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ISOELECTRIC FOCUSING STUDIES OF HUMAN PANCREATIC SECRETION

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SUMMARY

Pure bile, pancreatic and duodenal human juices have been analyzed by isoelectric focusing, either at rest or upon stimulation with caerulein. In rats, stimulation has also been performed with secretin. Twenty bands have been resolved and quantified in the pancreatic secretion. By developing zymograms, a number of isozymes have been identified: 6 isoamylases [p*I*'s 7.2, 7.1 and 6.6 (major) and p*I*'s 7.4, 6.7 and 5.8 (minor)], 3 lipases [p*I*'s 7.0 and 6.8 (major) and 6.4 (minor)], two major alkaline proteases (p*I*'s 9.8 and 8.4) and one major acidic protease (p*I* 4.3) and one band of RNAase activity (p*I* 8.6). The stimulation kinetics follow a mechanism according to Palade, indicating uniform response to secretogogues, parallel intracellular transport and parallel discharge of pancreatic exocrine proteins.

INTRODUCTION

The pancreas is a suitable organ for studies of mammalian cell function and differentiation at the biochemical level in terms of organ-specific enzymes [1—4]. Its proteolytic enzymes chymotrypsin and trypsin have been used as biochemical indicators for characterization of cellular differentiation and regulation of functional activity [1, 5]. These enzymes are also suitable indicators in studies of molecular evolution [6] or species variation [7].

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The exocrine pancreas is also an attractive biochemical model for the study of the induction and modulation of enzymes, since it synthesizes and secretes an impressive variety of proteins which span a broad range of molecular weights, isoelectric points and actual or potential activities [8]. In contrast to this pleotropic function, all of the exocrine cells of the pancreas are similar in appearance, each containing a reasonably homogeneous population of secretion (zymogen) granules [9]. It has been reported that the exocrine pancreas can apparently exert a long-term control over specific enzymes in response to specific diets [10, 11]. At present, there are two major lines of evidence in support of two opposite models: a model of "selective modulation" [12, 13] and a model of "parallel and synchronous discharge" [14, 15]. According to the first model, some stimuli, such as the presence of lysine in the intestine [12] or the injection of chymodenin [13] can elicit a rapid, selective, several-fold increase of some enzymatic activities in the pancreatic discharge granules. According to the second model, irrespective of stimulant (carbamylcholine, caerulein or 75 m*M* potassium chloride) and time of stimulation, the exocrine proteins are discharged in parallel and in constant proportions [14, 15].

The analysis of the pancreas discharge products can be also very useful in medical diagnosis and treatment, since it can be expected that pathological disturbances, such as chronic pancreatitis or carcinoma, can lead to alterations in the pattern of isoenzymes, as resolved by electrophoretic techniques. In order to obtain a deeper insight into these problems, we have applied the technique of isoelectric focusing (IEF) to the analysis of pure pancreatic juice, pure bile and duodenal juice in both rats and humans, in physiological conditions and under hormonal stimulation.

MATERIALS AND METHODS

This study was performed first in rats and subsequently in human patients admitted to a surgical ward for pathological conditions of the main biliary ducts, of the pancreas or of the upper gastrointestinal tract. In rats, pure bile and pure pancreatic juice were obtained by direct double cannulation of the main biliary duct (the pancreatic ducts discharge in its lower portion); attempts to obtain duodenal juice were unsuccessful. In human patients pancreatic secretion was collected by direct cannulation of the Wirsung duct during surgery for pathological conditions of the Vater papilla; pure bile was collected from patients bearing an external drainage (Kehr tube) and duodenal juice by cannulation with a Levine tube.

Stimulation of the pancreatic gland was in two different groups of animals by secretine alone (Booths, 12 U/kg) and by ceruletide alone (ceruleina Farmitalia, 0.33 μg/kg) with a single intravenous administration. Stimulation with a single substance was performed in order to demonstrate any possible difference in the IEF pattern: in view of the results obtained we used ceruletide alone in human patients, always with a single administration by endomuscular route (0.75 μg/kg). Samples were collected every 10 min during a 1-h period in the animals, during 90 min in duodenal cannulation and for approximately 40—50 min during surgery. Samples were immediately frozen in liquid nitrogen. Total protein content was determined by the method of Lowry et al. [16]. IEF

was carried out as previously described [17] using polyacrylamide gel plates containing 2% Ampholine (LKB, Bromma, Sweden) in the pH range 3.5—10. Urea (4 *M*) was added to the gel to avoid macromolecular aggregation. The samples were seeded in pockets precast in the gel [18]. At the end of the run (4 h) the plates were fixed in trichloroacetic acid (TCA) and the proteins stained with Coomassie Brilliant Blue R-250 [18]. Measurements of the isoelectric points of the protein bands were made with an Ir/IrO_2 electrode [19].

The gel scans were performed with an experimental apparatus, built in our laboratory, consisting of a flying spot (a red diode light source) moving over the Coomassie Blue stained gel. The scanner is piloted by a Digital PDP8/e computer, which also stores in a magnetic tape the gel signal, amplified by a solid-state photomultiplier. The data are displayed graphically on an X-Y plotter Plotmatic 815M (M.F.E.). The position of each peak and its relative area is typed on a teletype. This system is linear up to 3 A. In addition to Coomassie Blue staining, zymograms for amylase [20], protease [21], lipase [22] and RNAase [23] activities were developed in the screening of human samples. As we have also recently suggested and described in detail [24], best results were obtained by performing an agarose contact print.

RESULTS

Figs. 1A and B show the stimulation kinetics of rat pancreatic and biliary juices, respectively, upon injection with a single dose of the secretogogue caerulein. The total protein content in pancreatic secretion rises steeply, reaches a peak within 30 min and then declines steadily to basal levels within 50 min. The volume of secretion increases up to five times above the normal levels while its protein content is increased by a factor of three. Quite different kinetics are exhibited by bile juices: their volume increases only by 15%, while their protein concentration gradually decreases three-fold as compared with physiological levels.

Fig. 2A shows the IEF profiles of rat pancreatic juice upon stimulation with two different secretogogues, caerulein and secretin, at different time intervals. To avoid preferential adsorption of some protein components, the samples have been applied to the gel slab in pockets precast in the gel, from the anodic side, after 30 min of prefocusing. More than 20 protein components are resolved by IEF, and no material is precipitated or trapped within the pocket. A very similar protein distribution is observed both in the absence or in the presence of 4 *M* urea, suggesting that the IEF profile is not due to protein aggregates. As readily apparent by a visual inspection of the gel, and as shown by the scans in Fig. 2B, the protein patterns, and their relative distribution, are practically identical under stimulation with either secretogogue.

Figs. 3A and B show the the stimulation kinetics of pancreatic and biliary juices, respectively, from a normal human adult, upon injection of a single dose of secretin. In the case of pancreatic juices, while the overall stimulation pattern is similar to the one obtained in rats, the peak is reached in only 15—20 min, while the increase in protein concentration is only 50% above unstimulated levels. The pattern in the bile is almost identical to the stimulation profile in rats.

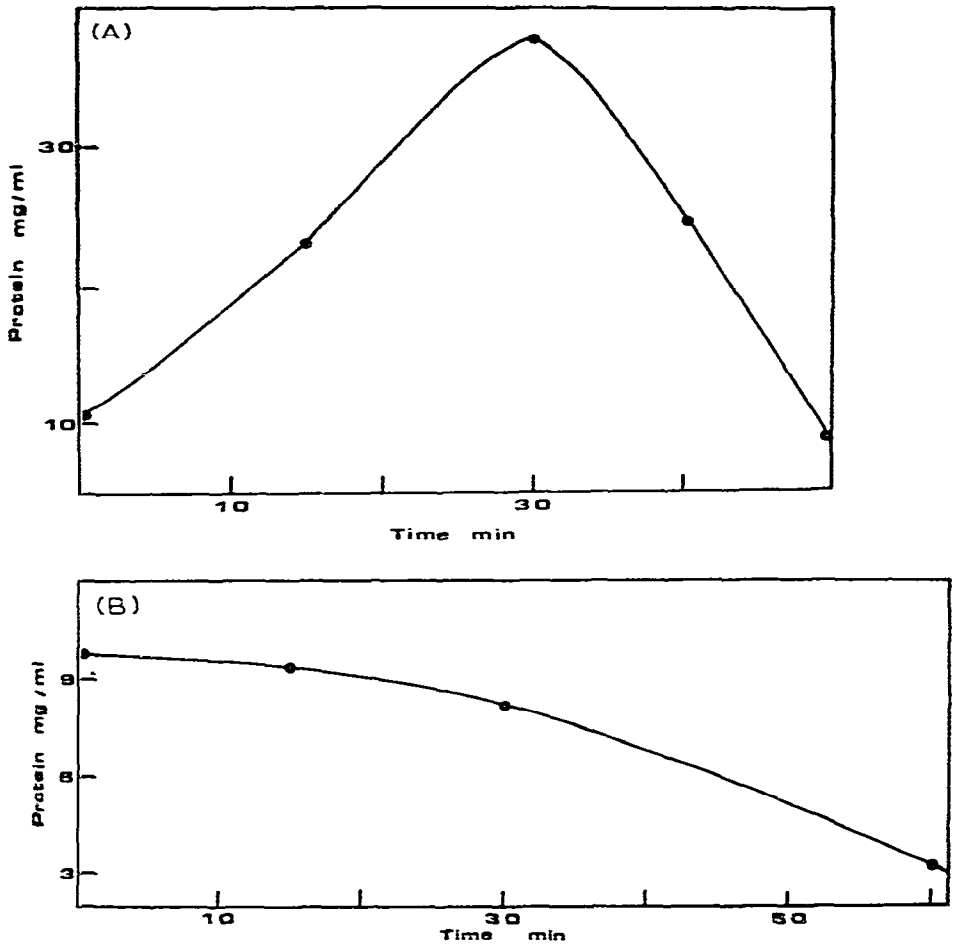
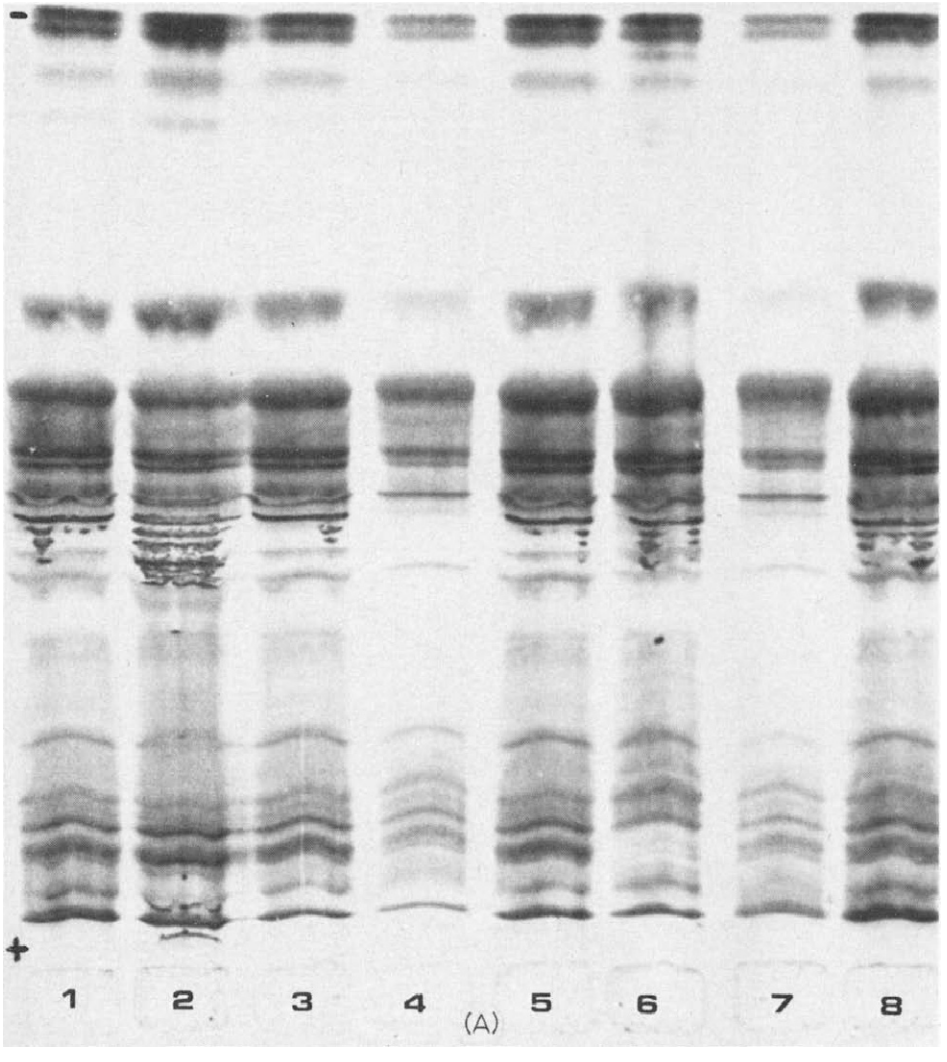
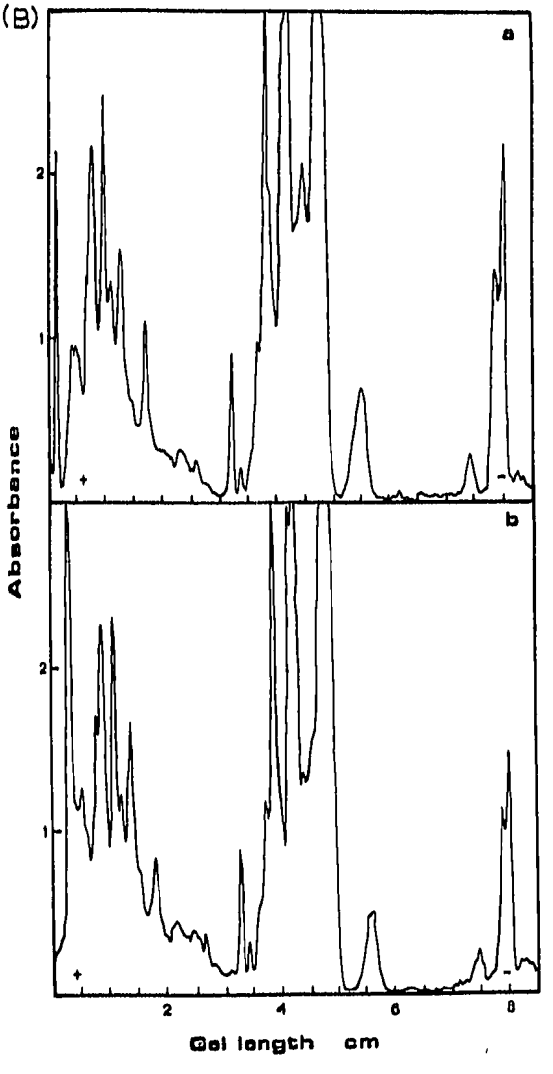


Fig. 1. Kinetics of pancreatic (A) and biliary (B) secretion in rat after caerulein stimulation (330 ng/kg intravenously). The protein concentration was determined according to Lowry et al. [16]; the possible interference by tensioactive substances from the bile was not investigated.

Fig. 2. (A) IEF of rat pancreatic juice. Samples 1—5 obtained by caerulein stimulation: 1, basal and 2—5, after secretogogue administration (2, 0—12 min; 3, 12—25 min; 4, 25—40 min; 5, 40—60 min). Samples 6—8 were collected after secretin stimulation (6, basal; 7, 0—30 min; 8, 30—60 min). Samples 4 and 7 were diluted 5 times, while all the others were diluted 3 times (in all cases, a 30-μl sample was applied in pockets precast in the gel). Experimental: polyacrylamide gel 5%T, 4%C_{Bis}, containing 2% LKB Ampholine pH 3.5—10 and 4 *M* urea; run: 4 h at 15 W constant; temperature, 4°C. Proteins were stained with Coomassie Brillant Blue R-250. (B) Scans at 600 nm of the patterns of samples 4 (a) and 7 (b) in Fig. 2A.



(A)



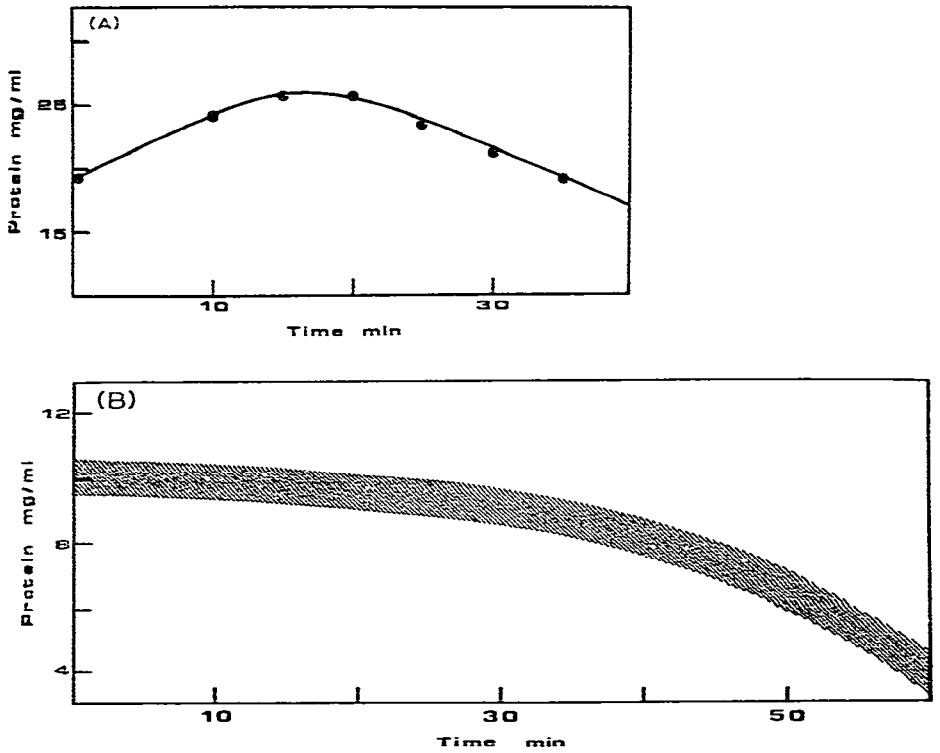


Fig. 3. Kinetics of pancreatic (A) and biliary (B) secretion in humans after caerulein stimulation (0.75 μg/kg). The protein concentration was determined according to Lowry et al. [16].

From a clinical point of view, it is of interest to obtain a reference IEF pattern of pure pancreatic juice from normal individuals, since it can be used as a basic reference for measuring possible qualitative or quantitative variations associated with pathological conditions. Fig. 4 exhibits the IEF profile of normal human adult pancreatic juice, obtained during surgery, upon stimulation with caerulein. A well-resolved pattern of about 20 components, distributed in the pH range 3.5—10, can be appreciated. As in the case of rat pancreatic juice, here too the qualitative and quantitative distributions of the various bands seem to be unaltered during the time of stimulation. This is also confirmed by the four scans of Fig. 5, which show the profiles of the 20 bands resolved by IEF in pancreatic juice. The 20 peaks have been numbered progressively and their relative abundance tabulated in Table I. It can be seen that the area of each peak remains remarkably constant during the period of stimulation, the distribution profile being practically identical to unstimulated samples.

We have also tried to demonstrate if any protein band could be detected in pure bile juice, obtained by cannulating either the choledochus or the cholecystis. Some faint protein bands can indeed be revealed (see Fig. 6), of which a few, indicated by arrows, appear to be in common with proteins of pure pancreatic juice. We cannot exclude, however, a partial contamination of bile secretion with the latter. Focusing of pure bile does not appear to be feasible,

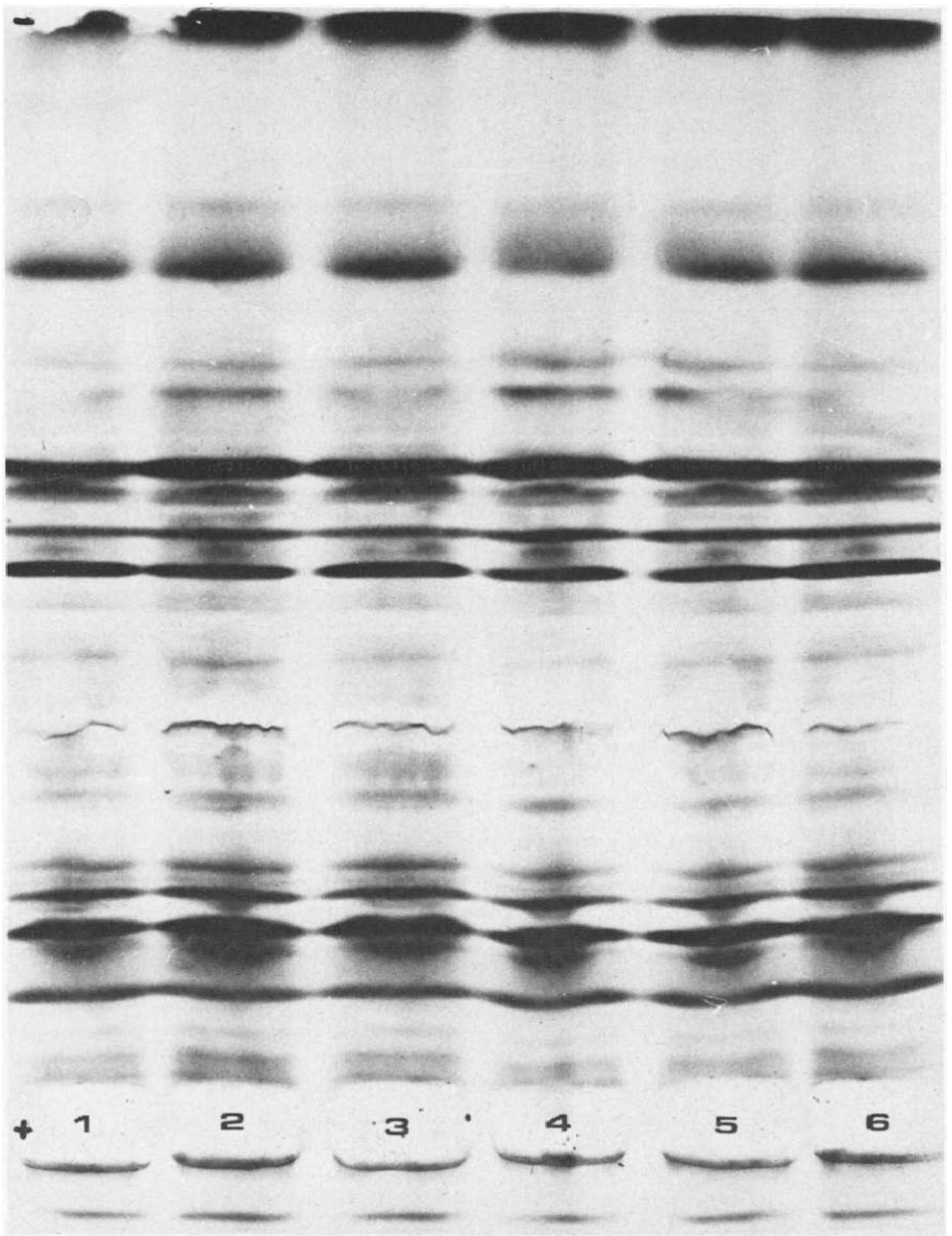


Fig. 4. IEF of human pancreatic juice obtained by surgical cannulation of the Wirsung duct, after caerulein stimulation. Samples: 1, basal secretion; 2, 0—10 min; 3, 10—15 min; 4, 15—20 min; 5, 20—25 min and 6, 25—30 min of discharge after stimulation.

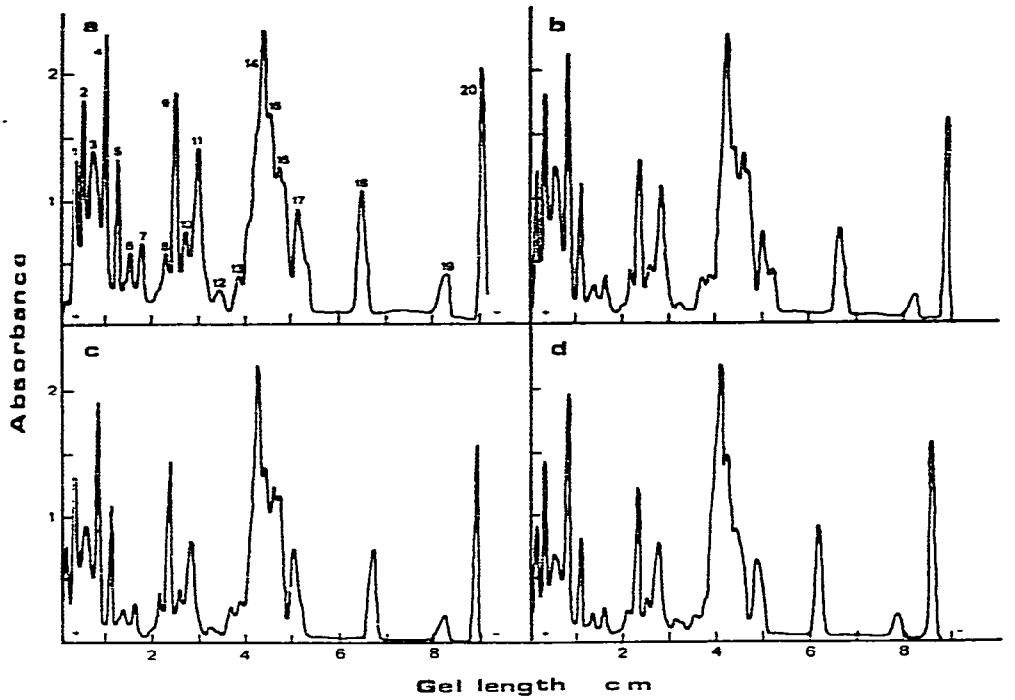


Fig. 5. Scans at 600 nm on IEF patterns from Fig. 4. (a) Sample 1; (b) sample 2; (c) sample 4; (d) sample 6.

TABLE I

RELATIVE PERCENTAGE OF PEAKS 1—20 OF FIG. 5

Data obtained by densitometry at 600 nm of the IEF patterns of human pancreatic secretion after stimulation with caerulein (Fig. 4) (S.D. = standard deviation).

Peak No.	Control	Time (min)				Mean	S.D.
		10	20	30	50		
1	3.7	4.0	3.6	3.5	3.8	3.7	0.172
2	5.7	6.6	5.2	5.5	6.0	5.8	0.477
3	8.0	8.6	8.1	8.3	8.8	8.4	0.300
4	6.5	6.1	7.0	6.8	6.4	6.6	0.314
5	3.4	3.1	3.2	3.0	3.3	3.2	0.140
6	1.1	1.0	1.0	1.1	0.9	1.0	0.075
7	1.6	1.8	1.5	1.5	1.4	1.6	0.136
8	2.4	2.0	2.0	2.2	2.1	2.1	0.163
9	5.1	4.9	4.6	4.8	4.5	4.8	0.213
10	2.4	2.2	2.2	2.3	2.0	2.2	0.132
11	6.0	7.0	5.5	6.5	6.2	6.2	0.500
12	1.9	1.7	1.6	1.8	1.5	1.7	0.141
13	2.0	1.9	1.8	2.0	1.6	1.9	0.150
14	17.6	16.6	18.5	16.6	17.0	17.3	0.720
15	7.6	8.1	8.5	7.4	7.5	7.8	0.417
16	6.6	5.3	7.0	6.5	6.0	6.3	0.585
17	5.3	5.2	6.0	5.8	5.9	5.6	0.326
18	5.1	5.0	5.3	5.6	5.8	5.4	0.300
19	2.9	3.0	2.8	3.1	3.4	3.0	0.206
20	6.2	6.0	5.6	6.4	5.9	6.0	0.271

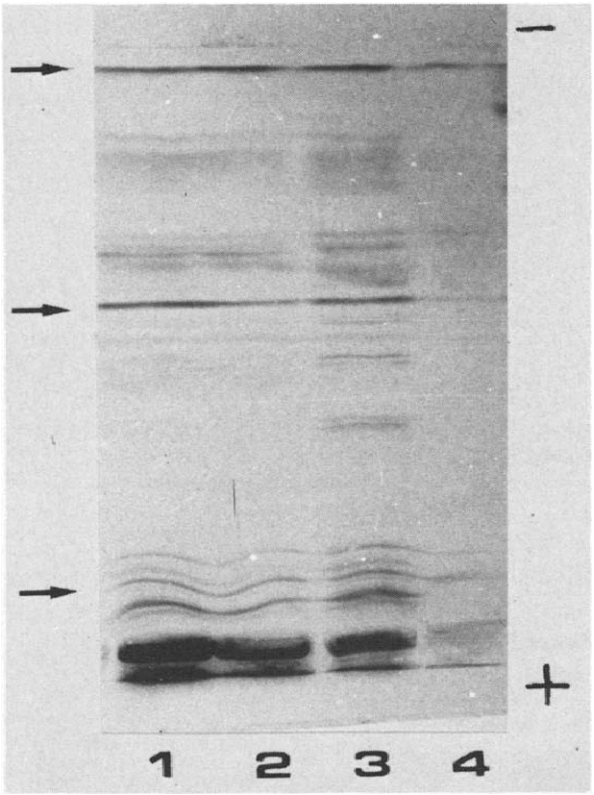


Fig. 6. IEF pattern of human bile: samples 1 and 2, collected from choledochus; 3 and 4 from cholecystis. 1 and 3, untreated, 2 and 4, dialyzed 12 h against Tris—Gly buffer, 10 m*M*, pH 8.2, then 24 h against 5 *M* urea in the above buffer. The arrows refer to protein bands common to pancreatic juice.

since often the IEF pattern is severely disorted by the high levels of biliary pigments and salts, which are not completely eliminated even after extensive dialysis (see samples 2 and 4 of Fig. 6). The biliary pigments collect at the anodic side into two heavy zones, the upper one of yellow colour, the lower one green (see samples 1—3 in Fig. 6), the former being the most abundant.

Besides analyzing pure pancreatic and biliary juices, we have also collected duodenal juice since, from a clinical point of view, this is the sample which can be more conveniently collected as it does not require surgery nor patient hospitalization. Fig. 7 shows the IEF profile of normal human adult duodenal juice taken at different time intervals, up to 60 min after stimulation with secretin. In agreement with what we have described so far, we can detect in this IEF pattern all the components of pancreatic secretion (see Fig. 4) mixed, especially in the acidic portion of the pH gradient with the few, minor protein components found in the bile juice (see Fig. 6). Scans and peak integration have

shown that the ratios of the components from pancreatic juice remain constant while the minor components, ascribed to bile products, in agreement with the stimulation kinetics of Fig. 3B, progressively decrease with time, together with the biliary pigments (1—2 sharp bands focusing below the application pocket; see Fig. 7). Also, in agreement with Fig. 3A, we can see the components from pancreatic juice increase in the duodenal juice, in the typical bell-shaped pattern, reaching a maximum after ca. 35 min of stimulation. The delay on the peak appearance (in pure pancreatic juice the plateau is reached in ca. 20 min) is most probably due to the dilution of the pancreatic juice by the bile secretion, with its considerably lower protein content.

We have also tried a characterization of the various enzyme activities present in the pancreatic secretion by developing a series of zymograms, for amylase, proteases, lipase and RNAase, as described under Materials and methods. The results are summarized in Fig. 8. Six amylase activities have been detected, three major bands having p*I*'s 7.2, 7.1 and 6.6 and three minor components,

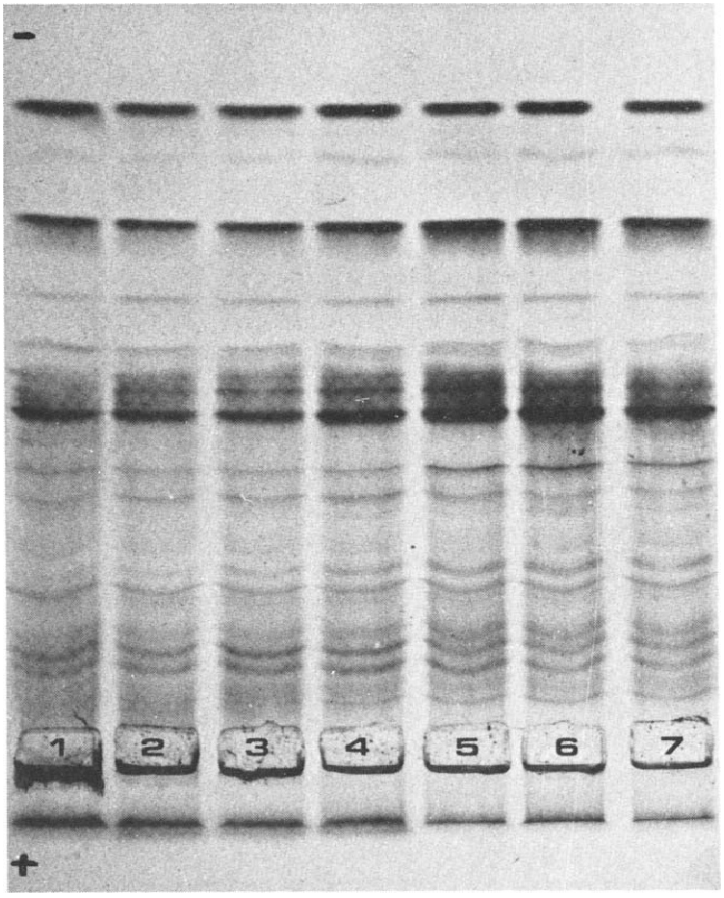


Fig. 7. IEF pattern of duodenal juice from humans after caerulein stimulation. Samples: 1, control; 2, 0—10 min; 3, 10—20 min; 4, 20—30 min; 5, 30—40 min; 6, 40—50 min; 7, 50—60 min after stimulation.

with p*I*'s 7.4, 6.7 and 5.8. These data are in general agreement with those of Berndt et al. [25] and Allan et al. [26]. Three lipase zones are evidenced, two major isozymes with p*I*'s 7.0 and 6.8 and a minor component with p*I* 6.4, in agreement with the IEF spectrum reported by Kurooka and Kitamura in sucrose density gradient columns [27]. Proteases are found with a wide spec-

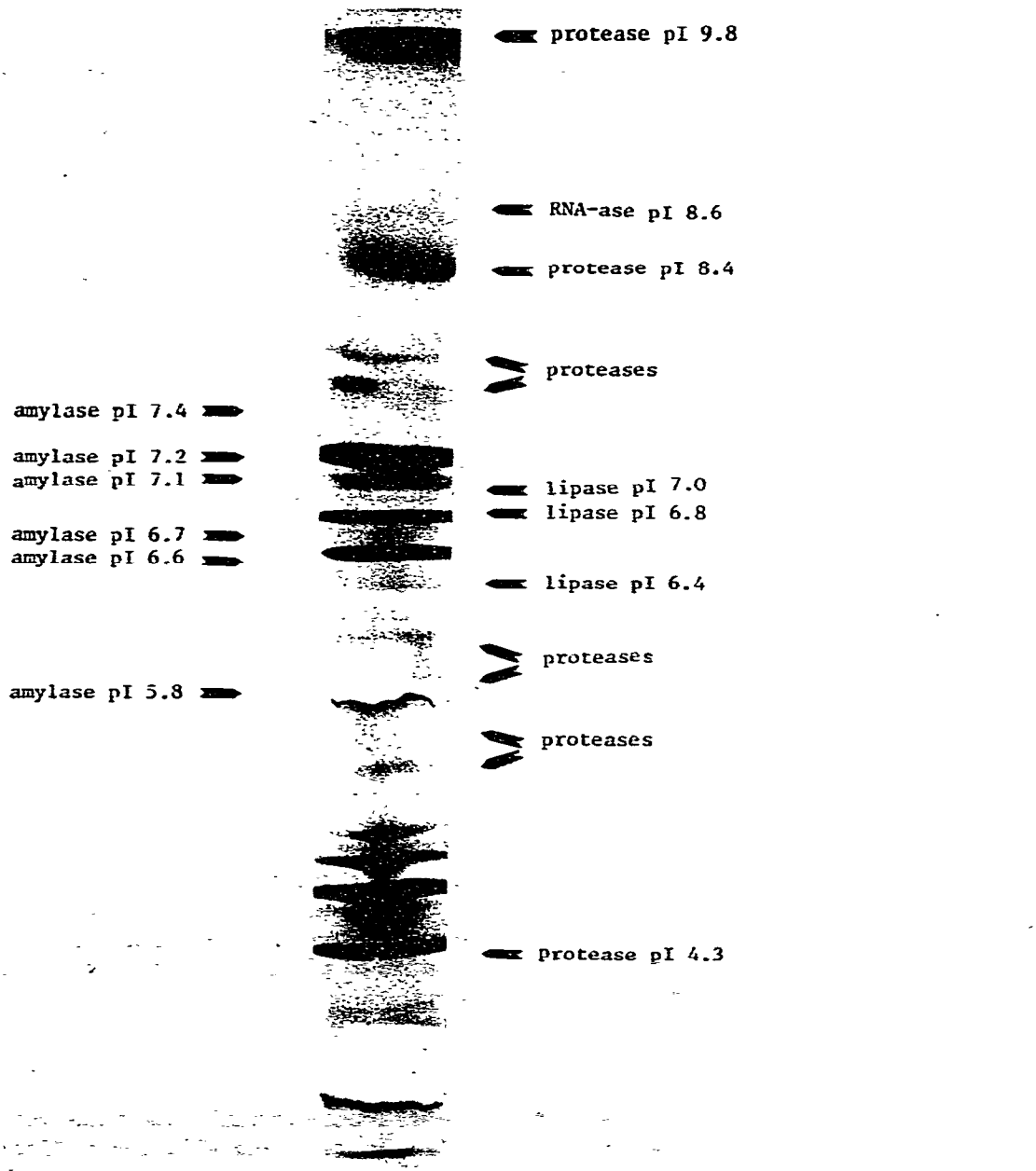


Fig. 8. Summary of the different enzymatic activities in pancreatic secretion as revealed by different zymograms (see Materials and methods).

trum of p*I*'s, in agreement with literature data on rat pancreatic secretion [28]. There are two heavy alkaline bands (p*I* 9.8 and p*I* 8.4) and a major acidic protease (p*I* 4.3). Several minor components are clustered around neutrality. One very sharp band of RNAase activity appears at p*I* 8.6. Some of the bands in the general Coomassie Blue pattern still remain to be identified.

DISCUSSION

In view of a future use of our IEF technique in clinical diagnosis, where the study of pancreatic secretion can be routinely accomplished only by duodenal tubage, it was necessary to study separately pure biliary, pancreatic and duodenal secretions, in order to obtain reference IEF patterns. Our data suggest that duodenal secretions, in which the majority of IEF bands are easily identifiable as pancreatic components, are indeed useful in producing a reference IEF profile of pancreatic juice discharge. Therefore, a future study of pancreatic function and possible alteration during disease can simply be accomplished by sampling and analyzing duodenal juices.

From a biochemical point of view, our data on rat and human pancreatic discharge, in normal conditions or under stimulation, fully support and expand the findings of Palade and co-workers [14, 15] on the synchrony of discharge of actual and potential enzyme activities from guinea pig pancreatic lobules. A parallel discharge also means a parallel intracellular transport. As already pointed out [14, 15], from the beginning of their appearance in condensing vacuoles, the major secretory proteins are already present in the same relative concentrations in which they are found later on in zymogen granules and in the discharge secretion. The fact that this discharge mechanism holds true for guinea pigs, rats and humans, even though the relative stimulation kinetics are somewhat different, suggests that the three major postulates of Palade and co-workers [14, 15]: (a) uniform response to secretogogues; (b) parallel intracellular transport; (c) parallel discharge, are indeed a general mechanism of pancreatic processing of exocrine proteins. However, this should not be taken to mean that the model of "selective modulation" of Rothman and co-workers [11—13] is necessarily wrong. The two models could in fact not be mutually exclusive, but could complement each other, for instance in terms of observation time. Our and Palade's data have only been obtained during a short period of observation or hormonal stimulation. However, over a long term, and in response to specific diets, it appears that the pancreas could alter the ratios among the different enzymes in the discharge granules [29, 30]. This could also happen in case of alteration of pancreatic function due to a disease. Further work is needed to elucidate these aspects.

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