added to the
medium in order to minimize adsorption of secretory proteins to
glass.

Processing of Discharged Secretion—At the end of the incubation,
the lobules were discarded, and the medium was collected, chiled,
supplemented with iPr2 P-F and benzamidine to a final concen-
tration of 1 mM (to prevent zymogen activation (19)), and cleared
of debris by a 30-min centrifugation at 105,000 × g at 3°. The
resulting pellet was very small and contained a negligible amount
of radioactivity. The cleared medium was desalted, the salt-free
medium in order to minimize adsorption of secretory proteins to
the gland. The resulting interstitial edema made possible the
processing of discharged secretion. At the end of the incubation,
the secretory process on a protein-specific basis.

For desalting, protein solutions and discharged secretion were
passed through a Sephadex G-25 ( coarse) column, measuring 2.5×
30 cm. The column was equilibrated and developed with 50
mM NH4HCO3, 0.1 mM iPr2 P-F, and 0.1 mM benzamidine at ap-
proximately 4° and 40 ml per hour. The effluent was monitored
for ultraviolet absorbance and radioactivity.

Cell Fractionation

Groups of lobules were minced, homogenized in 0.3 M sucrose,
and zymogen granule fractions were isolated from the homogenate
by the procedure of Tarzakoff and Jimenez (20).

Isoelectric Focusing Procedure

Isoelectric focusing was performed in the 110-m column as de-
scribed in the LKB manual 1-8100-E01 (LKB Produkter, A. B.,
Bromma, Sweden) with the cathode placed at the bottom and im-
mersed in 24 ml of 0.167% ethylenediamine in 1.5 M sucrose.
A 1% solution of ampholytes containing 0.1% Triton X-100 and,
in most cases, 0.1 mM iPr2 P-F was placed above the cathode.
The ampholyte was stabilized by a 1.0 to 0.2 M sucrose gradient. Ten
milliliters of 0.5% H2PO4 were placed above the gradient and
surrounding the anode. The sample, containing less than 1 mg
of protein in 2 ml of 1% ampholytes, 0.5% Triton X-100, 1 mM
iPr2 P-F and 0.5 M sucrose, was placed usually in the middle of
the column. In some experiments, the sucrose concentration in
the load was reduced so that the load could be positioned near
the anode but separated from it by a protein-free ampholyte solu-
tion.

The temperature was maintained at 6° and the voltage adjusted
to maintain the power output at approximately 1 watt. After 48
hours, when the resistance of the column had stabilized and the
voltage had been increased to 600 to 700 volts, the power was
turned off. The column was drained at a rate of approximately
50 ml per hour at 4° and 6 M ammonia solutions were collected into
tubes, each containing a solution of at least 20 μg of bovine plasma
albumin. The pH of the fractions was measured within 1 hour
while they were held in an ice bath. Aliquots of the effluent were
counted usually after mixing with NCS and 10 ml of 0.5% 2,4-
diphenylloxazol in toluene. For single label experiments, the
counting efficiency was 30% for 3H and 69% for 3C. In some cases,
effluents were counted with 1.1 ml of water and 10 ml of Aquasol. Counting efficiency was uniform across the
column effluents.

The procedure for running pH 9 to 11 columns differed somewhat
from that given above. The cathode solution was 0.1 N NaOH
in 45% glycerol; the gradient components were equal volumes of:
(a) 0.8% pH 9 to 11 ampholyte, 0.1% Triton X-100, and 0.1% iPr2
P-F, in 40% glycerol; and (b) 0.3% pH 9 to 11 ampholyte,
0.32% pH 6 to 8 ampholyte, 0.1% Triton X-100, and 0.1% iPr2
P-F in 40% glycerol; the anode solution was 0.86% H2PO4.

Gel Electrophoresis Procedure

Sodium dodecyl sulfate-gel electrophoresis was performed in 1-
mm and 6-mm thick slabs, using Maizel's (21) discontinuous "so-
dium dodecyl sulfate-disc" system and 13% acrylamide in the
running gel. The apparatus for 1-mm gels was that of Reid and
Bielawski (22) as modified by Enns (23). It made possible the
convenient fractionation and visualization of as little as 5 μg of
mixed pancreatic secretory protein. The apparatus for thicker
gels was a "Vertical Gel," E-C Apparatus Corp. (Philadelphia).

According to their nature, samples were prepared for electrop-
horesis in any of three ways: 1. Protein standards, desalted
lobe secretion, and resuspended zymogen granule fractions were
prepared by adding sodium dodecyl sulfate to a final concen-
tration of 1%, glycerol to 20%, 8-mecaptoethanol to 1%, and pH 6.7
tris-phosphate buffer to 60 mM, before boiling the samples for 1
to 2 min. 2. Some aliquots of secretion in KRB or TKC were
processed by the same procedure, except for the omission of the
counter buffer. The presence of KRB salts and amino acids posed
no problem. A precipitate (presumably a calcium salt of sodium
dodecyl sulfate) did form in the TKC-containing samples, but it

1 The abbreviations used are: Bz-Tyr-OEt, benzoyl-n-tyrosine
ethyl ester; butyl PBD: 2-(4'-4 butylphenyl)-5-(4'-biphenyl)-1,
3,4-oxidiazole; iPr2 P-F, diisopropylphosphofluoridate; hippuryl-
Arg, hippuryl-L-arginine; hippuryl-Phe, hippuryl-L-phenylala-
nine; IEP, isoelectric point; KRB, Krebs-Ringer bicarbonate
solution; Rapp, migration in the sodium dodecyl sulfate gels rel-
tive to bromophenol blue; Tos-Ang-OMe, p-toluenesulfonyl-L-
arginyl methyl ester; TKC, Tris-potassium calcium solution (100
mM Tris, 100 mM KCl, 20 mM CaCl2, pH 7.8, (x, y), protein with an
IEP = x and Rapp = y.

remained in the spacer gel and did not interfere with electrophoresis. 3. For other aliquots of incubation media as well as fractions from the focusing columns, the proteins were precipitated by adding carrier bovine plasma albumin and then trichloroacetic acid (to a final concentration of 10%) Control experiments performed with secretory proteins, biosynthetically labeled with [14C]leucine, showed that a bovine plasma albumin concentration of at least 100 μg per ml was required to ensure approximately 90% complete precipitation. After centrifugation for 10 min at 1000 X g at approximately 0°C, the tubes were inverted and thoroughly drained. The pellets were dissolved in sufficient volumes of 0.5 m pH 6.7 Tris-phosphate spacer gel buffer, or 1 m Tris base to neutralize the acidity. Sodium dodecyl sulfate, glycerol, and β-mercaptoethanol were then added, and the samples were boiled as described under "Gel Electrophoresis Procedure." In all of the cases, bromphenol blue was added as a tracker dye.

Gels were run overnight at room temperature, with constant voltage (40 to 50 volts for the thick slabs, and 20 volts for the thin slabs). Because the design of the thin slab apparatus permits a slow leak of electrode buffer from the cathode to the anode compartment, the buffer was circulated. The gels were stained for enzyme activity by adding carrier bovine plasma albumin and then trichloroacetic acid (to a final concentration of 10%). Control experiments were run to ensure that there was no activation of trypsinogen or any other zymogen. In this case, iPr2P-F was omitted from the isoelectric focusing columns used to localize chymotrypsinogen and trypsinogen, but was present in all of the others. It did not interfere with the activation of procarboxypeptidases presumably because of its slow rate of reaction with trypsin (30). The conditions used for activation of prophospholipase were the same as described by Pallas (10).

RESULTS

Isoelectric Focusing Patterns

Amino Acid-labeled Secretion—The separation of guinea pig secretory proteins by isoelectric focusing with wide range ampholytes, pH 3 to 10, is illustrated in Fig. 1a. Lobule secretion from four animals gave very similar patterns. In order to obtain better resolution of the proteins present in the secretion, narrow pH range ampholyte mixtures were used. Fig. 1b (pH 3 to 6 ampholytes) shows that the three most acidic components in the mixture have isoelectric points of 3.4, 4.8, and 5.1; the remainder of the radioactivity is at the bottom of the column. Fig. 1c (pH 6 to 8 ampholytes) reveals more detail than that observed in the pH 3 to 10 column; in addition to the large peaks at pH 6.8 and 7.2 observed in Fig. 1a, two minor peaks at pH 6.2 and 6.4 can be identified. When pH 9 to 11 ampholytes are used (Fig. 1d), none of the radioactivity extends into the cathode solution. The major component is a peak at pH 9.3 with apparent shoulders at pH 9.5 and 9.7; in addition, there are minor components at pH 11 and pH 12, and in some experiments only, at pH 8.0 and pH 8.4. The experiments illustrated in Fig. 1 indicate that the secretion labeled with radioactive leucine contains at least 12 components. A pattern similar to that given in Fig. 1a was obtained with the

<table>
<thead>
<tr>
<th>Enzyme or zymogen</th>
<th>Activation</th>
<th>Substrate</th>
<th>Standard</th>
<th>Quantities measured</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>No</td>
<td>Starch</td>
<td>Worthington porcine amylase (AA 2BA, 900 units/mg)</td>
<td>0.005-0.15</td>
<td>Bernfeld (24)</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>No</td>
<td>RNA</td>
<td>Worthington bovine RNase A (RASE OEA, 3,000 units/mg)</td>
<td>0.05-0.45</td>
<td>Kalnitsky (25)</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>Yes (16 hrs, 0°C)</td>
<td>Br-Tyr-OEt</td>
<td>Worthington bovine α-chymotrypsin (CDS-71A, 45 units/mg)</td>
<td>0.03-0.32</td>
<td>Walsh and Wilcox (26)</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>Yes (24 hrs, 24°C)</td>
<td>Tes-Arg-OME</td>
<td>Worthington bovine trypsin (TRL IGA, 180 units/mg)</td>
<td>0.003-0.03</td>
<td>Walsh (27)</td>
</tr>
<tr>
<td>Procarboxypeptidase A</td>
<td>Yes (3 hrs, 24°C)</td>
<td>Hippuryl-Phe</td>
<td>Worthington bovine carboxypeptidase A (specific activity, assumed, 35 units/mg)</td>
<td>0.0003-0.003</td>
<td>Greene et al. (1)</td>
</tr>
<tr>
<td>Procarboxypeptidase B</td>
<td>Yes (1 hrs, 0°C)</td>
<td>Hippuryl-Arg</td>
<td>Worthington porcine carboxypeptidase B</td>
<td>0.0003-0.003</td>
<td>Greene et al. (1)</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>No</td>
<td>DNA</td>
<td>Worthington bovine DNase I</td>
<td>0.1-0.4</td>
<td>Price et al. (28)</td>
</tr>
<tr>
<td>Phospholipase</td>
<td>Yes</td>
<td>Lecithin</td>
<td>None</td>
<td>None</td>
<td>Pallas (10)</td>
</tr>
<tr>
<td>Lipase</td>
<td>No</td>
<td>Tributyrin</td>
<td>None</td>
<td>None</td>
<td>Maylié (29)</td>
</tr>
</tbody>
</table>
FIG. 1. Column isoelectric focusing of secretion labeled with radioactive leucine. Samples a, b, and c: a set of lobules from the pancreas of one guinea pig was incubated for 140 min at 37°C in 25 ml of KRB containing all of the amino acids (except leucine), 5 μCi per ml of L-[4,5-3H]leucine (Schwarz-Mann L-[4,5-3H]leucine, 51 Ci per mmole), and 10⁻⁶ M carbamylcholine. For d, the conditions were the same except that the isotope was 5 FCi per ml of L-[U-¹³C]-leucine (New England Nuclear, L-[U-¹³C]leucine, 327 mCi per mmole). Under these conditions, radioactive leucine was incorporated by the tissue into secretory proteins concurrently with discharge. Newly synthesized protein started to appear in the medium 20 min after the beginning of amino acid incorporation. After incubation, the lobules were discarded and the medium examined by isoelectric focusing as described under "Materials and Methods." Each panel gives the pH gradient measured in the column effluent. The isoelectric points of the peaks of interest are identified. Proteins at the margin of each panel are poorly resolved because the pH change is abrupt.

Enzyme Localization in Effluents of Isoelectric Focusing Columns

An initial search was made for enzyme activity in the load and in the effluent of the pH 3 to 10 isoelectric focusing column. When rough positioning of the activity had been made, detailed tube by tube assays were conducted on the effluent from the high resolution columns illustrated in Fig. 1, b to d. Table II summarizes the results obtained in these assays.

Amylase and Ribonuclease—All of the amylase activity applied to the column (113%) was recovered in a single symmetrical peak centered at pH 9.7 (Fig. 3). The peak of ribonuclease no utilization of SO₄²⁻ for amino acid biosynthesis under the conditions studied. The absence of a label in the anode solution shows that no hydrolysis of ³⁵SO₄²⁻-labeled components occurs during isoelectric focusing. The possible function of this material is considered under "Discussion."

Enzyme Localization in Effluents of Isoelectric Focusing Columns

An initial search was made for enzyme activity in the load and in the effluent of the pH 3 to 10 isoelectric focusing column. When rough positioning of the activity had been made, detailed tube by tube assays were conducted on the effluent from the high resolution columns illustrated in Fig. 1, b to d. Table II summarizes the results obtained in these assays.

Amylase and Ribonuclease—All of the amylase activity applied to the column (113%) was recovered in a single symmetrical peak centered at pH 9.7 (Fig. 3). The peak of ribonuclease
FIG. 2. Column isoelectric focusing of secretion labeled with $^{35}$SO$_4^{2-}$. The lobules were incubated with $^{35}$SO$_4^{2-}$ [100 μCi per ml of $^{35}$SO$_4^{2-}$, New England Nuclear, 910 mCi per mmole] for 1 hour and then transferred to carbamylcholine-containing medium with normal levels of nonradioactive SO$_4^{2-}$. Discharge was allowed to continue for 2 hours. Isoelectric focusing was carried out in a pH 3 to 8 ampholyte. The distribution of $^{35}$S radioactivity (C--C) is compared with that of [H]leucine radioactivity (O----O) in a similar sample from another animal.

TABLE II
Isoelectric points of guinea pig pancreatic secretory proteins and estimated composition of zymogen granule extracts

<table>
<thead>
<tr>
<th>Zymogen Granule Protein</th>
<th>IEP</th>
<th>Activity Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ESTIMATES BASED ON ACTIVITY MEASUREMENTS*</td>
</tr>
<tr>
<td>Amylase</td>
<td>9.7</td>
<td>7.2</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>9.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>9.3</td>
<td>32.0</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>7.2</td>
<td>21.0</td>
</tr>
<tr>
<td>Procarboxypeptidase B</td>
<td>7.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Procarboxypeptidase A</td>
<td>4.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Activity estimates are based on enzyme assays carried out on a set of extracts of zymogen granule fractions. All of the enzymes were assayed in iPr$_2$ P-F containing extracts except chymotrypsinogen and trypsinogen, for which three (iPr$_2$ P-F)-free extracts were used. Total protein was determined by the method of Lowry et al. (32), using bovine plasma albumin as standard. The figures given for the quantity of zymogens present are underestimates because no corrections have been made for molecular weight changes expected to occur on activation.

$ Radioactivity estimates are based on sodium dodecyl sulfate-gel analysis of zymogen granule fractions labeled with mixed $^{14}$C-amino acids. Estimates for chymotrypsinogen and the procarboxypeptidases are upper limits because in both cases unidentified proteins run in the same region of the gel as the proteins of interest.

activity was coincident with that of amylase. The recovery of ribonuclease activity was 89%.

Chymotrypsinogen and Trypsinogen—The two zymogens were localized after activation with trypsin in the effluents of the pH 3 to 10 and pH 9 to 11 isoelectric focusing columns (Fig. 4). Here, iPr$_2$ P-F was present in the load, but not in the rest of the column; its omission from the ampholyte solution did not change the distribution of radioactive protein, indicating that little, if any, activation or proteolysis had occurred during isoelectric focusing. Because iPr$_2$ P-F is uncharged and was expected to remain in the load position, trypsin could be used to activate aliquots of effluent from the entire column except for the load region. Because of this situation, the recovery of the two zymogens after isoelectric focusing could not be estimated. Chymotrypsinogen was localized as a single peak, isoelectric at pH 9.5. Both zymogens appear to be present in the guinea pig secretion as single isoelectric components.

Procarboxypeptidase A and B—Potential carboxypeptidase A activity was detected as two isoelectric forms, pH 4.8 and pH 5.0, (Fig. 5). Of the loaded potential activity 87% was recov-
Fig. 5. Localization of procarboxypeptidase A in L-[3H]leucine-labeled secretion after isoelectric focusing in the pH 3 to 6 column shown in Fig. 4b. The conditions for the assay of carboxypeptidase A (O-O) after tryptic activation are given in Table I; •••• radioactivity.

Fig. 6. Localization of procarboxypeptidase B in L-[3H]leucine-labeled secretion after isoelectric focusing in the pH 6 to 8 column shown in Fig. 4c. The conditions for the assay of carboxypeptidase B (O-O) after tryptic activation are given in Table I; •••• radioactivity.

The peak at pH 5.0 is not coincident with the radioactivity peak at pH 5.1. The lack of coincidence can be accounted for by a contaminant protein which is not a carboxypeptidase precursor. This is suggested because the potential activity of combined, successive fractions from this region is additive (the inverse would be expected if inhibitors were present), and it is proven by the detection of a contaminant of different molecular weight by gel electrophoresis (cf. Figs. 10 and 11).

Procarboxypeptidase B was recovered, in 94% yield, as two isoelectric components at pH 7.0 and 7.2 (Fig. 6). It is not known whether the shoulder on the acidic side of each major peak represents additional components. Again, only one of the peaks coincides with a radioactivity peak; the results of mixing experiments similar to those carried out for procarboxypeptidase A rule out the presence of inhibitors. The results shown in

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of guinea pig lobule secretion and bovine pancreatic juice. Increasing concentrations (10 and 20 µg) of the two detergent samples were run simultaneously in a 1-mm slab gel. The guinea pig secretion had been prompted over a 2-hour period by 10⁻⁴ M carbamylcholine. In this gel and all of the others illustrated, the direction of electrophoresis is from top to bottom, and little or none of the spacer gel is included in the photograph. Despite the appearance of this photograph, no Coomassie brilliant blue staining material remains either in the spacer gel or at the spacer gel-running gel junction. The numbers in parentheses are the Rf values relative to bromphenol blue.

Fig. 10 demonstrate that a contaminant protein, not activatable to carboxypeptidase B, is present at pH 6.8.

Other Enzymes—DNase activity, present in trace amounts in granule extracts (Table II), could not be detected in the column effluent. Lipase activity was abundant in the secretion but not detectable in fractions from the isoelectric focusing column. Prophospholipase could not be detected in the secretion.

Sodium Dodecyl Sulfate-Gel Electrophoresis

Pancreatic Secretory Proteins—The pattern obtained when guinea pig pancreatic lobule secretion was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue is given in Fig. 7. The mobilities of the guinea pig proteins expressed relative to that of the tracking dye (bromphenol blue) are given in parentheses on the right side of the figure. This in vitro equivalent of guinea pig pancreatic juice is compared with bovine pancreatic juice collected in vivo. Both samples have a limited number of discrete bands. The patterns are similar but not identical; components corresponding to Bands A, B, and D (DNase) in the bovine juice were not detected in the guinea pig secretion. The sensitivity of the system is demonstrated by the staining intensity of bovine DNase and RNase (Bands D and G) which represent approximately 0.2% and 3% of the bovine secretory protein, respectively; hence, individual proteins at levels of 500 ng can be visualized easily in this system.
FIG. 8. Plot of mobility versus logarithm of molecular weight for a variety of proteins subjected to electrophoresis in a standard 1-mm sodium dodecyl sulfate-polyacrylamide gel (13%) acrylamide). The data from two independent runs are included, normalized to RNase mobility. In both runs, the ovalbumin and soybean trypsin inhibitor gave double bands. •, porcine amylase; ○, bovine plasma albumin; ●, bovine chymotrypsinogen A; ○, concanavalin A; §, bovine DNase I; ○, goat γ globulin heavy chain; ‡, ovalbumin; +, pepsin; X, bovine RNase A; □, soybean trypsin inhibitor; ○, bovine trypsinogen.

Band 1 in the guinea pig secretion is primarily the bovine plasma albumin added to the incubation medium to prevent adsorption of proteins to glass. However, Band 1 also contains the largest of the guinea pig secretory proteins which has the same mobility as plasma albumin in this system. This protein may be detected by Coomassie brilliant blue staining in the absence of albumin or by radioactivity when labeled secretion is examined.

There is no, or only a negligible amount of, Coomassie brilliant blue stainable material in the spacer gel or at the spacer gel-running gel junction in either guinea pig or bovine secretion. However, the sulfated macromolecular compound (isoelectric point ≈ pH 3.4) identified in the guinea pig secretion by the isoelectric focusing technique does remain largely in the spacer gel and can be detected by autoradiography when biosynthetically labeled with $^{35}$S SO$_4$$^2$.

Fig. 8 demonstrates the relationship between the logarithm of the molecular weight of several proteins and their mobility in 1-mm slab gels. As was expected, mobility is roughly proportional to molecular weight, but the lack of precise linearity indicates that more direct methods must be used for an accurate determination of the molecular weights of the pancreatic secretory proteins. The deviation of trypsinogen from a linear relationship has been noted before by other investigators (33).

Trypsin-activated Pancreatic Secretory Proteins—Because lobule secretion is collected at 37°C over periods up to 3 hours, it was necessary to determine the effect of deliberate trypsin activation on the sodium dodecyl sulfate-gel patterns. Zymogen granule extracts were activated by the addition of bovine trypsin and the appearance of trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B activity was determined with synthetic substrates as indicated under “Materials and Methods.” At both 0°C and 24°C the zymogens, with the exception of trypsinogen, were activated maximally within 3 to 6 hours. Maximum trypsinogen activation occurred after 24 hours at 24°C. In the absence of added trypsin, no activation of any of the zymogens was observed even in samples of granule content held at 24°C for 24 hours. This suggests the occurrence within zymogen granules of a pancreatic secretory trypsin inhibitor of the type found in several other species (34, 35).

The results given in Fig. 9 show that there are major alterations in the molecular weight of the pancreatic secretory proteins during trypsinic activation. The pattern obtained in 2 hours appears to remain stable for the remaining 22 hours. Band 2 (amylose) and Band 8 (RNase) persist, at least in part, throughout this entire period. A pair of new bands appears which moves slightly more slowly than Bands 5 and 6 and low molecu-
lar fragments are detected as a smear ahead of Band 8. Secretory protein held at 24°C for 24 hours without added trypsin gave essentially the same pattern as the control sample in agreement with the results of the activation assays already mentioned. The results given in Fig. 9 indicate that the sodium dodecyl sulfate-gel system can detect effectively activation and autolysis of the proteins secreted by the guinea pig pancreas.

Cross Correlation Experiments—After resolving the secretory proteins of the guinea pig pancreas into two sets of fractions according to two different parameters, i.e. either isoelectric point or size, we carried out a series of cross-correlation experiments in which successive isoelectric focusing fractions were analyzed by sodium dodecyl sulfate-gel electrophoresis. With this approach, it should be possible: (a) to identify gel bands with specific enzymes or zymogenes; (b) to estimate the isoelectric point and molecular weight of each protein; and (c) to assess the extent of the fractionation achieved by each of the two procedures.

The experiments were carried out as follows: 14C-labeled secretion was run on pH 9 to 11 and pH 3 to 10 isoelectric focusing columns and the effluents were selectively sampled by taking 0.3-ml aliquots (each spanning less than a 0.05 pH unit) from all peaks of radioactivity or enzymatic activity, or both.

Fig. 10 is a radioautogram of a stained and dried gel. The results of this and a variety of similar experiments are combined into a matrix in Fig. 11, which also illustrates, in both dimensions, the distribution of radioactivity derived from mixed 14C-amino acids. The positioning of the most acidic species (isoelectric point \( \approx 3.4 \)) was established by virtue of its \(^{35} \)S content (cf. Fig. 12) and is indicated only in the matrix (Fig. 11). The radioautogram (Fig. 10) shows a high level of resolution, best appreciated by a systematic description of its pattern.

The pH 9.7- and 9.6-aliquots gave an intense band with \( R_{\text{BB}} \approx 0.15 \) and a faint one with \( R_{\text{BB}} \approx 0.32 \). The first band (9.7; 0.18) is identified as amylase by its amount and position in isoelectric focusing and the identification is supported by the finding that its migration rate is the same as that of porcine amylase. The estimated molecular weight is 52,000. The faint band (9.7; 0.32) remains unidentified.

Near pH 9.5, there is a major band with \( R_{\text{BB}} = 0.36 \) which spreads somewhat to adjacent fractions as do most of the other bands (in this case, from approximately pH 9.6 to 9.0). This (9.5; 0.36) band is identified as chymotrypsinogen by its position in isoelectric focusing. It has the same mobility as bovine chymotrypsinogen, and therefore its molecular weight can be estimated at approximately 26,000.

At pH 9.3, there is a major band with \( R_{\text{BB}} = 0.5 \), and two fainter bands with \( R_{\text{BB}} = 0.22 \) and 0.09. The major band (9.3; 0.5) is identified as trypsinogen by its amount and position in isoelectric focusing. It has the same mobility as bovine trypsinogen and therefore its molecular weights can be estimated at approximately 25,000. The fainter bands (9.3; 0.22) and (9.3; 0.09) remain unidentified.\(^5\) The slower moving of the two was found only in this one experiment.

At pH 9.1 and 9.0, the trypsinogen band is still strong while the chymotrypsinogen and the unidentified polypeptide bands become progressively fainter. No new bands are detected between pH 9.0 and 7.1.

At pH 7.2 and 7.0, there is a doublet of major bands with \( R_{\text{BB}} = 0.21 \) to 0.22 and a broader band with \( R_{\text{BB}} \approx 0.35 \), the latter being slightly more acid than the doublet. On each \( R_{\text{BB}} \) region, minor satellite bands are detected, as indicated in Fig. 11. The bands of the doublet (7.2 to 7.0; 0.21) and (7.0; 0.22) are identified provisionally as procarboxypeptidase B on the basis of their position in the isoelectric focusing column and for other reasons covered under "Discussion." The faster moving band (7.0; 0.35) has not been identified. The approximate molecular weights are 40,000 and 22,000 for the procarboxypeptidases B and the unidentified protein, respectively.

At pH 5.0, there is a major band with \( R_{\text{BB}} = 0.13 \) (which has a slightly faster moving satellite) and another major band with \( R_{\text{BB}} = 0.22 \). The first of these bands (5.0; 0.13) has not been identified, but the latter (5.0; 0.22) is thought to be procarboxypeptidase A on account of its position in the isoelectric focusing column. The (5.0; 0.22) protein has the same mobility as bovine plasma albumin and therefore has a molecular weight of \( \approx 68,000 \); procarboxypeptidase A has a molecular weight of \( \approx 40,000 \).

At pH 4.8, the (5.0; 0.22) band is still present and an additional major band with \( R_{\text{BB}} = 0.21 \) appears. Although the evidence is incomplete, both bands are tentatively identified as procarboxypeptidase A.

At pH 3.4 no \(^{14} \)C-labeled bands are visible; but the most acidic component of the separation (isoelectric point \( \approx 3.4 \) (cf. Fig. 2)) can be positioned provisionally in the matrix (Fig. 11) by virtue of its \(^{35} \)S content. The experiment illustrated in Fig. 12 shows that this component scarcely penetrates into the running gel.

As far as the resolution of the gels is concerned, there is no overlap within the major Bands 1, 2, 5, 6, 7, and 8; extensive overlap exists only in the region of Bands 3 and 4.

The characteristics and the identity of the proteins in the major bands can therefore be summarized as follows:

<table>
<thead>
<tr>
<th>Band</th>
<th>Characteristics</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IEP, ( R_{\text{BB}} ))</td>
<td>unidentified protein</td>
<td>amylase</td>
</tr>
<tr>
<td>1 (5.0; 0.13)</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>2 (5.0; 0.13)</td>
<td>unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>3 (5.7; 0.18)</td>
<td>unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>4 (9.3; 0.22)</td>
<td>unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>5 (9.7; 0.22)</td>
<td>unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>6 (5.0; 0.36)</td>
<td>unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>7 (9.3; 0.50)</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
<tr>
<td>8 (9.7; 0.59)</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
<td>procarboxypeptidase A, procarboxypeptidase B, unidentified protein</td>
</tr>
</tbody>
</table>

In Fig. 10, no radioactive band was detected at the position expected for RNase. The possible reasons are given under "Discussion." There are several indications that Band 8 in the gels is the guinea pig pancreatic RNase.

\(^{35} \)S-Labeled Material—\(^{35} \)S-Labelled secretion has also been analyzed by sodium dodecyl sulfate-gel electrophoresis. Fig. 12 illustrates a typical result and shows that most of the radioactivity remains in the spacer gel, only minor amounts reaching as far as the first Coomassie brilliant blue positive band. A similar radioactivity pattern was obtained when a \(^{35} \)S-labeled zymogen granule fraction was subjected to electrophoresis.

\( \left(9.7; 0.59\right) \) band is identified as chymotrypsinogen by its position in the isoelectric focusing column; i.e. much more than that suggested by the density of the corresponding radioautographic band.
FIG. 10. Radioautograms from a single 1-mm gel used for a cross-correlation experiment. Aliquots of 14C-labeled fractions from an isoelectric focusing column were precipitated with trichloroacetic acid in the presence of carrier bovine plasma albumin. Control experiments showed that approximately 87% of the radioactivity of the samples was precipitated under the conditions used, i.e., in the presence of Triton X-100 and ampholytes. In all cases, except for the pH 3.4 fraction, centrifugation gave firm, manageable pellets which were redissolved, reduced, denatured with sodium dodecyl sulfate, loaded at the top of the gel, and subjected to electrophoresis as given under "Materials and Methods." After staining and destaining, the gel was dried and radioautographed. Exposure times for the upper and lower radioautograms were 2 and 6 weeks, respectively. At the left edge of the figure is an indication of the mobilities of several protein standards. The 14C-labeled fractions come from the effluent of the pH 9 to 11 isoelectric focusing column illustrated in Fig. 1d and from a pH 3 to 10 pattern similar to Fig. 1a, but labeled with mixed amino acids.

Protein Composition of Zymogen Granule Content

With the composition of the acinar secretion partially elucidated, it became important to know whether the same mixture of proteins exists within the zymogen granules. So far, the equivalence, pancreatic secretion (juice) zymogen granule content has been established only in the bovine (1, 2) and canine (10) pancreas. Fig. 13 illustrates a gel loaded with trichloroacetic acid-precipitated guinea pig lobule secretion, trichloroacetic acid-
Cross-Correlation Matrix Relating IEF Fractions to SDS-Gel Bands

Percent of total radioactivity
(\(^{14}\text{C}\)-mixed amino acids)

<table>
<thead>
<tr>
<th>pH</th>
<th>1.6</th>
<th>7.4</th>
<th>6.3</th>
<th>19.5</th>
<th>32.1</th>
<th>27.3</th>
<th>7.6</th>
<th>5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>54.5</td>
<td>6.8</td>
<td>9.6</td>
<td>9.0</td>
<td>11.9</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Stainable bands

FIG. 11. Diagrammatic representation of cross-correlation data. As in Fig. 10, the horizontal axis represents isoelectric pH and the vertical axis represents mobility in sodium dodecyl sulfate gels. The figures on the top and the right of the graph give the radioactivity distribution among secretory proteins as per cent of the total. The cross-hatched bands represent compounds not detected in the radioautographs in Fig. 10; the (9.7; 0.6) component is ribonuclease, and the component at pH 3.4 is the sulfated compound shown in Figs. 2 and 13.

FIG. 12. \(^{35}\text{SO}_4\)-labeled secretion analyzed in a 6-mm slab gel. The arrows indicate the positions of the principal secretory proteins (numbered as in Fig. 7) which were stained with Coomassie brilliant blue before slicing and counting. The labeling protocol was the same as for Fig. 2. The isotope was 500 \(\mu\text{Ci}\) per ml carrier-free, neutralized \(\text{H}_2\text{SO}_4\) (Schwarz-Mann, Lot YR-1031).

The two sets of estimates (Table II) agree reasonably well with each other; e.g. the activity and radioactivity estimates are 7.2% and 6.2%, respectively, for amylase and 21% and 25%, respectively, for trypsinogen. However, because the resolution of the protein mixture in the gels is partly incomplete, several of the radioactivity-based estimates must be considered as upper limits useful for comparative estimates rather than for precise comparison.

zymogen granule fraction was lysed by resuspension in 60 mM 
NaCl, 130 mM NaHCO₃, pH 8.4 and its membranes were eliminated 
by centrifugation at 105,000 × g for 30 min as in Ref. (1):

The large aggregates and the smear at the top of the gels is due to 
the added bovine plasma albumin. The load for each standard 
was 2 μg. "The lipase sample proved to be grossly impure. The 
zymogen granule fraction was lysed by resuspension in 60 mM 
NaCl, 130 mM NaHCO₃, pH 8.4 and its membranes were eliminated 
by centrifugation at 105,000 × g for 30 min as in Ref. (1).

DISCUSSION

The mixture of secretory proteins synthesized and discharged 
in vitro by guinea pig pancreatic lobules has been resolved in a 
number of fractions according to net charge (by isoelectric focusing) 
or size (by sodium dodecyl sulfate-gel electrophoresis). The 
first procedure does not denature proteins; hence, enzymic activities 
could be assayed in the eluant and assigned to a number of 
distinct fractions. In the second procedure, proteins are 
denatured, but more satisfactory separation of a number of molecular 
species could be obtained. When used in cross-reference experiments, the two procedures allowed a partial characterization 
by isoelectric point and approximate molecular weight of 
all of the secretory proteins so far resolved; they also made it possible the identification of the actual or potential enzymic activities of most of the proteins of the secretory mixture.

Amylase, chymotrypsinogen, trypsinogen, and RNase have been identified reliably on the basis of their enzymic activities, 
relative amounts, and electrophoretic mobilities which proved to be similar to those of homologous bovine and porcine proteins.

All of these proteins occur as single species according to one or both separation procedures.⁷

The procarboxypeptidases A and B have been detected by enzyme activity (after trypsin activation), but their identification 
with specific protein species is less reliable because of the presence of other secretory proteins in the same regions of both 
isolectric focusing columns and sodium dodecyl sulfate-gel electrophorograms.

By analogy with the situation encountered in other species the polypeptides of 40,000 molecular weight found in the pH 
7.2 to 7.0 and pH 5.0 to 4.8 isoelectric focusing fractions are 
tentatively identified as procarboxypeptidase A and procarbox-
peptidase B, respectively, but definitive identification will 
require better resolution than that so far achieved. For each 
procarboxypeptidase, two molecular species of slightly different 
isolectric point, and probably slightly different size, have been 
found. In other species (e.g. ox), multiple forms of procarbox-
peptidase A have been recorded and accounted for as allotypic 
variants (3) or aggregates with other secretory proteins (33). 
Another possible source of multiplicity (which applies to secre-
tory glycoproteins only) is microheterogeneity in glycosylation (13); but so far there is no evidence that the procarboxypepti-
dases are glycoproteins in the guinea pig or in other species.

Two major bands remain unidentified. The first, which has an estimated molecular weight of 68,000, could be lipase because 
the secretory mixture has lipolytic activity and because the por-
cine and bovine homologues are relatively large glycoproteins 
of approximately 48,000 molecular weight (36). The apparent 
difference in size may be explained by a larger amount of carbo-
hydrate in the guinea pig enzyme. The second band could be 
proelastase, because it has the size of the porcine homologue (37), i.e. 22,000 molecular weight. The secretion was not assayed, however, for elastolitic activity. Many minor components (at least 5) detected at the present level of resolution also remain unidentified.

In general, the results show that the pancreatic secretion of the guinea pig contains the usual set of digestive enzymes and 
zymogens known to occur in other mammals (1-6, 10). The similarities are extensive, but differences are nonetheless demonstrable. By comparison with the bovine pancreas, the 
guinea pig gland produces little D'Nase and apparently no anti-
omic chymotrypsinogen. Of a total of eight major bands detectable by sodium dodecyl sulfate-gel electrophoresis, only four 
have similar mobilities in the two species.

The alkaline extract of the zymogen granule fraction can be 
equated to the content of the zymogen granules on the basis of 
evidence already published (1, 2, 10). The results presented 
show that the protein composition of the extract is qualitatively 
identical with that of the discharged secretion, and data in a 
subsequent paper⁶ indicate that the identity is also quantitative. 
These findings add the guinea pig to the short list of species 
(e.g. ox (1, 2) and rat (10)) in which an equivalence has been 
found.

⁷ RNase was not detected in all gel electrophoretograms; it 
gave a stainable band, e.g. in Figs 7 and 13, but in Fig. 10 no 
radioactivity was recorded at the corresponding level. Other 
experiments with labeled amino acids showed that a radioactive pro-
tein with the sodium dodecyl sulfate-gel mobility of RNase is 
both synthesized and secreted by guinea pig pancreatic lobules. 
The absence of detectable RNase in certain secretion samples 
could be explained by individual variations, inadequate gel resolution 
(small amounts, t.e less than 1/10 of the load, of a fast moving 
protein diluted beyond detection in a large gel volume), or losses incurred during some preparatory step prior to electrophoresis 
(e.g. trichloroacetic acid precipitation).

---

**Fig. 13. Sodium dodecyl sulfate-polyacrylamide gel (1 mm) 
analysis of lobule secretion, zymogen granule content, and perti-
nent standards.** The lobule secretion and the zymogen granule 
content were precipitated by trichloroacetic acid after the addi-
tion of carrier bovine plasma albumin, and were run at several 
load levels (5, 10, 15, and 20 μg, carrier albumin not included). 
The large aggregates and the smear at the top of the gels is due to 
the added bovine plasma albumin. The load for each standard 
was 2 μg. The lipase sample proved to be grossly impure. The 
zymogen granule fraction was lysed by resuspension in 60 mM 
NaCl, 130 mM NaHCO₃, pH 8.4 and its membranes were eliminated 
by centrifugation at 105,000 × g for 30 min as in Ref. (1).
established between the protein composition of the pancreatic juice and that of the zymogen granule content.

A large fraction of the secretory proteins of the guinea pig was found to be cationic; the average isoelectric point of the secretory mixture is 9.2 and approximately 60% of its protein mass is isoelectric above pH 8.4. Because the secretion was also found to contain a sulfated polysaccharide component, possibly a sulfated peptidoglycan, ionic interactions can be anticipated between the latter and the cationic proteins within zymogen granules or in the juice. Recent work has shown that the concentration of secretory proteins (which starts in the condensing vacuoles of the Golgi complex and is maintained in the zymogen granules) is not an energy-dependent process (38). Hence, it seems plausible that water content in these compartments is reduced by a decrease in the osmotic activity of their content which could be caused by a large scale formation of macromolecular aggregates by ionic interactions of the type mentioned above. Autoradiographic experiments have demonstrated the incorporation of $^{35}S$ (from $^{35}SO_4^{2-}$) into macromolecular components in the Golgi complex and is maintained in the zymogen granules of the pancreatic exocrine cell of the mouse (31).

The procedures described in this paper make possible an inclusive analysis of all of the high molecular weight secretory products of the exocrine pancreas of the guinea pig; in addition, they are applicable on a microscale and hence make possible a rather detailed investigation of events occurring within the pancreas of a single animal, thereby eliminating individual variations. In a subsequent paper, these procedures are used to investigate discharge and intracellular transport at the level of the exocrine pancreatic exocrine cell of the mouse (31).

REFERENCES

Studies on the Guinea Pig Pancreas: FRACTIONATION AND PARTIAL
CHARACTERIZATION OF EXOCRINE PROTEINS
Alan Tartakoff, Lewis J. Greene and George E. Palade


Access the most updated version of this article at http://www.jbc.org/content/249/23/7420

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/23/7420.full.html#ref-list-1