1962

Comparison of the Protein Contents of Bovine Zymogen Granules and of Pancreatic Juice

Lewis Joel Greene

COMPARISON OF THE PROTEIN CONTENTS OF BOVINE ZYMOGEN GRANULES AND OF PANCREATIC JUICE

A thesis submitted to the Faculty of The Rockefeller Institute in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Lewis Joel Greene, B.A.

Acceptable for publication,

G. E. Palade

Professor, The Rockefeller Institute

C. H. W. Hirs,

Affiliate, The Rockefeller Institute

May 1962

The Rockefeller Institute

New York, New York

ACKNOWLEDGMENT

It is with gratitude and appreciation that I acknowledge the educational opportunities which I have been privileged to receive at The Rockefeller Institute as a Graduate Fellow and as a recipient of the Academic Press Fellowship (1959-1960).

I wish to express my great debt to my research advisors Dr. G. E. Palade and Dr. C. H. W. Hirs for their unfailing support and guidance throughout.

I am grateful to Drs. W. H. Stein, S. Moore, M. Kunitz, G. E. Perlmann, and R. D. Hotchkiss for the research experience I have gained in their laboratories.

The research described in this thesis was performed in the laboratory of Dr. D. D. Dziewiatkowski, and I wish to thank him for his generosity in allowing me the use of the facilities of his laboratory. Dr. P. Siekevitz provided the facilities for the cell fractionation work. I wish also to thank Miss S. Westerhoff for her invaluable technical assistance in the preparation of the material for the electron microscopy and for the photographs presented in this thesis. Dr. D. A. Yphantis performed the ultracentrifugal studies and was kind enough to instruct me in the theory and practice of the procedure he has developed.

Dr. N. O. Nielsen and Dr. T. H. Plummer of Brookhaven National Laboratory provided the pancreatic juice used in the research and I thank them for their indispensable co-operation.

Miss R. Mandlebaum and Mr. R. F. Carter were responsible for the preparation of the extensive illustrative material contained in this thesis. I appreciate both the technical skill and the efforts which they expended on my behalf. My thanks also to Miss J. Pemberton and Miss C. Gillespie for conscientiously typing the manuscript and to Miss S. Wohl for verifying the bibliography.

ABSTRACT

A zymogen granule fraction has been isolated from bovine pancreas by differential centrifugation under conditions designed to favor homogeneity at the expense of yield. The fraction has been examined in the electron microscope and found to contain a limited amount of contamination represented by clearly recognizable microsomes and mitochondria. These contaminants could account for a part of the 5% phospholipid and 1% nucleic acid found in the fraction by Schneider partition. The zymogen granule fraction was composed mainly of protein, about 95%.

The protein content of the granules has been solubilized by exposure to pH 8, after which a membrane fraction could be isolated by centrifugation. This fraction is assumed to represent the smooth surfaced membranes which bound *in situ* the zymogen granules.

The proteins present in the lysate of the granules and in the pancreatic juice have been fractionated by chromatography on DEAE cellulose and IRC-50 by the procedures employed by Keller, Cohen and Neurath. Trypsinogen, chymotrypsinogen A, ribonuclease, amylase, chymotrypsinogen B, procarboxypeptidase B, deoxyribonuclease and procarboxypeptidase A have been located in the chromatograms as 13 distinct peaks on the effluent curves. Complete recovery of the protein in pancreatic juice has been attained in this procedure; the recovery of the protein from the granule preparations was 10% less. Exact correspondence between the two chromatograms (juice and zymogen granule lysate) in terms of peak position, peak area, and enzyme specific activity has been observed. The chromatograms have revealed the presence of multiple forms of ribonuclease, in particular ribonuclease B; and have proved the existence of a new precursor of carboxypeptidase A, designated as procarboxypeptidase A', the molecular weight of which is approximately 25,000 less than procarboxypeptidase A.

The quantitiative measurement of proteins of the juice and lysate provides a nearly complete and direct proof of the Heidenhain hypothesis; that the intracellular storage site of the protein of the pancreatic secretion is the zymogen granule.

TABLE OF CONTENTS

SECTION I

INTRODUCTION

1. Pancreatic Zymogen Granules

a. Visualization of Granules in Acinar Cells: The outstanding morphological feature of the exocrine pancreas is the large number of granules located in the apical region of the acinar cell. The granularity of this region was first illustrated by Claude Bernard (1856) as a grainy, intracellular track bordering the lumina of the secretory cavities and it was subsequently described in further detail by Langerhans and Heidenhain (Heidenhain, 1875). Later, distinct granules were demonstrated in the apical zone by staining procedures. Finally, the fact that they were seen in the pancreas of living rabbits in spread mesenteric preparations (Kühne and Lea, 1882) proved that they were not artifacts of histological preparation but normal constituents of the acinar cell.

b. Heidenhain's Hypothesis: Secretory Proteins are Stored in Zymogen Granules: Heidenhain (1875, 1883) was the first to establish experimentally the relationship between the presence of the granules in the acinar cells and the functional state of the gland. No doubt he was influenced by the studies of Goodsir who, on various glands, including the ink pouch of the cuttlefish, had demonstrated that the secretion products first appear within the glandular cell (1844).

Heidenhain's conclusion that the granules were the secretory products synthesized by the cell was based on the following experimental observation: dogs copiously fed after a fast of 48 hours were sacrificed at different times during digestion and fragments of pancreas were fixed for histological examination. Shortly after the meal, when the discharge of the pancreatic juice had started, the granular inner zone of the cell was markedly reduced. After several hours, when the flow of pancreatic juice had stopped, the granular inner zone again increased in size, apparently by the formation of new granules. Complete restitution of the granular zone did not occur until 14 to 16 hours after the feeding. The variations in the protease content of the gland, as demonstrated in glycerol extracts, paralleled the amount of granular material observed in the histological preparations at different stages of the secretory cycle. Heidenhain proved that the granules were not

fat, as believed by histologists of that time, by demonstrating that they were soluble in water and dilute alkali. Finally, Kühne and Heidenhain showed that extracts of the gland did not contain active proteolytic enzymes but rather a substance capable of generating them. For this reason the material present in the cell was named zymogen and the granules were named zymogen granules. On the basis of this evidence Heidenhain concluded that the zymogen was stored in the granules until the time it was secreted as part of the pancreatic juice.

Support for this hypothesis came from the studies of Kühne and Lea (1882) who observed that in the pancreas of living animals the granules in the immediate vicinity of the glandular lumen disappeared during secretion. Following Heidenhain's lead, many other investigators have correlated, in both living and fixed preparations, the discharge of zymogen granules with the secretion of a protein rich pancreatic juice. The following stimulants have been shown to bring about the discharge of the granules: (1) direct electrical stimulation of the vagus and splanchnic nerves, (2) parasympathomimetic drugs, such as pilocarpine and choline chloride, (3) the presence of foodstuff in the small intestine and (4) the hormone, pancreozymin (cf. Thomas, 1950, pp. 129-132).

Reviews of previous investigations into the physiology, cytology and biochemistry of the pancreas can be found in the monograph of Babkin (1950) and the articles by Palade et al. (in press) and Desnuelle and Rovery (1961). No attempt will be made to present a detailed review of this literature; only the papers that are particularly relevant to the present study shall be discussed. In this section we shall consider: a. direct immunochemical and biochemical evidence that the zymogen granules contain at least some of the secretory proteins; b. current information about the secretory cycle of the pancreas at the cellular level; c. properties of the well characterized pancreatic digestive enzymes; and d. separation of pancreatic juice proteins.

c. Recent Evidence that Granules Contain the Secretory Protein: The studies cited present only the circumstantial evidence to support Heidenhain's hypothesis. Some direct but incomplete evidence indicating that the zymogen granules do, in fact, contain secretory protein has been brought forward by the following recent studies.

(1) Histological Localization by Means of Fluorescein-labeled Antibodies

Marshall (1954) used fluorescein-labeled antibodies to visualize the intracellular localization of secretory protein in the bovine exocrine pancreas. Antibodies prepared against chymotrypsinogen A and carboxypeptidase A concentrated in the apical region of the cell, where the zymogen granules are most numerous. The lumina and ducts, which contained protein already secreted by the acinar cells, were also heavily labeled. Antisera to ribonuclease and deoxyribonuclease reacted with the zymogen granule region in a similar manner, but also stained the rest of the cytoplasm, though less intensely. These differences in distribution between the proteolytic enzymes and the nucleolytic enzymes have also been noted in cell fractionation studies and will be discussed later. The magnification of the light micrographs presented in Marshall's paper is too low to allow a satisfactory evaluation of his claim that the method yields results at a resolution of 0.5 μ. Marshall stated that all cells and zymogen granules were alike in their reaction to the antibodies against the two proteolytic enzymes. The apparent lack of cytological specificity may be interpreted to mean that each granule contains the full complement of digestive enzymes.

(2) Cell Fractionation Studies

Table 1 summarizes the results of recent experiments which employed differential centrifugation to isolate fractions containing zymogen granules from pancreatic homogenates. The symbol + indicates that the enzyme activity was demonstrated in the fraction. In two cases an enzyme activity could be separated from most of the protein in the fraction by means of ion-exchange chromatography. No information is available about the proteolytic enzymes of the species listed in the table. By analogy to the well characterized bovine pancreatic enzymes we could expect three endopeptidases, trypsinogen, chymotrypsinogen A, chymotrypsinogen B, and two exopeptidases, procarboxypeptidase A and procarboxypeptidase B, to be present in the general class denoted by the term protease. Endopeptidases make the greatest contribution to the measured activity when hemoglobin or casein are used as substrates. With the exception of the guinea pig

TABLE 1

ACTIVITY OF DIGESTIVE ENZYMES DEMONSTRATED IN ZYMOGEN GRANULE FRACTIONS

	AMYLASE	LIPASE	PROTEASE*	CHYMOTRYP-SINOGEN A	RIBO-NUCLEASE	DEOXYRIBO-NUCLEASE	REFERENCE
Dog	+	+	+				Hokin, 1955
Guinea Pig (1)			+	+ (2),(3)	+		Siekevitz and Palade, 1958a,b; 1960
Guinea Pig	+	+	+				Hansson, 1959
Mouse	+				+ (3)	+	Dickmann and Morrill, 1959 Van Lancker and Holtzer, 1959
Rat	+						Laird and Barton, 1957

* Activity measured by digestion of hemoglobin or casein after activation by trypsin or enterokinase.

(1) Electron microscopic examination of fraction.

(2) Identified on basis of activity toward acetyl-L-tyrosine ethyl ester. Chromatographic behavior indicates protein is cationic.

(3) Activity separated from most of protein in the fraction by ion exchange chromatography.

protein having properties similar to bovine chymotrypsinogen A* (Siekevitz and Palade, 1958a,b; 1960), the individual proteolytic enzymes were not identified in the fractionation studies summarized in Table 1. No enzymatic determinations were performed in search of the activities corresponding to the open areas of the table. It may be concluded that many, but not all, of the enzymatic activities usually associated with the classical pancreatic enzymes have been demonstrated to be present in this fraction.

The most convincing evidence for the localization of the secretory protein within the zymogen granules comes from specific activity measurements. The zymogen granule fraction had the highest specific activity for each enzyme when compared to the other cell fractions isolated (Hokin, 1955; Siekevitz and Palade, 1958a,b; Dickman and Morrill, 1959; Van Lancker and Holtzer, 1959). Siekevitz and Palade showed that this result is not an artifact due to cross contamination of fractions or mechanical disruption of cell organelles, two factors which must be considered in this system. Using the electron microscope, these authors examined the zymogen granule fraction during successive purification steps and found that the specific activity for ribonuclease and trypsin activatable protease increased as the amount of mitochondrial and microsomal contamination decreased. Although these data indicate that the zymogen granules contain some of the secretory protein, the experiments cited cannot be considered a complete test of the Heidenhain hypothesis because only a few of the secretory proteins were determined and a quantitative comparison was not made with pancreatic juice.

The results of the cell fractionation studies bear on other problems concerning the pancreatic secretory process, many of them as yet unresolved. It is beyond the scope of this review to discuss in detail all of these results and therefore they will be only summarized. In a recent paper Palade *et al.* (1961) discuss these data within the general context of the pancreatic secretory cycle.

* The protein could be chromatographed on IRC-50 and, after activation with trypsin, it catalyzed the hydrolysis of acetyl-L-tyrosine ethyl ester but did not hydrolyze benzoyl-L-arginine ethyl ester to any appreciable extent.

In addition to the demonstration that the zymogen granules contain secretory protein, the two salient findings brought forward by the work on pancreatic cell fractions are: (1) the secretory proteins, determined by enzyme assay, are found in all fractions; though the zymogen granules fraction usually contained the largest percentage of the recovered activity; (2) the individual secretory proteins do not have the same pattern of distribution among cell fractions. The interpretation of data derived from distribution studies is complicated by a number of factors related to the cell fractionation procedure itself, the methods used for the determination of secretory protein (enzyme activity), and the existence of a secretory cycle in which the digestive proteins move from compartment to compartment within the cell.

Some, perhaps all, of the secretory protein recovered in the nuclear and mitochondrial fractions may be attributed to contamination by intact zymogen granules. Centrifugation procedures, when used in conjunction with distribution studies, succeed only in concentrating the components present in the homogenate rather than separating them completely into homogeneous fractions (see, for example, electron microscope studies of Siekevitz and Palade, 1958a). During homogenization and centrifugation, spurious localizations may arise due to the disruption of vacuoles (zymogen granules, Golgi vacuoles) and other compartments present in the intact cell, and the subsequent adsorption of secretory protein on to various structures. The adsorption of chymotrypsinogen A by pancreatic microsomes (Keller and Cohen, 1961) and of hemoglobin by ribosomes (Petermann and Pavlovec, 1961) may be cited as examples of this phenomenon.

The determination of secretory protein in cell fractions solely by means of enzyme activity measurements presents serious problems, especially when carried out on mixtures of proteins some of which are active proteolytic enzymes. Even if complete activation of the proteolytic zymogens were achieved, the subsequent autolysis of the mixture of protease makes the determination uncertain unless properly controlled. The extent of activation and some of the enzyme assays may also be affected by enzyme inhibitors, trypsin inhibitor (Siekevitz and Palade, 1958a), deoxyribonuclease inhibitor (Van Lancker and Holtzer, 1959) and ribonuclease inhibitor (Dickmann and Morrill, 1959), demonstrated to be unequally distributed among cell fractions. The determination of amylase (Stein and

Fischer, 1958) and lipase (Marchis-Mouren et al., 1959) by means of enzymatic activity is very sensitive to experimental conditions and under certain conditions these enzymes are inactivated by proteolytic enzymes.

Analysis of the kinetics of the specific radioactivity distribution in cell fractions (counts per minute/mg protein) after a pulse of radioactive amino acids indicates that the secretory proteins are synthesized on the attached ribonucleoprotein particles (isolated in the microsome fraction) and then later appear in the mature zymogen granules (Siekevitz and Palade, 1960). For this reason the amount of secretory protein recovered in these fractions will depend on the secretory state of the gland. One aspect of species variations in the secretory cycle relates to the intracisternal granules of the guinea pig. Fractionation studies combined with electron microscopy have shown that one of the intermediate stages of secretory protein transfer from ribonucleoprotein particles to mature zymogen granules is represented by the concentrations of secretory protein (not limited by a membrane as the zymogen granules) found within the intracisternal spaces of the endoplasmic reticulum in the intact cell and within the circular membranes derived from the endoplasmic reticulum recovered in the heavy microsome fraction (Palade, 1956; Siekevitz and Palade, 1958b).

Although most of the evidence supports the view that secretory protein is stored and extruded from the cell using a mechanism involving zymogen granules, it is not certain that this pathway is mandatory for all enzymes under all physiological conditions. Redman and Hokin (1959), on the basis of fractionation studies, have suggested that during conditions of continuous stimulation and secretion a second pathway exists for amylase involving the tranfer of soluble protein directly from the cytoplasm through the cell membrane into the acinar lumen. The secretory protein located in the cytoplasm by virtue of this mechanism might be found in several fractions depending on its physical state in the cytoplasm.

(3) Autoradiography Combined with Electron Microscopy

Some of the uncertainties inherent in the interpretation of the data from cell fractionation experiments have been removed by recent autoradiographic studies (Caro and Palade, 1961). After the injection of a pulse of tritiated leucine to recently fed guinea pigs, the distribution of

label in subcellular structures was determined by autoradiography combined with electron microscopy. Because of the large difference known to exist between the rate of synthesis of protein for secretion and the rate of synthesis of other cellular protein immediately after stimulation of pancreatic secretion (Hansson, 1959), the distribution of radioactivity was interpreted to represent the distribution of newly synthesized secretory protein. The secretory cycle determined by this method has been restricted to the passage of secretory protein from

rough endoplasmic reticulum → smooth endoplasmic reticulum (Golgi) → zymogen granules.

The significant information from the kinetic data of the autoradiographic experiments is the role of the Golgi complex in the secretory cycle.* These data remove mitochondria and nuclei from the direct pathway of synthesis or transport of secretory protein and eliminate the confusion in the fractionation results due to intercontamination of fractions. The time course for the distribution of the label favored a single site for protein synthesis, rough endoplasmic reticulum, and identified the granules as storage sites for the secretory protein.

d. Pancreatic Secretory Cycle: In general there is good agreement between the fractionation and autoradiographic results concerning the major characteristics of the secretory cycle. Though many of the details of the processes involved are unknown, the current interpretation of the available data suggests the following outline for the pancreatic secretory cycle (Palade et al., in press).

1) Protein is synthesized on the ribonucleoprotein particles attached to the membrane of the endoplasmic reticulum.
2) Protein is released from the particles and transported across the membrane of the endoplasmic reticulum to the intracisternal spaces.

* The participation of the Golgi apparatus in the secretory process had been suggested by light microscope (cf. Junqueira and Hirsch, 1956) and electron microscope studies (cf. Kurosumi, 1961). However, the kinetic data present the first biochemical support for the microscopic observations.

3) Protein is transported within the cavities of the endoplasmic reticulum to the smooth surfaced vacuoles of the centrosphere region (Golgi). The progressive filling of the vacuole with newly synthesized protein and its subsequent movement to the apical region of the cell corresponds to the first appearance of the zymogen granule.
4) The protein may remain stored in the granules for several hours until discharge is stimulated by the intake of food.
5) Discharge of the contents of the zymogen granules involves coalescence of the zymogen granule membranes with the cell membrane and the subsequent extrusion of the contents.

The studies reviewed have utilized the techniques of cell fractionation, autoradiography, and fluorescein labeled antibody localization, and are in general agreement that, for at least the enzymes tested, the zymogen granules actually contain secretory protein. Kinetic evidence derived from the first two methods indicates that the zymogen granules represent storage sites in a complicated intracellular secretory cycle. However, it is clear that a direct and complete test of the Heidenhain zymogen granule hypothesis (that the granules are precursors of pancreatic juice protein) would require the qualitative and quantitative characterization of the separated proteins of pancreatic juice and zymogen granules.

In the remainder of this section the properties of the well characterized secretory proteins and recent advances in their separations and purification will be discussed.

2. Pancreatic Secretory Proteins

a. The Properties of the Well Characterized Pancreatic Digestive Enzymes: The pancreas plays a major role in the process of digestion by synthesizing many of the enzymes which catalyze the hydrolysis of fat, carbohydrate and protein in the small intestine. Many of the enzymatic properties of extracts of the whole gland and pancreatic juice were recognized in the last century; but only recently has it been possible to isolate these proteins in sufficiently pure form to permit their chemical and enzymatic characterization. The major stimulus for the modern study of the pancreatic secretory proteins came from the now classic investigations of

TABLE 2

ENZYMES SECRETED BY THE PANCREAS

ZYMOGEN OR ENZYME	JUICE[1]	SPECIES[2]	GENERAL ENZYME ACTIVITY	SPECIFICITY	SUBSTRATE IN THIS STUDY	ENZYMATIC ACTIVATION	METAL	MOLECULAR WEIGHT	ISO-ELECTRIC POINT
Trypsinogen	+	Bovine	Endopeptidase	Basic amino acids	TAME	Trypsin[3]	-	23,800	9.3
Chymotrypsinogen A	+	Bovine	Endopeptidase	Aromatic amino acids	ATEE	Trypsin	-	25,000	9.1
Chymotrypsinogen B	+	Bovine	Endopeptidase	Aromatic amino acids	ATEE	Trypsin	-	24,000	5.2
Procarboxypeptidase A	+	Bovine	C-terminal exopeptidase	Aromatic amino acids	CGP	Trypsin	Zn	94,000	< 4.5
Procarboxypeptidase B	+	Bovine	C-terminal exopeptidase	Basic amino acids	HA	Trypsin	*Zn	-	-
Ribonuclease	+	Bovine	Endonuclease	Pyrimidine (3')	RNA	Not required	-	13,700	9.2
Deoxyribonuclease	+	Bovine	Endonuclease	No base specificity	DNA	Not required	-	40,000	4.7
Amylase	+	Porcine	Endoamylase	α 1 $\rightarrow$ 4 glucosyl linkages	STARCH	Not required	Ca	45,000	5.4
Lipase	+	Porcine	Esterase	Emulsified triglycerides	Olive oil emulsion	Not required	-	-	5.2

1 Demonstrated in pancreatic juice.

2 Species: origin of well characterized protein.

3 Enterokinase, mold kinase.

* Carboxypeptidase B, molecular weight 34,000.

Northrop and Kunitz who crystallized trypsinogen, chymotrypsinogen A, ribonuclease and deoxyribonuclease from acid extracts of bovine pancreas (Northrop et al., 1948).

The literature concerning these proteins is so large that it cannot be completely reviewed in this section and therefore only a limited amount of information will be presented. Table 2 summarizes some of the enzymatic and physical properties of the well characterized pancreatic proteins, all of which have been demonstrated in the pancreatic juice. The information in Table 2 is derived from the review articles listed in the text.

Proteolytic Enzymes: The ability of pancreatic juice to digest protein was observed by Bernard and Corvisart, while the active principle was named trypsin by Kühne (Oppenheimer, 1925). It was known to Kühne and Heidenhain that the enzyme was present in the gland and pancreatic juice in an inactive form which rapidly underwent conversion into the active proteolytic enzyme (Heidenhain, 1883). We now know that pancreatic juice contains at least five proteolytic zymogens. In articles by Desnuelle and Rovery (1961) and Neurath (1960) recent studies of the pancreatic proteolytic enzymes are discussed.

Trypsinogen: Trypsinogen was first isolated by Kunitz and Northrop who demonstrated that the conversion to the active enzyme was catalyzed by trypsin, enterokinase or penicillium kinase (Northrop et al., 1948). The autocatalytic conversion of bovine trypsinogen to trypsin is associated with the hydrolysis of one peptide bond located at the N-terminal end of trypsinogen, with liberation of the hexapeptide, Val $(Asp)_4$Lys (Davie and Neurath, 1955). The same or similar events appear to take place during the activation of trypsinogen mediated by enterokinase (Yamashina, 1956), aspergillus saitoi protease (Gabeloteau and Desnuelle, 1960) and penicillium kinase (Hofmann, 1960). Trypsin is the only enzyme known possessing the ability to activate all the other proteolytic zymogens present in the pancreatic juice. The events occurring during the physiological activation of pancreatic juice are: (1) activation of trypsinogen by enterokinase present in the small intestine, and (2) activation of trypsinogen and other zymogens by trypsin liberated from (1).

Trypsin has endopeptidase activity which is maximally active near pH 8.

It is specific for amide or ester bonds to which the carbonyl group is contributed by arginine or lysine (cf. Green and Neurath, 1954, for a review of specificity of proteolytic enzymes). The ester substrates are hydrolyzed more rapidly than amides and are employed for routine assay using potentiometric or spectrophotometric methods to monitor the reaction. The amino acid sequence of trypsinogen is currently being investigated (Walsh et al., 1961) and it appears that the molecule is a single polypeptide chain with no detectable C-terminal residue (Pechère and Neurath, 1957).

Chymotrypsinogen A: The existence of a second pancreatic proteolytic enzyme was suggested by two observations of Vernon (1913) who partially separated the milk clotting activity from the protein solubilizing activity present in pancreas, and noticed that the total activatable protein solubilizing activity obtained by enterokinase activation was less than that generated by using recently activated pancreatic extracts. The protein, chymotrypsinogen A, was discovered by Kunitz and Northrop (1934) and found to be a zymogen which could be activated by trypsin but not by enterokinase. The active enzyme differs from trypsin in that it clots milk but does not clot blood; it resembles trypsin in its ability to digest denaturated protein. A comparison of the physical and enzymatic properties of trypsinogen, chymotrypsinogen A, and chymotrypsinogen B may be found in the review article by Desneulle and Rovery (1961).

The activation of chymotrypsinogen A involves a series of enzymatic reactions and results in the formation of three related proteins, π δ and α chymotrypsin. The π and δ forms are considered to be intermediates in the production of α chymotrypsin. The activation conditions can be manipulated to obtain any one of these proteins. All three forms are enzymatically active and have the same specificity, but the π and δ forms have a higher specific activity than does α chymotrypsin (Rovery et. al. 1957).

Like trypsin, the chymotrypsins are endopeptidases, which are maximally active at pH 8. They exhibit high but not absolute specificity for ester and amide bonds of aromatic amino acids. Amino acids with large side chains, such as leucine, methionine and glutamine, are also substrates for chymotrypsin. Recent studies indicate that chymotrypsinogen A possesses a single polypeptide chain with half cysteine as N-terminal and

asparagine as C-terminal residue. Its amino acid composition has been determined and the amino acid sequence is being investigated (Hartley, 1961).

Chymotrypsinogen B: In 1947 Laskowski isolated a new proteolytic zymogen from bovine pancreas. The enzymatic properties of the active enzyme were similar to those already described for chymotrypsinogen A, i.e. (1) ability to digest casein and clot milk;(2) ability to hydrolyze peptide linkages involving the carbonyl group of aromatic amino acids. It was named chymotrypsinogen B (Keith et al., 1947).

Although the two bovine chymotrypsinogens have similar molecular weights, 25,000, and N-terminal residue, half cysteine, they differ from each other in chemical properties. Chymotrypsinogen A, a cationic protein isoelectric at pH 9.1 can be separated from anionic chymotrypsinogen B, isoelectric at pH 5.2, by means of chromatography on DEAE cellulose (Keller et al., 1958b). The amino acid composition of the zymogens differs significantly in proline, hydroxyamino acid and amide content.

Both chymotrypsinogens are activated by trypsin at approximately the same rate. The chemical events in the activation of chymotrypsinogen B are unknown. Although their specificity is similar, the enzymes can be distinguished by comparison of the rates of hydrolysis of acetyl-L-tyrosine ethyl ester and acetyl-L-tryptophan ethyl ester in 30% methanol (Keller et al., 1958b).

Procarboxypeptidase A: Waldschmidt-Leitz and Porr (1929) demonstrated that extracts of autolyzed pancreas catalyzed the hydrolysis of carboxyl-terminal peptide bonds. The enzyme, carboxypeptidase, was crystallized by Anson (1937) and subsequently shown to have high specificity for unsubstituted carboxyl-terminal aromatic and branched aliphatic amino acids. Hippuryl-phenyllactic acid, the ester analogue of hippuryl-phenylalanine, is also hydrolyzed by the enzyme (see Green and Neurath, 1954 for a discussion of specificity). The crystalline enzyme is a metalloprotein containing 1 gram atom of zinc per mole. The zinc-free enzyme is inactive but zinc and other metals of the first transition period restore part or all of the activity. A review of the specificity and specific activity of carboxypeptidase containing zinc and other metals has recently been written by Vallee (1961).

Although the existence of the zymogen was demonstrated in 1929, procarboxypeptidase A was not isolated until 1956 (Keller et al.). The chemical events associated with the trypsin mediated activation of procarboxypeptidase A, molecular weight 96,000, to carboxypeptidase A, molecular weight 34,000 are known only in part (Brown et al., 1961)*. The molecular characteristics of procarboxypeptidase A and carboxypeptidase A are reviewed by Neurath (1960).

Procarboxypeptidase B: Folk and Gladner (1958) found a new carboxypeptidase, which was demonstrated to have the same general structural requirements for specific substrates as carboxypeptidase A with the exception that the C-terminal amino acid must be contributed by arginine or lysine residues. Procarboxypeptidase B, which was purified by these authors, was later chromatographed by Keller (1958b). Substitution of the zinc in porcine carboxypeptidase B with cobalt enhances the peptidase activity and diminishes the esterase activity; whereas cadmium in place of zinc has the opposite effect on the relative specificity of the enzyme (Folk and Gladner, 1961).

Nucleolytic Enzymes

Ribonuclease: Jones and Perkins (1923) observed that boiled extracts of pancreas transformed yeast ribonucleic acid to acid soluble products without the concomitant liberation of purine and pyrimidine bases or inorganic phosphate. Kunitz (1940) crystallized the enzyme and it was later chromatographed by Martin and Porter (1951) and by Hirs et al. (1953). Ribonuclease is an endonuclease which catalyzes the hydrolysis of the phosphodiester bond between 3'- and 5'- hydroxyl groups of the ribose moieties in the ribonucleic acid chain liberating 3'- phosphomono esters. It is maximally active at pH 7-7.5, and exhibits specificity for pyrimidine nucleosides bonded at the 3'- position to phosphate (Schmidt, 1955). The covalent structure of bovine pancreatic ribonuclease is known in detail (Hirs et al., 1960). The cationic protein, isoelectric at pH 9.2, consists of a single polypeptide chain of 124 amino acids crosslinked by 4 disulfide bonds.

Anfinsen and White (1961) have reviewed current knowledge concerning tertiary structure, chemical modifications, and the mechanism of ribonuclease action.

* The activation of procarboxypeptidase A is discussed in greater detail in Section IV, 3a.

Deoxyribonuclease: In 1950 Kunitz crystallized from pancreatic extracts an endonuclease which acted on deoxyribonucleic acid. The ability of pancreatic juice to liquify gels of deoxyribonucleic acid had been observed by Abderhalden in 1906 and by other investigators (see Kunitz, 1950, for crystallization and historical review). Deoxyribonuclease is an endonuclease which catalyzes the hydrolysis of the phosphodiester bonds of deoxyribose polynucleotides. The enzyme differs from ribonuclease by requiring bivalent cations for activity and by displaying no absolute specificity for either purine or pyrimidine bases adjacent to the susceptible linkage. Another significant difference distinguishing these enzymes is that the products of deoxyribonuclease digestion are 5'-phosphomono esters rather than 3'-mono esters (Schmidt, 1955).

Deoxyribonuclease is an anionic protein of molecular weight 40,000. The amino acid composition has been reported but little chemical information is available about this protein (cf. review of Laskowski, 1961).

Lipase

Bernard (1849) was the first to call attention to the importance of the pancreas for the digestion of fat by demonstrating that pancreatic juice catalyzed the hydrolysis of neutral fat to glycerol and fatty acid.

In a review article, Desneulle (1961) describes the physical and enzymological properties of porcine pancreatic lipase, which was recently isolated in pure form. The outstanding feature of lipase, which distinguishes it from other esterases present in the gland, is that the enzyme exhibits maximum activity when adsorbed at an oil/water interface (Sarda and Desnuelle, 1958). When adsorbed on an oil/water interface, lipase is inhibited by diethyl-p-nitrodiphenyl phosphate, but not when it is in solution. It has been suggested that lipase in solution may be considered a zymogen which, like trypsinogen and chymotrypsinogen, is inactive. Conversion to the active enzyme is brought about by some limited change in tertiary structure, presumably as the result of interfacial adsorption rather than by limited proteolysis which is the primary event for the proteolytic zymogens (Desneulle, 1961).

Amylase

The ability of pancreatic juice to solubilize starch was demonstrated by Bouchardat and Sandras (1845). Although saliva also contains an enzyme

capable of catalyzing the same reaction, Bernard (1856) showed that the pancreatic enzyme was the more important one in digestion.

The pancreatic enzyme belongs to the class of α amylases which randomly hydrolyze the internal 1 $\longrightarrow$ 4 glycosidic linkages of starch and glycogen. This endoamylolytic action results in a rapid reduction of viscosity and molecular weight: the primary products are oligosacharides (dextrins). The β amylases of plant and microbial origin, by contrast, attack the non-reducing outer chain and in a regular manner hydrolyze every other glycosidic bond to produce maltose.

Recent chemical and enzymological information about amylases may be found in the review article by Fischer and Stein (1960). The best characterized pancreatic amylase, crystallized by Fischer and Bernfeld (1948), is of porcine origin. α amylases are metalloenzymes which contain at least one gram-atom of firmly bound calcium/mole of enzyme, which is required for enzyme activity. It has been demonstrated that calcium protects the enzyme from inactivation by chymotrypsin and trypsin (Stein and Fischer, 1958). Fischer and Stein believe that the chelated calcium maintains the protein in proper configuation for biological activity.

b. Enzyme Activities Demonstrated in Pancreas or Pancreatic Juice: In addition to the well characterized pancreatic enzymes or zymogens many other enzyme activities have been demonstrated in tissue extracts or pancreatic juice. It is not certain at the present time that all the enzyme activities present in tissue extracts are components of the exocrine secretion. Like the erepsins, some of these enzymes may be present in tissue extracts but not in pancreatic juice (Lebreton and Mocoroa, 1931). Some of these enzymes are listed in Table 3. They have not been studied in the present investigation.

The concentration of the proteins in the gland (or juice, or granules) has not been determined. For this reason their significance in terms of the protein composition (by weight) is not known. Since our results and those of Keller et al. (1958b, 1961) account for 80% of the total protein present in zymogen granule extracts and pancreatic juice in terms of the enzymes listed in Table 2, the proteins listed in Table 3 may account for 20% of the protein in the exocrine secretion. This value (20%) is a maximum estimate based on the assumption that Tables 2 and 3 list all the protein in the exocrine secretion.

TABLE 3

ENZYME ACTIVITIES DEMONSTRATED IN TISSUE EXTRACT OR PANCREATIC JUICE*

Proelastase	Grant and Robbins (1955)
Elastase	Lamy et al. (1961)
Pancrinogen	Soejima and Shimura (1958)
Pancrin	Grant and Robbins (1956)
Cholesterol Esterase	Swell et al. (1955)
Esterase	Sarda and Desneulle (1958)
Phospholipase A	Rimon and Shapiro (1959)
Phospholipase B	Shapiro (1953)
Phosphodiesterase	Davis and Allen (1956)
Trypsin Inhibitor (cationic)	Kunitz and Northrop (1936)
Trypsin Inhibitor (anionic)	Kazal et al. (1958)

* Not included in the present investigation.

c. Separation of Proteins Present in Pancreatic Juice

(1) By Free Electrophoresis and Paper Electrophoresis

Several attempts to separate pancreatic juice proteins by free electrophoresis, paper electrophoresis or ion-exchange chromatography have been reported. Munro and Thomas (1945) used free electrophoresis to isolate five fractions from dog pancreatic juice. Later, Byrne et al. (1951) and Grossberg et al. (1952) using different buffer systems found six and ten components respectively. Paper electrophoresis of dog and rat pancreatic juice resulted in six and seven fractions respectively (Delcourt and Delcourt, 1953; Rotshild and Junqueira, 1956). Hansson (1951) found at least seven components in cat pancreatic juice. Although most of these investigators were able to demonstrate that enzyme activity was associated with many of the fractions, the experiments were of limited usefulness because: (1) the separations were not complete enough to permit quantitative estimation of individual proteins; and (2) little or no detailed information about the pancreatic proteins of these species was available.

(2) By Ion-Exchange Chromatography

Ion-exchange chromatography proved to be the most efficient separative procedure. When Keller et al. (1958b) applied these methods to bovine pancreatic juice, excellent resolution of the major protein components was achieved.

Chromatography had been used to prepare and purify bovine chymotrypsinogen A (Hirs, 1953), ribonuclease (Hirs et al., 1953) and trypsinogen (Keller et al., 1958b; Tallan, 1958) on the cation exchange resin IRC-50. Keller et al. applied the cellulose ion-exchanger DEAE,developed by Peterson and Sober (1956), for the separation of the anionic proteins, procarboxypeptidase A, procarboxypeptidase B, chymotrypsinogen B and deoxyribonuclease. With the cellulose ion exchanger and IRC-50 they were able to achieve a chromatographic separation of the principal protein constituents of bovine pancreatic juice. The seven pancreatic digestive enzymes listed in Table 4 were qualitatively identified in the chromatographic effluent by means of enzyme activity measurements. Quantitative information about the absolute proportions of the pancreatic enzymes was obtained by determining the peak area by absorbancy measurements at 280 mμ

TABLE 4

COMPOSITION OF BOVINE PANCREATIC JUICE

COMPONENT	PER CENT TOTAL PROTEIN	
Proteolytic		
Trypsinogen	14	72%
Chymotrypsinogen A	16	
Chymotrypsinogen B	16	
Procarboxypeptidase A	19	
Procarboxypeptidase B Carboxypeptidase B	7	
Nucleolytic		
Ribonuclease	2.4	4%
Deoxyribonuclease	1.4	
Amylolytic*	< 2%	
Lipolytic*	Very low	
Unidentified	10%	

* Not located on chromatograms.

Data taken from Keller *et al*. (1958b).

and using extinction coefficients. Table 4 lists the composition of bovine pancreatic juice determined by this method. A more complete discussion of these quantitative estimations will be given in Section IV, 3b where the results of the present investigation are discussed.

3. Present Research

In order to determine the relationship between the zymogen granules and pancreatic juice, a complete comparative analysis of the enzymes, zymogens and other proteins present in each of the two mixtures is required. A protein analysis of the zymogen granules can be meaningful only if a pure or reasonably pure fraction of zymogen granules is isolated from the glandular tissue. The comparison must be carried out on a species whose pancreatic enzymes are well characterized. This provision restricts the choice to bovine pancreatic juice and zymogen granules.

To carry out this project, which amounts to a direct and complete test of the Heidenhain hypothesis, improved preparative methods were developed for the isolation of zymogen granules from homogenates of bovine pancreas. Pancreatic juice was obtained by cannulating the pancreatic duct of cows and yearling steers. The proteins extracted from the granules and proteins in the pancreatic juice were compared after separation by ion exchange chromatography. Specific substrates were used qualitatively to identify the enzymes or zymogens after activation. Quantitative determinations of the individual proteins present in these mixtures were made on the basis of enzyme activity and absorbancy measurements.

After these experiments had been initiated, Dr. Keller informed me that similar research was under way in her laboratory at the University of Washington. Some of the results of this work have already been published (Keller and Cohen, 1961), and I am indebted to Drs. Keller and Cohen for the courtesy of sending me the manuscript prior to publication.

The remainder of this thesis is organized in the following manner:

Section II. Isolation and Characterization of Bovine Zymogen Granules.

Section III. Collection of Bovine Pancreatic Juice.

Section IV. Comparison of the Protein Composition of Bovine Zymogen Granules and Pancreatic Juice.

SECTION II

ISOLATION AND CHARACTERIZATION OF BOVINE ZYMOGEN GRANULES

1. Introduction

a. Structural Features of Zymogen Granules as Determined by Light and Electron Microscopy: The zymogen granules in the acinar cells of the pancreas are demonstrable with the light microscope in both living and fixed preparations (Kühne and Lea, 1882). Gage systematically surveyed the size and shape of the granules in at least one member of each vertebrate class. He reported that granules, released by crushing cells fixed in formalin-saline, are spherical bodies; the average diameter for all vertebrate forms studied is 1-2 micron (Gage, 1945).

Information from studies using the electron microscope on sectioned material confirms the spherical shape of about 1 micron in diameter. In osmium tetroxide fixed preparations, zymogen granules are electron opaque masses limited by a smooth membrane of about 70 Å (Sjöstrand and Hanzon, 1954). There are no structural elements visible within the granule. Their content appears to be homogeneous or finely granular at the present level of resolution and under the conditions of fixation and staining currently employed. Since the granules are more than 90% protein, on a dry weight basis, it can be concluded that zymogen granules are membrane limited vacuoles filled with a highly concentrated solution of protein. The appearance of the granules isolated from sucrose homogenates of guinea pig pancreas by differential centrifugation is identical to that found *in situ* (Siekevitz and Palade, 1958a). Secretory granules of more or less similar appearance are found in many protein secreting glands (*cf.* Kurosumi, 1961, p.12).

b. Isolation of Zymogen Granules from Pancreatic Homogenates

(1) Contribution of Cell Types to Pancreatic Homogenate

The zymogen granules are isolated from a homogenate of the pancreas, a gland which has a heterogeneous cell population. Since the granules are found only in the acinar cells, it is of interest to: (1) estimate the proportion of acinar cells in the whole cell population; and (2) examine the possibility of contamination of the zymogen granule fraction by granules derived from other cell types.

If it is assumed that ducts, blood vessels and the associated connective tissue account for 5% of the volume of the pancreas, the data for human pancreas (Ogilvie, 1933) may be recalculated to give the results presented in Table 5. It is clear that one cell type is predominant in the intact gland and that the homogenate represents primarily acinar cells.

Since only acinar and islet cells contain secretory granules, the situation is even more favorable when the granules alone are considered. The islet Alpha and Beta cells contain spherical granules 0.2 to 0.3 microns in diameter. In some species the Beta cell granules have rectangular profiles (cf. Lacy, 1957; Stoeckenius and Kracht, 1958). The contamination of the zymogen granule pellet by islet cell granules, when isolated by differential centrifugation, depends on the relative concentration of each type of granule in the homogenate and their sedimentation rate. The sedimentation rate, in turn, is determined by the size and density of the granules (De Duve and Berthet, 1954). In view of the small size of their granules and the low concentration of islet cells in the tissue (Table 5), we can conclude that the zymogen granule fraction should be essentially free of contamination from granules derived from islet cells.

(2) Isolation of Zymogen Granules by Centrifugation Procedures

Centrifugation has been used in several laboratories to isolate fractions containing zymogen granules (Claude, 1943; Hokin, 1955; Laird and Barton, 1957; Siekevitz and Palade, 1958a; Van Lancker and Holtzer, 1959). These investigations were concerned primarily with the determination of the intracellular distribution of the enzyme activity of a limited number of secretory proteins. With the exception of Siekevitz and Palade who also used gradient centrifugation, the technique of differential centrifugation was used in all of these studies.

Our primary aim was to obtain a fraction of zymogen granules free of contaminating nuclear fragments, mitochondria and microsomes. This requirement precluded any attempt to recover all of the granules present in the homogenate or to obtain the maximum yield per gram of tissue. The situation was further complicated by the fact that large amounts of protein, 100-200 mg, were needed for chromatographic analyses.

TABLE 5

CELL TYPES PRESENT IN PANCREATIC TISSUE

	PERCENT OF TOTAL VOLUME OF PANCREAS
Acinar Cells*	84
Islet Cells	1.8
Adipose and Connective Tissue	9
Ducts, Vessels and Surrounding Connective Tissue	(5)

* Includes centroacinar cells; their proportion is unknown but can be considered small.

Differential centrifugation was selected from the several methods currently used to isolate cellular constituents from tissue homogenates. Although gradient differential centrifugation and isopycnic gradient centrifugation should yield more homogeneous preparations, these methods could not be used because of the limited rotor capacity of equipment available at this time. The combination of differential centrifugation and differential gradient separation was not employed because resuspension of the pellet disrupted and damaged some of the granules (Siekevitz and Palade, 1958a). The procedure which was adopted, therefore, represents a compromise between the requirements for a homogeneous preparation, and for large amounts of material necessary for chromatographic analyses.

2. Methods

a. Isolation of Zymogen Granules: The number of sedimentations, the duration and centrifugal field used for the isolation of zymogen granules were empirically determined using electron microscopy to identify the contaminating material and estimate its amount.

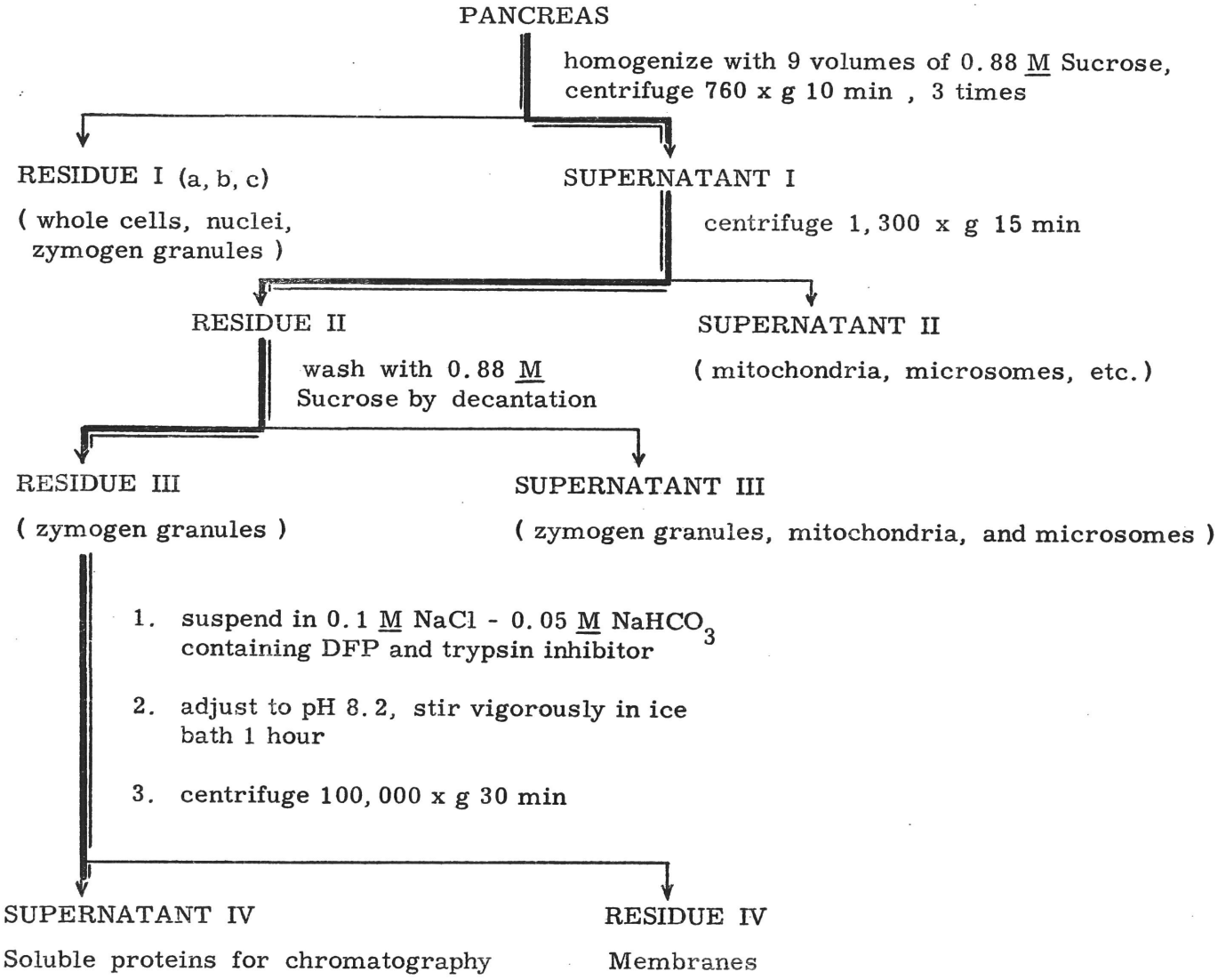
Three successive low speed centrifugations removed all the rapidly sedimenting cell debris, red blood cells and disrupted nuclei from the suspension. Some of the zymogen granules were also sedimented and therefore lost by this procedure. The result, however, was that the zymogen granule pellet subsequently obtained was always free of the more rapidly sedimenting contaminants. The macroscopic heterogeneity of the zymogen granule pellet, a compact white mass overlaid by a fluffy tan layer, was utilized to obtain another step of purification which did not require resuspension or resedimentation of the pellet. The tan fluffy layer containing zymogen granules, mitochondria, and microsomes was separated from the white mass of zymogen granules by swirling the tube in the presence of fresh solvent (Hokin, 1955). Figure 1 is a schematic outline of the preparative procedure for the isolation of zymogen granules.

(1) Preparation of Homogenate

Calf pancreas was obtained from a local abattoir 15 minutes after the death of the animals. The glandular tissue of 6 to 8 animals was pooled, placed in a dry polyethylene bag and stored on crushed ice. Thirty minutes were required to transport the material to the laboratory before homogenization was begun.

Fig. 1. Isolation of zymogen granules by differential centrifugation.

ISOLATION OF ZYMOGEN GRANULES BY DIFFERENTIAL CENTRIFUGATION



All operations were carried out in a room at 0-4°C. Homogenization of the gland was facilitated by preliminary dissection of connective tissue, fat and large blood vessels. Portions of 10 grams of tissue, minced with scissors, were homogenized in 9 volumes of 0.88 M sucrose in a glass homogenizer fitted with a teflon pestle. The suspension was filtered through gauze and stored in an ice bath until 100 grams of tissue had been homogenized.

(2) Isolation of Zymogen Granules by Differential Centrifugation

Sedimentation of Nuclei and Cell Debris: In order to remove material more dense than zymogen granules, the filtered homogenate was centrifuged three times at 2100 rpm (760 x g) for 10 minutes in an International Centrifuge (Model SVB, Type 1). Before decanting the supernatant (Supernatant I), the fat floating on top of the fluid was removed by means of a spatula or pipette. The pellets (Residues, Ia, Ib, Ic) were discarded.

Isolation of Zymogen Granules: Supernatant I was centrifuged 5500 rpm (1300 x g) for 15 minutes in the #40 rotor of a Spinco Model L preparative ultracentrifuge. The supernatant fluid was decanted and discarded (Supernatant II). Fat was removed by wiping the sides of the lusteroid tube with gauze. The pellet (Residue II) was made up of a compact white mass covered by a tan fluffy layer. Three ml of 0.88 M sucrose were added to each tube and the fluffy layer was mobilized and dispersed into the sucrose by gentle swirling motion (Supernatant III). This "top wash" was repeated 2 or 3 times. Excess sucrose was drained by inverting the tube and the inside wall was wiped with gauze.

(3) Lysis of Granules - Preparation of pH 8 Soluble Proteins

The zymogen granule pellets (Residue III), about 84 centrifuge tubes/100 grams wet weight, were resuspended in 0.17 M NaCl by means of a motor driven teflon pestle. Four to five ml were used for each batch of 12 tubes. The milky suspension immediately cleared upon the addition of 10 ml 0.2 M $NaHCO_3$ pH 8.4, 4×10^{-3} M in diisopropylphosphofluoridate (DFP). While mixing, the solutions were vigorously stirred by a teflon coated magnetic stirring bar at 0°C. When all the pellets had been resuspended in this manner, an additional aliquot of 0.005 ml of DFP was added (final volume about 45 ml and final DFP concentration 10^{-3} M) and the pH was adjusted from 7.8 to 8.2 with 0.1 N sodium hydroxide. Vigorous stirring

was continued for 45 minutes to assure the complete lysis of granules, solubilization of protein and inactivation of trypsin. Two to four milligrams of soybean trypsin inhibitor were finally added and the slightly turbid solution was centrifuged for 30 minutes at 40,000 rpm (100,000 x g) in 4 tubes. The clear solution, which had a trace of yellow color, was carefully removed from the tubes with s syringe fitted with polyethylene tubing. This solution was designated as pH 8.0 soluble zymogen granule protein (Supernatant IV). The pellet, Residue IV, consisted of two opaque tan layers, of which the uppermost was darker.

b. Electron Microscopy: The zymogen granule and membrane pellets (Residue III and Residue IV) were fixed *in toto* and processed by a modification of the method of Palade and Siekevitz (1956) currently used in their laboratory.

Five ml of 1% osmium tetroxide in 0.88 M sucrose were carefully added to the centrifuge tube containint the pellet. After fixation for 18 hours at 4°C, the OsO_4 solution was replaced by 70% ethanol; 15 minutes later the tube was cut and the pellet removed with a spatula. The pellet was cut into strips of known orientation and thereafter dehydrated in ethanol and embedded in a mixture of n-butyl methacrylate/methyl methacrylate (80:20 V/V) (Newman *et al.*, 1949).

When the top wash of the granule preparation (Supernatant III) or the third residue of the low speed centrifugation (Residue Ic) was to be examined in the electron microscope, the residue or wash solution was resuspended by means of a motor driven teflon pestle in 0.88 M sucrose and centrifuged in the Spinco Model L preparative ultracentrifuge for 15 minutes at 15,000 rpm (14,000 x g). The pellet obtained was processed in the same manner as the zymogen granule and membrane pellets. Bovine pancreatic tissue was fixed in osmium tetroxide at pH 7.3-7.5 (Palade, 1952) and embedded in n-butyl methacrylate. Thin sections were cut on a Porter-Blum microtome, stained with lead hydroxide (Watson, 1958) and examined in the RCA EMU-2b electron microscope.

c. Chemical Analysis of Cell Fractions

(1) Schneider Partition

Aliquots of pellet suspensions and supernatant fluid were treated with cold trichloroacetic acid (TCA) to a final concentration of 10%. After 2

hours at 0°C, the precipitate was washed twice with cold 5% TCA and was extracted according to the method of Schneider (1957) to yield fractions corresponding to "phospholipid", nucleic acid, and "protein".

The ribonucleic acid (RNA) content of the nucleic acid fraction was determined by the orcinol method (Mejbaum, 1939). A sample of yeast RNA containing 0.090 mg P/mg was used as a standard. Deoxyribonucleic acid (DNA) in the same fraction was measured by the diphenylamine reaction (Dische, 1930). Standard curves were prepared by relating absorbancy at 660 mμ and 595 mμ to micrograms of total RNA-phosphorus and DNA-phosphorus, respectively. Aliquots of the nucleic acid fraction were also digested in 10 N sulfuric acid and total phosphorus was estimated by the method of Fiske and Subarrow (1925). The phosphorus content of the phospholipid fraction was determined after digestion in 10 N H_2SO_4.

The final residue "protein" was subjected to sulfuric acid digestion, and total nitrogen was determined by means of Nessler's reagent (Umbreit, 1945). Protein was also estimated on the residue from the extraction procedure by the method of Lowry et al. (1951).

(2) Correction for Protein in Phospholipid Fraction

In the Schneider procedure phospholipids are removed from the TCA precipitate by successive extractions with 95% ethanol at room temperature, ethanol-ether (3:1) at 50-60°C and ether at 40°C. These extracts are combined and called the phospholipid extract. It has been reported that protein (which is neither lipoprotein nor phosphoprotein) can be extracted from the TCA precipitate under these conditions*. For example, TCA precipitated albumin (Korner and Debro, 1956) and insulin (Light and Simpson, 1956) are soluble in absolute ethanol and ether, respectively, when small amounts of water and acid are present. Trace amounts of water and TCA trapped in the precipitate are sufficient to cause solubilization (Korner and Debro, 1956). The ability of TCA to increase the solubility of insulin in organic solvents had been demonstrated by Harfenist and Craig (1952). TCA in low concentrations greatly increased the partition ratio of insulin in the two phase system, 2-butanol/water.

* I am grateful to Dr. Joel Rothschild for bringing this point to my attention.

Since the zymogen granule fraction contains more than 90% protein, and at least four of the secretory proteins have a molecular weight of 25,000 or less, it seemed desirable to check whether, in fact, such a loss of protein to the phospholipid fraction did occur. Extracts corresponding to phospholipid, nucleic acid and protein were prepared from washed TCA precipitates of zymogen granule fractions and pancreatic juice.

The pancreatic juice did not contain detectable amounts of phospholipid phosphorus; nor nucleic acid phosphorus, orcinol positive material or diphenylamine positive material in the nucleic acid fraction. Protein was determined by the procedure of Lowry (1951) and nitrogen after digestion by Nessler's reagent (Umbreit, 1945) in both the phospholipid and protein fractions. The sum of the protein in both fractions was called total recovered protein (Lowry); similarly the sum of the nitrogen contents of the phospholipid and protein fractions was called total recovered nitrogen. When expressed this way, the results for nitrogen and protein (Lowry) were the same, indicating that we were measuring protein in the phospholipid fraction by either of these methods. The phospholipid fraction (of both the juice and granules) contained 22-25% of the total recovered protein (Lowry) or nitrogen. In the granule preparations the amount of nitrogen associated with the phospholipid was too small to affect the nitrogen distribution when the values for juice and granules were compared. When the temperature at which the 95% ethanol extraction was conducted was increased to 70-80°C (the other extraction temperatures were not changed), 31-35% of the protein or nitrogen was recovered in the phospholipid fraction. On the basis of these results, the amount of protein found in the protein fraction was multiplied by an appropriate factor to correct for this loss of protein to the phospholipid fraction. The protein loss to the nucleic acid fraction was not further investigated. No attempt was made to determine which secretory proteins were extracted by the organic solvents.

3. Results

a. Electron Microscopy of Fractions: Examination of the fractions in the electron microscope was facilitated by the known orientation of the material during sectioning and the use of specially constructed supporting grids (athene type) which permitted continuous observation of large areas of the

grid in the absence of supporting wires. With these technical advantages, it was possible to obtain a series of photographs at low magnification of the entire depth of the pellet.

Zymogen granule fractions from ten preparations of bovine pancreas examined in this manner appeared to be identical, thus attesting to the reproducibility of the preparative method. The zymogen granule fraction (Residue III), top wash (Supernatant IV), bottom (Residue Ic) and membrane fraction (Residue IV) will be discussed in this section.

(1) Zymogen Granule Pellet (Residue III)

The zymogen granules isolated by this procedure had an appearance similar to that of the granules found in the cell. Their limiting membrane was visible and the contents appeared homogeneous (cf. Plate I and Plates III and IV). Damaged or disintegrated granules were rarely, if ever, observed. No stratified contaminants were found, and in this sense the pellets were homogeneous. There was, however, a small amount of contaminating material distributed more or less evenly throughout the pellet. Swollen and extracted mitochondria, small fragments of the endoplasmic reticulum and chromatin were the morphologically recognizable contaminants. Their area represented approximately 5% of the area occupied by the zymogen granules in the preparation. Representative areas of such preparations are illustrated in Plates II, III and IV. A description accompanies each plate.

(2) Top Wash (Supernatant III)

The tan fluffy material overlaying the compact white pellet was mobilized, resuspended and resedimented. The pellet thus derived from Supernatant III was fixed *in toto*. When examined in the electron microscope it was found to be heterogeneous, for it contained recognizable zymogen granules, extracted mitochondria, membranes derived from the endoplasmic reticulum and fragments of nuclei containing recognizable chromatin. Free chromatin and smooth membranes were also present. The pellet was markedly stratified, the lower third contained intact zymogen granules in the same relative concentration as found in the granule fraction. The middle third of the pellet contained pockets of granules and mitochondria apparently trapped between clumps of membranes. The top of the pellet had few granules and was composed primarily of small membranes and chromatin. The observed stratification of material was imposed by the second sedimentation.

(3) Bottom Wash (Residue Ic)

When Residue Ic was resuspended and resedimented, a heterogeneous stratified pellet was also obtained. Zymogen granules were found at all levels of the pellet but were concentrated at the bottom where red blood cells were occasionally found. Recognizable fragments of nuclei still possessing a membrane and filled with chromatin were found in the bottom and middle of the pellet. In addition to cell membranes, swollen, extracted mitochondria and aggregates of chromatin, there was present some fibrillar material presumably derived from basement membranes. The results of the electron microscopic observation of these pellets, zymogen granule, top wash and bottom wash were used as guides in the development of the isolation procedure.

(4) Membrane Fraction (Residue IV)

This fraction was obtained by sedimentation following lysis of the granules in NaCl-$NaHCO_3$ at pH 8.2 The pellet occasionally contained a few intact zymogen granules. There were small fragments of amorphous material, similar to the content of the zymogen granules, associated with some of the membranes. Smooth membranes, which in many cases appeared as circular profiles with no visible internal structure, were the major component of the pellet. This is consistent with the view that the zymogen granules are the major source of membranes in the preparations. The collapsed and packed membranes in the center of the pellet are difficult to identify (Plate V). Those membranes still intact are more easily identifiable as the zymogen granule envelope; compare Plate VI with Plate I. Probably all the membranes, with the exception of recognizable mitochondria, represent sections of the zymogen granule membrane left behind after the extraction of the protein content.

b. Gross Chemical Composition of Zymogen Granule Fractions: The amount of nucleic acid, phospholipid and protein in the zymogen granule fractions and tissue homogenate are presented in Table 6. Only preparations corresponding to Residue III, zymogen granules after lysis and sedimentation of the membranes, were used in the chromatographic studies for comparison with pancreatic juice. Related fractions, Residue Ic and Supernatant III, are presented for the purpose of comparison.

PLATE I

Electron micrograph showing the apical region of two adjacent bovine pancreatic exocrine cells. Part of the lumen (l) of a pancreatic acinus is visible in the upper right corner. Numerous large granules (ZG) are found in the cytoplasm. The granules are limited by a smooth membrane (m). Their oval shape is due to deformation during sectioning. Some of the contents of the granules seem to be partially or completely extracted (arrow) as indicated by the clear halo surrounding two granules near the center of the field and a large, apparently empty vacuole, present in the lower left corner of the micrograph. Elements of the endoplasmic reticulum (er) bearing attached particles and free particles (p) are prominently distributed in the cytoplasm. The extraction of some of the granules and the apparent disorganization of the endoplasmic reticulum are probably post mortem changes.

Magnification: 31,000

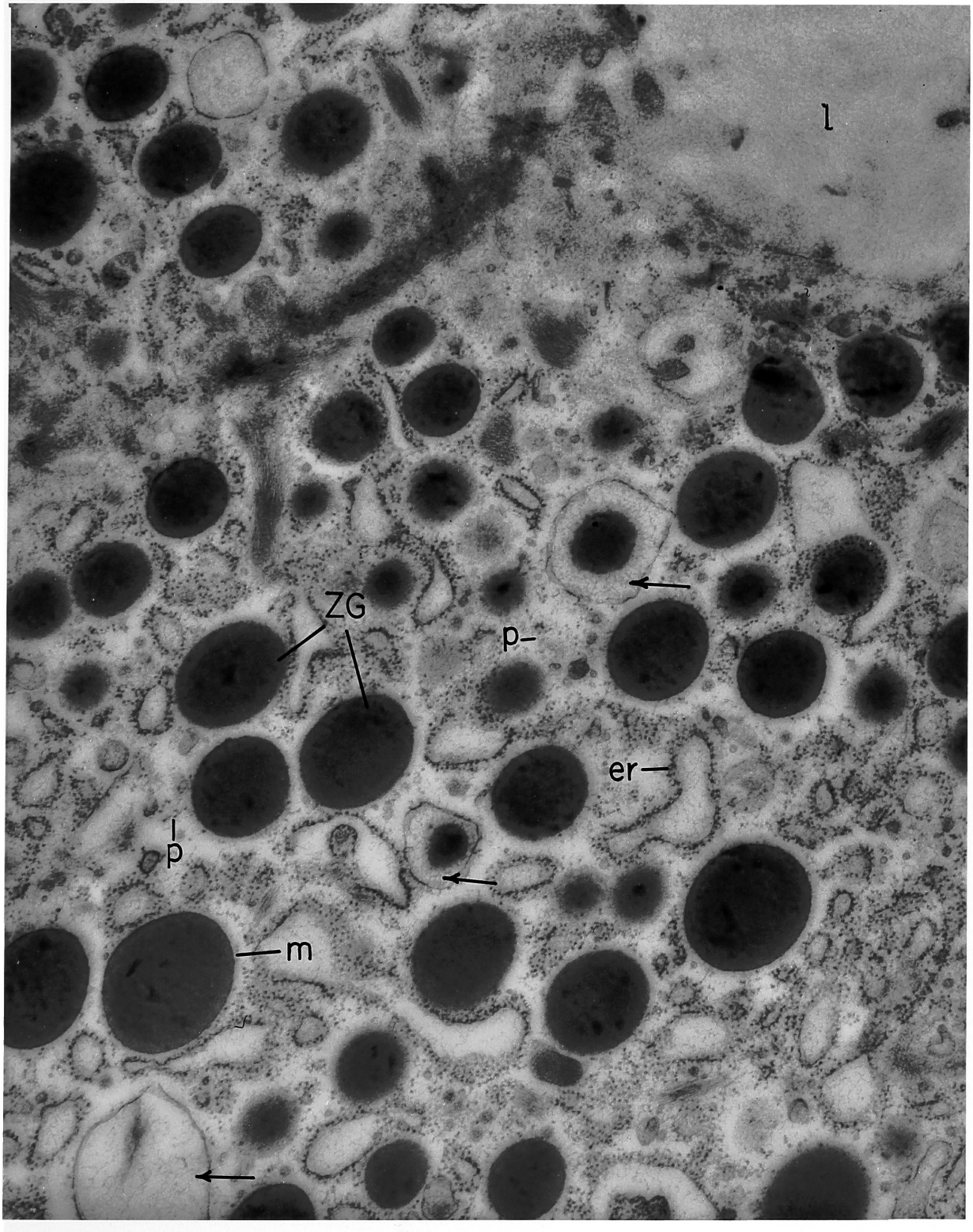

l
ZG
p
er
p
m

PLATE II

Electron micrograph showing a large field of a section through a zymogen granule pellet. The pellet consists primarily of zymogen granules; the contaminants are recognizable only at higher power (Plate III). This micrograph demonstrates the overall homogeneity of the preparation.

Magnification: 5,000

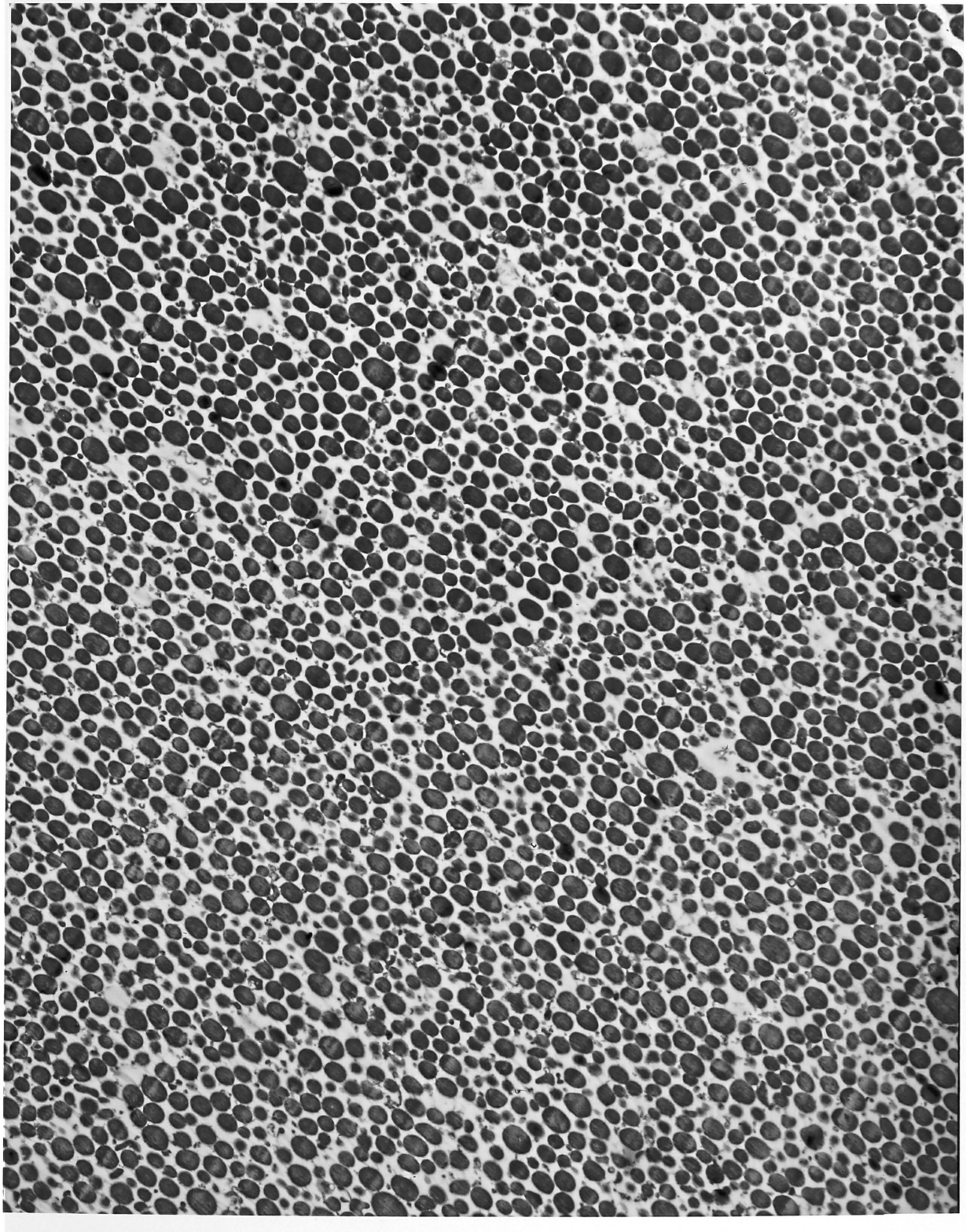


PLATE III

Electron micrograph of a section through a zymogen granule pellet demonstrating recognizable extracted mitochondria (m_1) and fragments of the endoplasmic reticulum (arrow) bearing attached particles. The apparently rough surface of the granules is due to sectioning artifact. The intact mitochondrion on the left side (m_2) appears as dense as the granules but its internal membranes are recognizable.

Magnification: 25,000

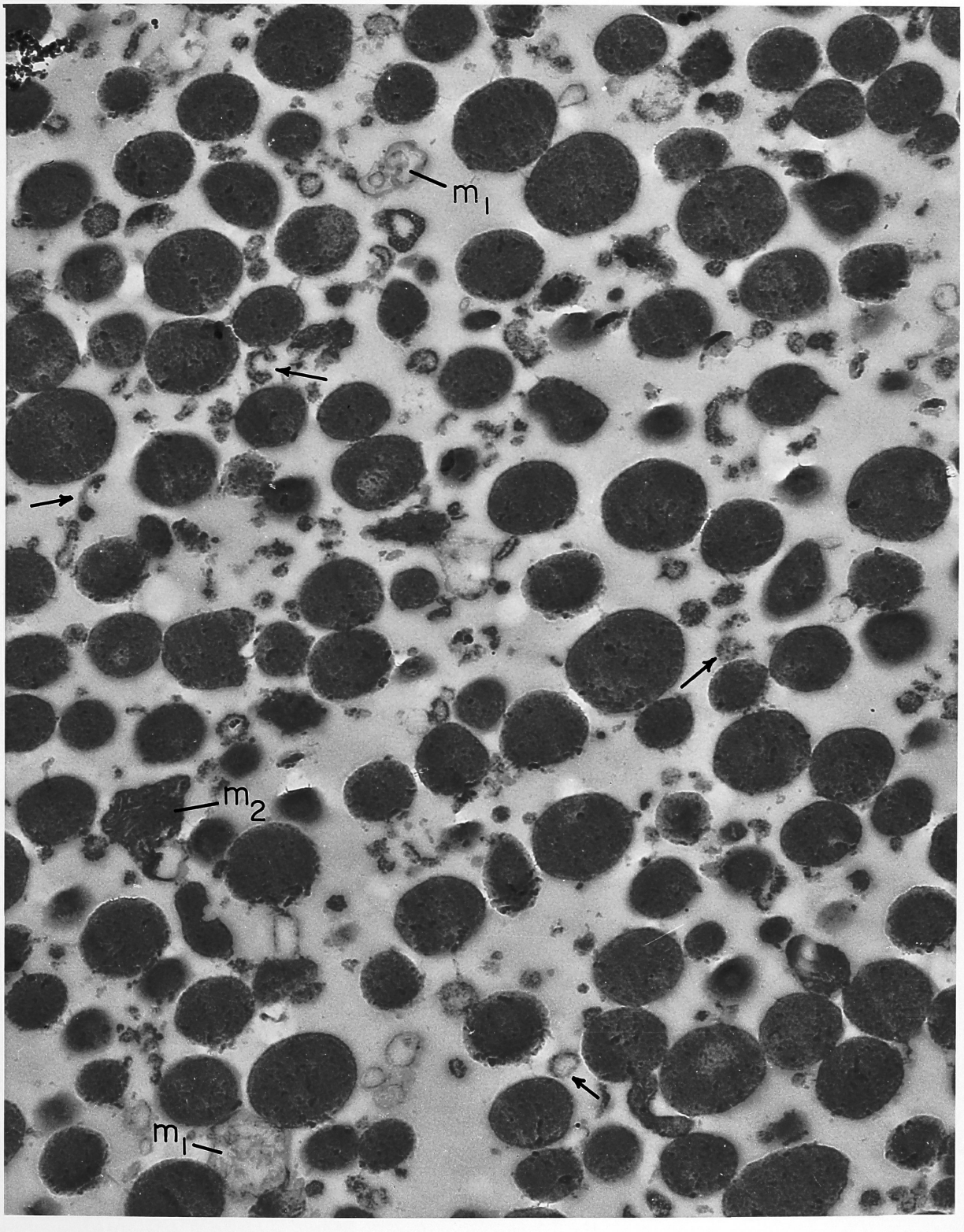

m1
m2
m1

PLATE IV

Electron micrograph showing a small field of a thin section through a zymogen granule pellet. This demonstrates the single smooth membrane (m) limiting each granule. The content of the granule appears to be finely textured and free of internal structural elements. The black crystalline material (arrow) over some of the granules is contamination from lead stain used to increase contrast. The oval shape of the granules is due to deformation during sectioning.

Magnification: 68,000

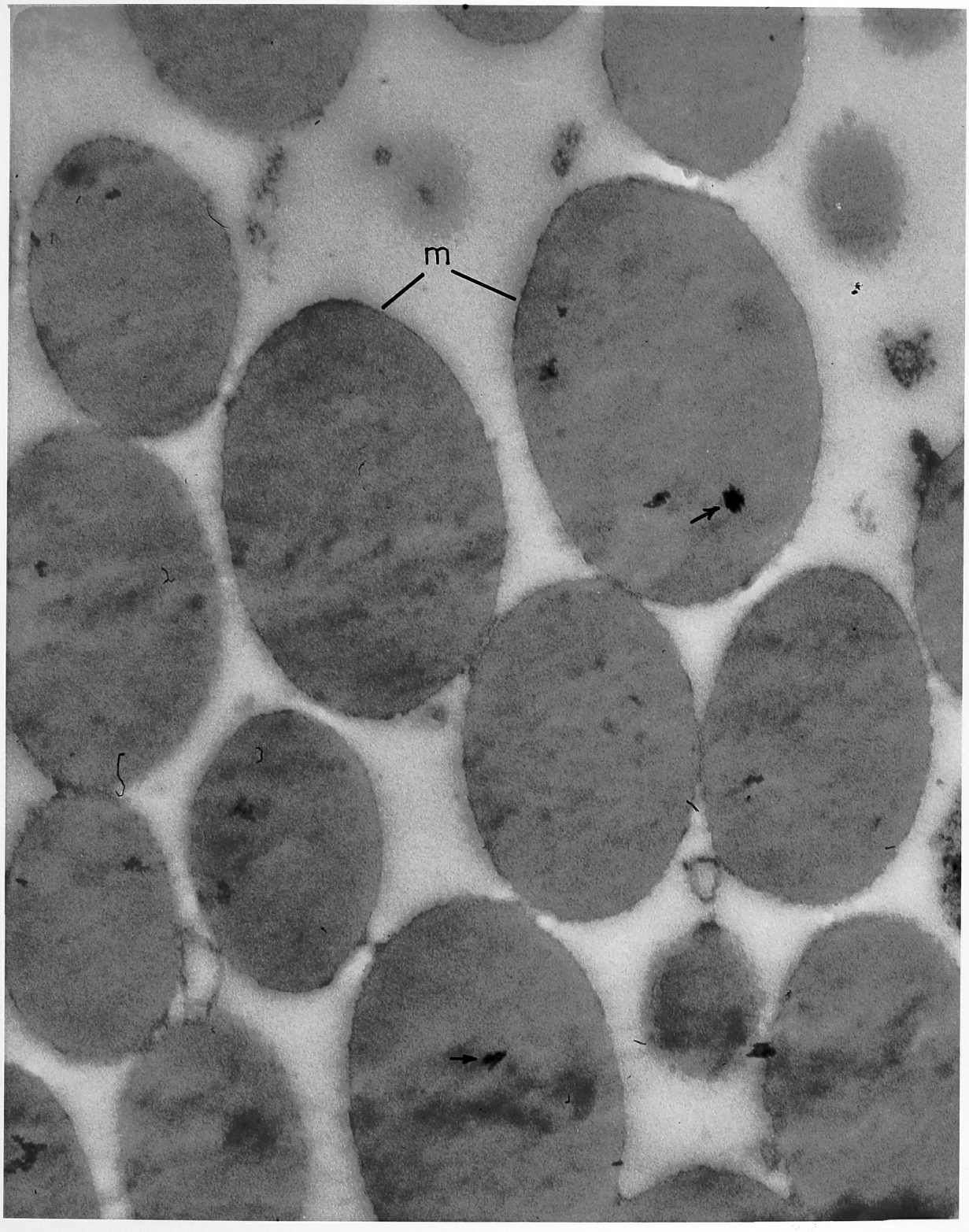

m

PLATE V

Electron micrograph showing the center of the pellet obtained by sedimentation following lysis of the zymogen granule fraction. The membranes in this part of the pellet are very tightly packed and for this reason their origin is difficult to identify. Some appear to be damaged and extracted mitochondria (m) as suggested by their double membranes and residual internal structures. Most of the others are assumed to be deformed zymogen granule membranes (arrow).

Magnification: 26,000

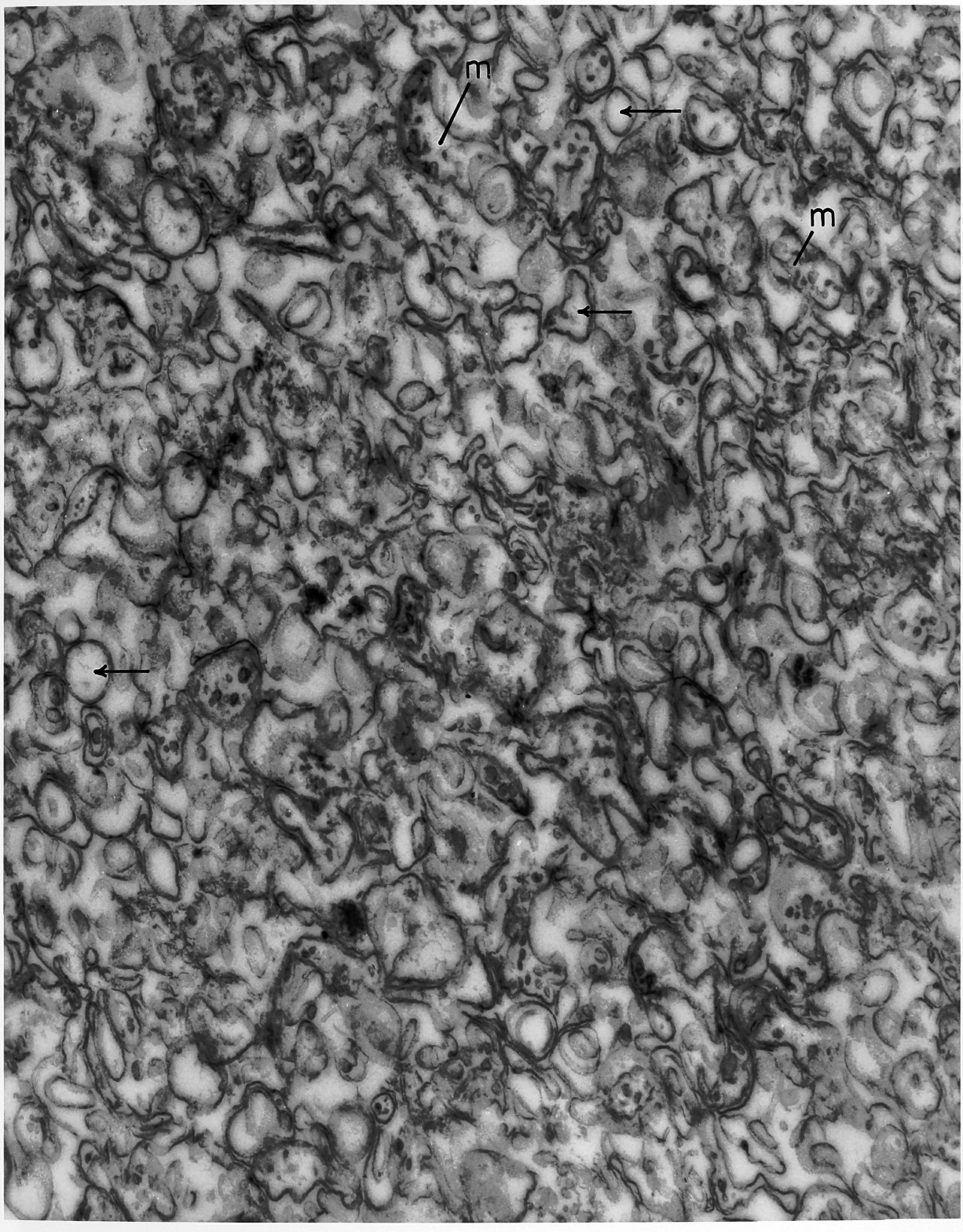
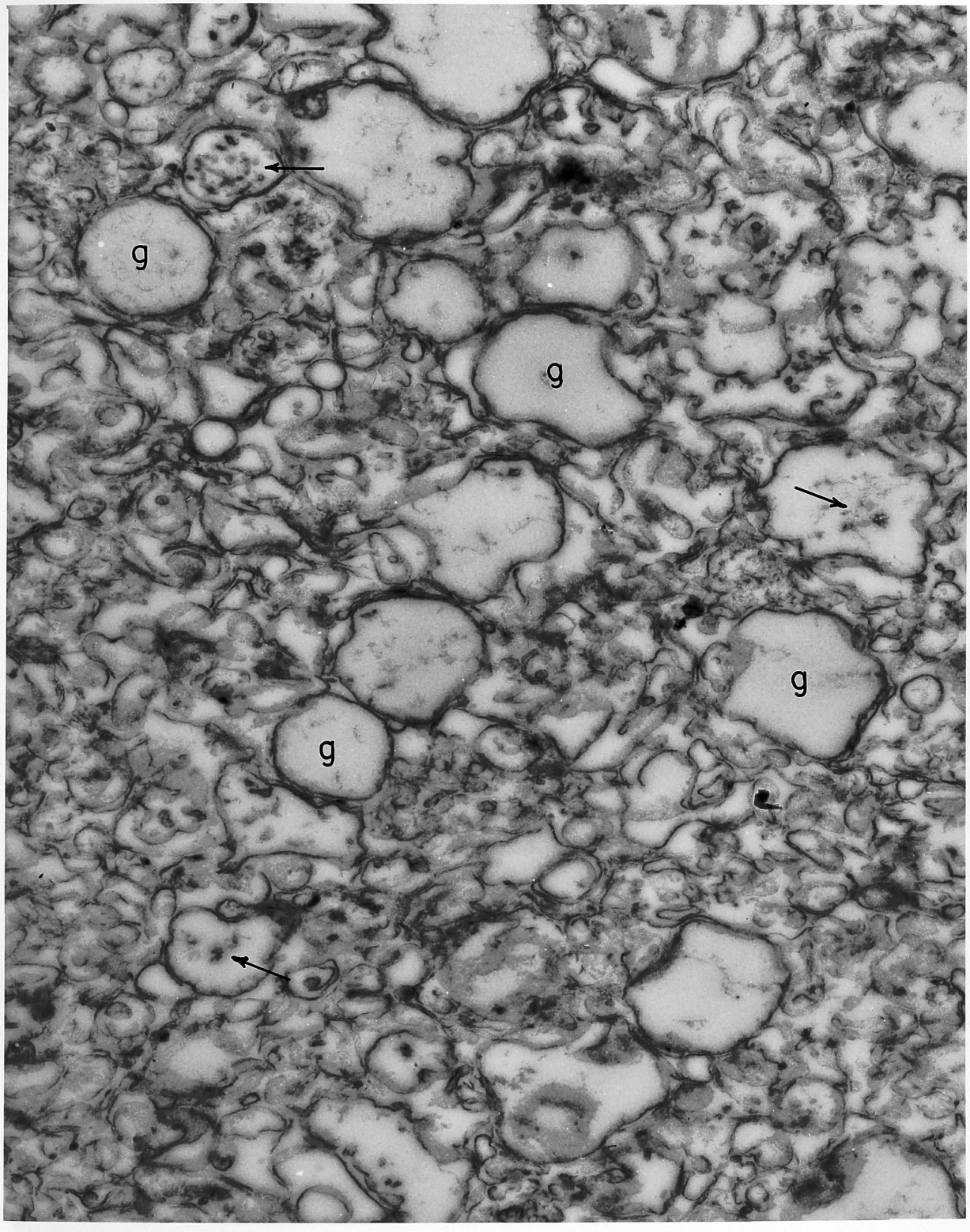

m
m

PLATE VI

Electron micrograph showing the top of the pellet obtained by sedimentation following lysis of the zymogen granule fraction. The field contains many circular or subcircular profiles having no visible internal structure. The amorphous material within the profiles (arrow) may represent unextracted zymogen. Most of the membranes in this field appear to be derived from zymogen rather than mitochondria.

Magnification: 26,000


g
g
g
g

(1) Nucleic Acid

The amounts of RNA and DNA were nearly equal in all Residue III preparations and were appreciably different from the ratio DNA/RNA = 0.29 determined for the homogenate. This enrichment of DNA relative to RNA is due to the fact that the granules sediment immediately after the disrupted nuclei. The absolute amount of nucleic acid, however, is small when compared to the protein content of the fraction. The nucleic acid in the fraction represents 0.7 to 1.1% of the protein (by weight) as compared to 14.7% in the homogenate. The purification of the protein with respect to individual species of nucleic acid is apparent when one considers that the granule fraction contains 2% of the protein but only 0.2% of the RNA and 0.1% of the DNA present in the tissue homogenate.

In these experiments the spectrophotometric methods gave higher values (10-30%) for nucleic acid phosphorus than did direct analysis on acid digests of aliquots of the same sample (compare A+B to C). This result is not uncommon and is usually attributed to difficulties with the RNA determination by the orcinol reaction (Hutchinson and Munro, 1961). Interference due to the presence of hexoses, sucrose, heptoses and other material has been reported. The apparent specificity of the orcinol reaction for purine bound ribose makes the method sensitive to the base composition of the standard. It would have been preferable to use bovine pancreatic ribonucleic acid as a standard. Neither of these explanations has been experimentally tested.

It is likely that the small amount of nucleic acid in the granule fraction is due to contamination. The electron micrographs indicate that the preparation does contain microsomes and chromatin. The absence of nucleic acid in the pancreatic juice (Daly and Mirsky, 1952; our work) is strong evidence that there is no nucleic acid within the granules.

(2) Protein

The zymogen granule fraction represents about 2% of the protein present in the homogenate. The composition of the fractions is presented in Table 7 which includes the factors used for calculation. The results are limited to those classes of compounds determined by the Schneider procedure. No determination of the content of water, electrolytes, TCA soluble non-electrolytes or neutral lipid was performed. The data for the homogenate

and the results of Hokin for dog zymogen granules (recalculated from his data) are presented for the purpose of comparison.

It is apparent that the zymogen granule fractions have a high relative protein concentration and that the zymogen granule, on a dry weight basis, is primarily protein.

(3) Phospholipid

The phospholipid of the fraction is derived from zymogen granule membranes as well as microsomal and mitochondrial contamination. An accurate estimate of the proportion of the phospholipid derived from the granule membrane cannot be made at this time. The value of 0.055 for the ratio phospholipid/protein was determined for the zymogen granule fraction, whereas it was 0.106 and 0.095 for fraction Residue Ic and Supernatant III respectively. It is interesting to note, however, that if conditions are assumed to be similar to those found for human red blood cells (Ponder, 1948) a zymogen granule of 0.7μ diameter would have a phospholipid/protein ratio of 0.035. This estimate is based on the following assumptions: (1) the amount of lipid is proportional to the surface area; (2) the phospholipid content of the respective membranes is the same; and (3) the amount of protein is proportional to the volume. In fact the ratio for the red blood cell is 0.0077; the factor of 4.5 difference is the result of an increase in the area/volume ratio. While bearing in mind the sensitivity of the calculation to the diameter of the granule and the assumptions that must be made, we may tentatively conclude that about 50-60% of the phospholipid in the zymogen granule fraction is derived from the zymogen granule membrane. The remainder may be assigned to contaminating membranes derived from mitochondria and microsomes.

c. Lysis and Solubilization of the Protein Content of Zymogen Granule: This procedure was based on the observation of Hokin (1955) that suspensions of dog zymogen granules cleared when the pH was 7.2 or higher. In our experiments the granules were lysed at pH 8.2 in a solution of NaCl and $NaHCO_3$. The pH and electrolytes were chosen to reproduce as closely as was possible the inorganic composition of pancreatic juice. Under these conditions 95% of the protein was soluble and did not sediment when centrifuged at 100,000 x g for 30 minutes. The chemical composition of the membrane fraction has not been investigated.

TABLE 6

GROSS CHEMISTRY OF ZYMOGEN GRANULE FRACTIONS

Two determinations were performed in parallel on each fraction. Value given represents average value of duplicate analyses for each determination. Fractions partitioned by the procedure of Schneider (1957).

FRACTION	EXP.	Protein-N* mg/gm (1)	µg RNA-P/ mg. Protein-N* (A)	µg DNA-P/ mg Protein-N* (B)	µg NA-P/ mg Protein-N* (C)	µg PL-P/ mg Protein-N (D)	$\frac{NA}{Protein^*} \times 10^2$ (E)
R-Ic (bottom)	1	0.145	8.0	6.7	12.7	26.9	2.0
	2	0.163	5.8	4.3	13.5	26.2	2.2
R-III (zymogen granules)	1	0.310	3.1	2.4	4.3	14.0	0.69
	2	0.294	3.7	1.8	4.8	13.7	0.77
	3	0.208	4.7	3.1	6.7	15.3	1.07
S-III (top)	1	0.112	8.5	10.4	16.5	34.8	2.7
	2	0.308	7.5	4.9	11.0	22.6	1.8
Homo-genate	2	14.9			90	79	14.3
	3	13.3	79	22.7	94	86	15.0

* Corrected for protein in phospholipid extract.

(1) Per gram wet weight pancreas pulp.

(A) Orcinol reaction on nucleic acid extract, corrected for DNA, expressed as RNA-phosphorus.

(B) Diphenylamine reaction on nucleic acid extract expressed as DNA-phosphorus.

(C) Phosphate determination on nucleic acid extract after acid digestion.

(D) Phosphate determination on phospholipid extract after acid digestion.

(E) NA = NA-Px10

$Protein^* = Protein\text{-}N^* \times 6.25$

TABLE 7

CHEMICAL COMPOSITION OF PANCREATIC HOMOGENATE AND CELL FRACTIONS

Values given as per cent by weight of recovered material (1),(2).

	HOMOGENATE*	BOTTOM* R-1c	ZYMOGEN GRANULES* R-III	TOP* S-III	DOG ZYMOGEN GRANULES (3)
% Nucleic Acid (RNA+DNA)	10	1.8	0.8	1.9	0.5 (4)
% Phospholipid	22	9.5	5.2	8.5	2.8
% Protein	68	89	94	90	97

(1) Nucleic Acid + Phospholipid + Protein = 100%.

(2) Calculated from data presented in Table 5.

Nucleic acid = Na-Px10
Phospholipid = PL-Px25
Protein = Nx6.25

(3) Calculated from data of Hokin (1955). Total N reported.

(4) RNA only.

* Average value from data of individual preparations given in Table 6.

SECTION III

PANCREATIC JUICE

Pancreatic juice was collected at Brookhaven National Laboratory from yearling steers (Holstein) and from one cow (Ayreshire) aged approximately 7 years. The quantitative enzyme activity determinations described in this thesis were performed with juice samples collected from the cow over a period of approximately five weeks. The results obtained in terms of chromatographic elution profiles were identical with samples collected from all animals.

1. Collection of Pancreatic Juice

Pancreatic juice was run from the animal continuously, and an average flow rate of 250 ml per hour of a secretion containing from 0.5% to 0.7% protein was maintained. For a particular collection, the juice was allowed to flow into a 500 ml erlenmeyer flask cooled in an ice bath and equipped with a magnetic stirrer. During the collection of juice 0.1 ml volumes of 10^{-2} M DFP in isopropanol were added at intervals of 50 ml. At the termination of collection, crystalline soybean trypsin inhibitor was added (3% by weight of juice protein) and the flask was sealed in an ice container prior to transportation to New York. Approximately two hours elapsed between collection and receipt of the sample in the laboratory. Upon receipt the juice was stirred with DFP sufficient to attain a concentration of 10^{-3} M for a period of 1 hour at 4°C.

The surgical and physiological technique of Butler *et al.* (1960) was employed to achieve cannulation of the bovine pancreatic duct. The technical details of this procedure are outside the scope of this thesis. The animals usually lost the pancreatic fistula after a month, at which time they were sacrificed, and it could be demonstrated that no pancreatic pathology existed.

SECTION IV

COMPARISON OF THE PROTEIN COMPOSITION OF BOVINE ZYMOGEN GRANULES AND PANCREATIC JUICE

1. Introduction

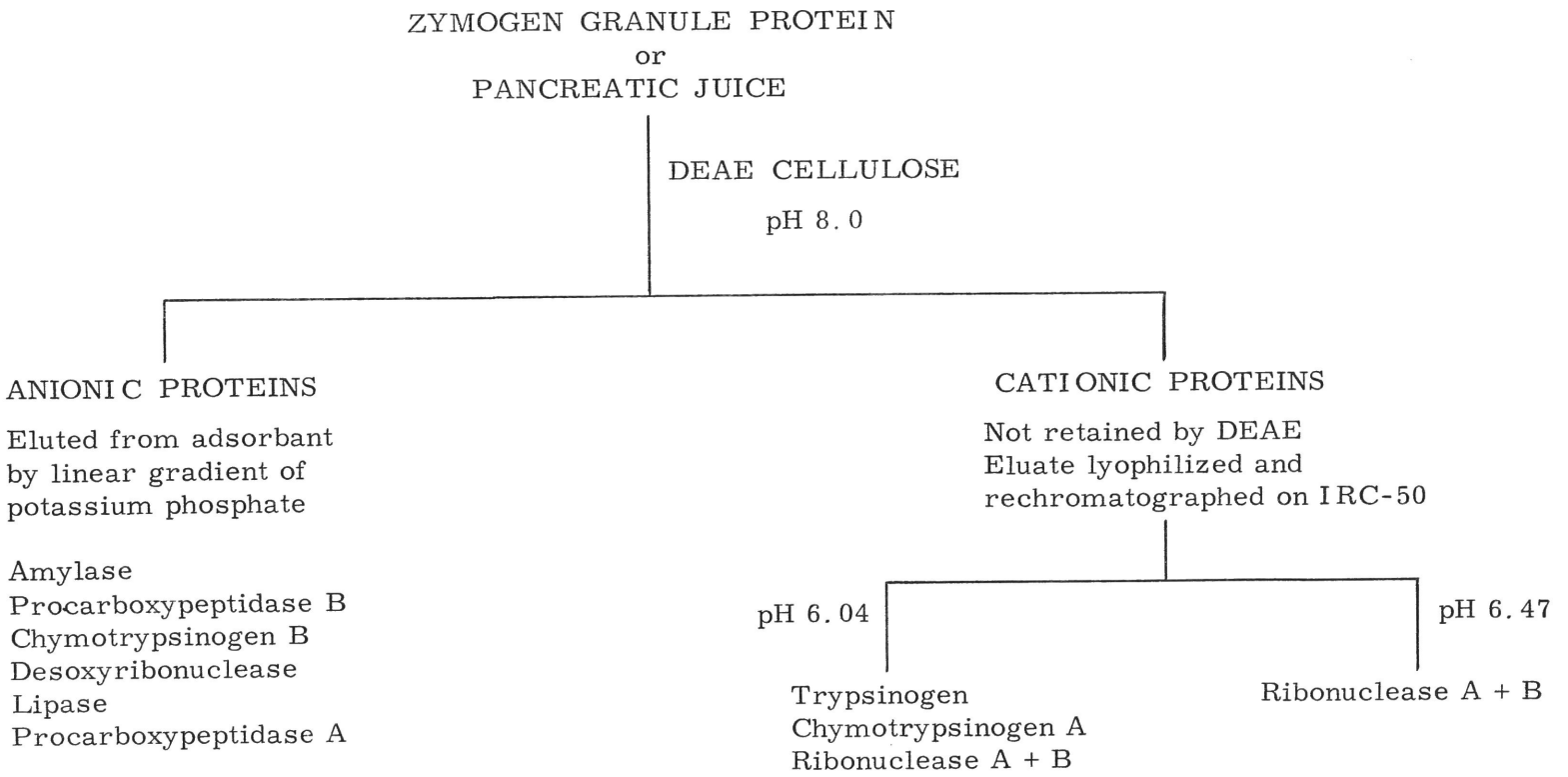
Procedures for the isolation of bovine zymogen granules and for the collection of bovine pancreatic juice have been described in Sections II and III. In this section a comparison of the protein composition of these mixtures will be presented.

A summary of the chromatographic procedures which were used to separate the pancreatic proteins is presented in Figure 2. This combination of chromatographic methods was used by Keller et al. (1958b) when they examined bovine pancreatic juice. DEAE cellulose is an adsorbent which is prepared by introducing diethylamino-ethyl groups into cellulose (Peterson and Sober, 1956). These basic groups are responsible for the adsorption of anions. When a solution of pancreatic juice protein or zymogen granule protein properly equilibrated by dialysis against dilute buffer (0.005 M potassium phosphate) is applied to a DEAE cellulose column at pH 8, the anionic proteins amylase, procarboxypeptidase B, chymotrypsinogen B, deoxyribonuclease, lipase and procarboxypeptidase A are adsorbed to the resin, whereas the cationic proteins trypsinogen, chymotrypsinogen A, and ribonuclease A and B filter through the column and emerge as an unretarded cationic peak. This filtration step therefore separates the protein into two major fractions, anionic and cationic. The anionic proteins are eluted from the adsorbent by a linear concentration gradient of potassium phosphate (pH 8.0). The cationic fraction is subsequently resolved by rechromatography on the cation-exchange resin IRC-50.

The molecular interactions responsible for the separation of proteins by chromatographic processes are largely unknown. It is fairly certain that ionic interactions play a part in that cationic proteins are adsorbed by cation-exchangers and anionic protein by anion-exchangers. As pointed out by Boardman and Partridge (1955), secondary short range forces are also involved in the adsorption of the protein molecules to the adsorbent. These authors attributed the strong interaction of carboxylic resins with proteins to the formation of hydrogen bonds between the undissociated

Fig. 2. Chromatographic separation of pancreatic proteins.

CHROMATOGRAPHIC SEPARATION OF PANCREATIC PROTEINS



carboxylic acid groups and the side chains of the proteins. The rapid increase in the extent of such secondary interactions with decrease of pH is probably the reason why many proteins are completely adsorbed and completely desorbed in a very narrow pH range. With a polyacrylic acid resin like IRC-50 this range is less than 0.1-0.2 units of pH for most proteins, and the resin is therefore of limited versatility in protein fractionation. In addition adsorption on IRC-50 is frequently accompanied by denaturation. This limitation does not apply to ion-exchange adsorbents based on cellulose. Carboxymethyl cellulose, for example, shows similar adsorption-desorption properties to IRC-50, but denaturation is significantly less, a property which may be attributed to the high degree of hydration of cellulose exchangers.

The desorption of proteins from ion-exchangers may be effected either by change of pH to desorption conditions or, more commonly, by increase of the ionic strength of the percolating solution. The wide range of adsorption-desorption behavior introduces a high degree of selectivity for fractionation of proteins. Although the application of eluents under non-equilibrium conditions may present difficulties in the theoretical interpretation of the chromatographic process, extremely useful practical results are achieved.

In the present work DEAE cellulose has been used with salt gradient elution and the positions taken by zones in the chromatograms have been found to be reproducibly determined to a high precision by the salt concentration in the effluent. By contrast IRC-50 was used under equilibrium conditions with the cationic proteins because of the relatively wide range of pH in which these proteins are able to interact with the resin. The results obtained with the IRC-50 columns are therefore presented in terms of effluent volumes.

2. Methods

a. Ion Exchange Chromatography: All chromatographic separations were performed in a room at 4°C. The columns were constructed from glass tubes fitted at the bottom with a perforated teflon disc for DEAE cellulose chromatography, or a ceramic plate for chromatography using IRC-50. Fractions were collected by means of a fraction collector utilizing a photoelectric drop counting device.

(1) Chromatography on Diethylaminoethyl (DEAE) Cellulose

Preparation of Adsorbent: Adsorbent columns, 1.0 x 70 cm were prepared from DEAE cellulose which had been previously equilibrated with potassium phosphate (0.005 M, pH 8.0) according to the procedure of Peterson and Sober (1956). The resin was obtained from a commercial source.

Preparation of Sample: Thirty to forty ml of pancreatic juice or zymogen granule extract, containing 3 to 5 mg protein/ml were equilibrated by dialysis with 4 liters of potassium phosphate buffer (0.005 M, pH 8.0). Preliminary treatment of the 23/32 dialysis casing in 50% acetic acid removed most of the water soluble, ultra-violet absorbing material normally found in commercial casings. The buffer, containing 10^{-4} M DFP, was changed four times at two hour intervals. A 0.15 M NaCl solution had a 50% escape time of 45 minutes under these conditions. After dialysis the pH and the salt concentration of the protein solution were adjusted, if necessary, to pH 8.0 with 0.1 N NaOH and by appropriate dilution to a conductivity equivalent to that of the equilibrating buffer solution.

Operation of Columns: As the equilibrated protein solution was added to the top of the column the collection of fractions, 3.2 ml/tube, was begun. After several washes with 3 ml portions of potassium phosphate (0.005 M, pH 8.0), a continuous flow of the same buffer was applied to the column. At fraction number 50 a linear concentration gradient of potassium phosphate was applied and elution was continued until the experiment was terminated at fraction number 250 (0.25 M potassium phosphate in effluent).

A concentration gradient was established (Alm et al., 1952) by introducing a solution of 2.4 M potassium phosphate (containing 2.26 M K_2HPO_4 and 0.14 M KH_2PO_4) into a constant volume mixing chamber, fitted with a teflon coated magnetic stirring bar, containing 6.4 liters of potassium phosphate (0.005 M, pH 8.0). The concentration of potassium phosphate pH 8.0 in the effluent was determined by conductivity measurements with the aid of a bridge previously standardized with solutions of known composition. In the range of volumes used in this study (10% of mixing chamber capacity) the concentration gradient was linear.

Flow rates of 8-12 ml/hour were achieved by means of an adjustable hydrostatic head 30 to 60 cm above the top of the column. All connections were constructed of glass and polyethylene tubing.

Cationic Fraction: The effluent solution corresponding to tubes 16 to 38, Figures 3 and 4, was collected directly into a volumetric cylinder containing a concentrated solution of soybean trypsin inhibitor; 3% by weight of the total protein applied to the DEAE cellulose column was used routinely. The fraction was lyophilized immediately after collection and stored at -20°C.

(2) Chromatography on IRC-50

Preparation of Columns: Amberlite IRC-50 fractionated by the hydraulic procedure of Hamilton (1958) was used throughout. The resin was equilibrated and columns prepared by methods described in detail for the chromatography of ribonuclease and chymotrypsinogen A. For the separation of trypsinogen, chymotrypsinogen A and ribonuclease, columns 0.9 x 70 cm were prepared from resin equilibrated at pH 6.04 (Hirs, 1953). The quantitative estimation of ribonuclease was performed on columns 0.9 x 30 cm at pH 6.49 as described by Hirs et al. (1953).

Preparation of Sample: The lyophilized powder containing 20-60 mg protein and inorganic salt was dissolved in 3 ml of 0.2 M NaH_2PO_4 and dialyzed at 4°C in 23/32 casing against sodium phosphate (0.2 M, pH 5.8) in a thin film apparatus of the kind described by Craig and King (1955). Fresh buffer (70 ml) was placed in the outside chamber four times at hourly intervals. The dialysis system had a 50% escape time of 30 minutes for 0.1 M sodium phosphate, pH 6.0. After dialysis the protein solution was adjusted to a final volume of 5 ml and an aliquot was chromatographed immediately on IRC-50 using 0.2 M sodium phosphate at pH 6.04 as eluent. The remaining solution was frozen and stored at -20°C for 2 to 4 weeks, when a second aliquot was chromatographed on IRC-50 at pH 6.49 with 0.2 M sodium phosphate as eluent.

Operation of Columns: A flow rate of 2 ml/hour was attained by means of a hydrostatic head 5-20 cm above the top of the columns. Fraction number 1 was collected as the sample went into the top of the column. For each experiment a 3 ml aliquot of dialyzed cationic protein containing 4-15 mg/ml was chromatographed at 4°C on a column 0.9 x 67 cm at pH 6.04. As soon as possible after the fractions were collected (1 ml/tube) 2 ml of 0.025 N HCl were added to each tube to adjust the pH to 3.1-3.3.

For experiments at pH 6.04, 1 ml aliquots of the dialyzed cationic proteins were chromatographed on a column 0.9 x 30 cm. The effluent fractions, 0.5 ml/tube, were diluted with 1.0 ml distilled water after collection.

b. Activation of Proteolytic Enzymes: The five proteolytic zymogens, trypsinogen, chymotrypsinogen A, chymotrypsinogen B, procarboxypeptidase A and procarboxypeptidase B, in pancreatic juice and zymogen granule extracts required enzymatic activation for conversion to the active forms. In order to study the entire effluent from the chromatograms, procedures were developed to facilitate the rapid and quantitative activation of these zymogens. Preliminary experiments were performed to determine the rate of activation and the stability of the enzyme activities in solutions containing large amounts of trypsin. Within the time limits to be defined for each enzyme, it was found that there was no need to control precisely the ratio of trypsin to zymogen. In order to minimize the number of manipulations and losses due to lyophilization, the activations and assays were performed directly on aliquots removed from the collecting tubes as soon as possible after collection. Aliquots of the effluent containing chymotrypsinogen B, procarboxypeptidase A and procarboxypeptidase B were added to a solution containing a constant amount of trypsin. The necessary dilution of effluent solution, 5-20 fold, was determined by the results of absorbancy measurements at 280 mμ.

Chymotrypsinogen A: 0.5 ml of the effluent from the IRC-50 column, pH 6.04, which had been acidified to pH 3.1-3.3 immediately after collection, was neutralized to pH 7.2 by the addition of an equal volume of 0.055 M K_3PO_4. An aliquot of 0.010 ml trypsin (3 mg/ml in 10^{-3} M HCl) was added to each sample. The weight ratio trypsin/chymotrypsinogen A was 0.05 to 0.10. After standing at 4°C for 48 hours the solutions were assayed for chymotryptic activity. The activity was stable for 48-96 hours at 4°C.

Chymotrypsinogen B: 0.010 ml of a trypsin solution (3 mg/ml in 10^{-3} M HCl) were added to 1.0 ml of the effluent from the DEAE chromatograms which was about 0.06 M in phosphate, pH 8. After 24-48 hours at 4°C, the chymotryptic activity was determined. The maximum zymogen concentration activated, was 1 mg/ml. Enzymatic activity was stable for at least 50 hours under these conditions.

Procarboxypeptidase A and Procarboxypeptidase B: Aliquots containing 0.05 to 0.20 ml of the effluent from the DEAE column were added to 1 ml of 0.05 M tris (hydroxymethyl) aminomethane pH 7.6 containing 5% NaCl. 0.010 ml of a trypsin solution (3 mg/ml in 10^{-3} M HCl) was added and the tubes were incubated at 37°C. Under these conditions procarboxypeptidase B was activated within 30 minutes, and the activity was stable for 3 hours (cf. Folk and Gladner, 1958). After 6 hours the activity was 80% of maximum. The maximum concentration activated was 100 μg/ml.

The activity of carboxypeptidase A reached maximal values at 2-4 hours and was stable for at least 8 hours. Therefore activity measurements were made 4-8 hours after the trypsin had been added. The maximum procarboxypeptidase A concentration in the activation mixture used was 200 μg/ml.

Trypsinogen: The method of McDonald and Kunitz (1941) for the activation of trypsinogen in the presence of $CaCl_2$ could not be used because the effluent from the IRC-50 column contained phosphate ions. For this reason an acid protease preparation* isolated from aspergillus was used at pH 3.1-3.3. The enzyme is probably similar to trypsinogen kinase (Nakanishi, 1959). The aspergillus protease did not catalyze the hydrolysis of TAME under the conditions employed for the determination of trypsin. 0.025 ml of a 1% solution of the aspergillus acid protease was added to 0.500 ml aliquots of the acidified effluent from the IRC-50 columns. Maximal activity was reached after 2 to 4 hours at 4°C but started to decrease after 6 hours. The maximum trypsinogen concentration in the activation mixtures was 2 mg/ml.

c. Determination of Enzyme Activity: The procedures used for quantitative determination of the pancreatic enzymes by activity assays were standard procedures or slightly modified forms thereof. For this reason, only a summary of the assay conditions will be presented. In all cases the measurements were made under conditions previously demonstrated and checked in this laboratory to give a linear relationship between measured activity and enzyme concentration. Whenever possible, chemically defined synthetic substrates were employed. This procedure assured the unequivocal identification

* I wish to thank Dr. H. Schleich for this preparation. He has indicated that the sample placed at my disposal was a research sample and is not a product of Wallerstein Laboratories. It is no longer available.

of the enzymes and permitted accurate and reproducible quantitative estimates of enzyme concentrations. The protocols for the chromatography, activation and assay of the pancreatic enzymes were the same for both pancreatic juice and zymogen granule extracts.

The synthetic substrates used were obtained from a commercial source and/or synthesized by procedures described in the literature. In both cases they were of sufficient purity for the purpose of this investigation.

Proteolytic Enzymes

Trypsin, Chymotrypsin A and Chymotrypsin B: Activity both before and after appropriate activation was determined by measuring the hydrolysis of specific ester substrates. The rate of hydrolysis was monitored by titration at constant pH using a Beckman Model G pH meter fitted with external electrodes. The temperature was kept at 25°C by circulating water from a thermostat through the jacketed reaction vessel. A micro burette was used to deliver a standardized solution of NaOH to the reaction mixture. No precautions to exclude CO_2 were taken because the assay solutions contained 0.005 M buffer. Aliquots of 0.025 to 0.250 ml of enzyme solutions were assayed in 3.0 ml of substrate solution. The consumption of sodium hydroxide was measured for the first 2 to 3 minutes of reaction. The initial rate of reaction was determined graphically using at least six points, and determinations were performed in duplicate at two enzyme concentrations. At the concentrations of enzyme employed, 5 to 20 μg per assay, the contribution of spontaneous hydrolysis to the measured rate was negligible.

Trypsin: The hydrolysis of p-toluenesulfonyl-L-arginine methylester (TAME) was measured at pH 7.9. The substrate solution contained 0.010 M TAME, 0.04 M NaCl and 0.005 M tris-(hydroxymethyl)-aminomethane (THAM) (Rovery et al., 1953). Calcium was not added because the sample solution contained phosphate ions. Activity unit: μmoles/min, TAME hydrolysis (initial rate).

Chymotrypsin A and B: The chymotrypsin determinations were performed in aqueous solution rather than 30% methanol to eliminate the problems associated with electrode equilibration and spontaneous hydrolysis. The assay system had the following composition: saturated solution of acetyl-L-tyrosine ethyl ester (ATEE) about 0.08 M in 0.1 M KCl, 0.01% bovine serum

albumin buffered at pH 7.8 with 0.005 M sodium phosphate (personal communication, Dr. B. H. J. Hofstee). Activity units: μmoles/min, HA hydrolysis (initial rate).

Carboxypeptidase A and B: The hydrolysis of the C-terminal peptide bonds of carbobenzoxyglycyl-L-phenylalanine (CGP) and hippuryl-L-arginine (HA) was used to determine the enzyme activity of carboxypeptidase A and carboxypeptidase B respectively. Zero time samples were used as blanks and enzyme blanks (no substrate) were determined. 0.025 to 0.050 ml aliquots of enzyme solution before and after appropriate activation were added to 0.500 ml of assay solution at 25°C, and the reaction was followed through an interval that permitted the measurement of initial reaction velocities (estimated from A_{280} and preliminary experiments). Once calibrated, duplicate samples were used to obtain a zero time point and the reaction time point (3 to 25 minutes). Aliquots of the assay solution, 0.100 ml, were added to 0.90 ml 0.05 N HCl to stop the reaction. The amount of amino acid liberated (phenylalanine and arginine) was determined by the ninhydrin reaction (Moore and Stein, 1954).

Carboxypeptidase A: The final composition of the assay solution was 0.02 M CGP in 0.020 M sodium veronal, pH 7.5 (Keller, 1956); Activity unit: $\frac{1000}{t} \log \frac{0.02}{0.02-x}$ where x is concentration of phenylalanine at time t (min.).

Carboxypeptidase B: 0.010 M HA in 0.025 M tris-(hydroxymethyl)-aminomethane (THAM), pH 7.65 (Folk and Gladner, 1958). These authors used 0.025 M HA but also demonstrated that the enzyme was saturated with respect to substrate at lower initial concentrations. Activity units: μmoles/min, HA hydrolysis.

Nucleolytic Enzymes

Ribonuclease: The activity of ribonuclease was determined by the liberation of acid soluble nucleotides from yeast ribonucleic acid according to the procedure of Kalnitsky (1959). Yeast ribonucleic acid purified by precipitation and dialysis was kindly provided by Dr. T. H. Plummer. Activity unit: $\Delta A_{260} = 1.0/4$ minutes.

Deoxyribonuclease: Aliquots containing 0.010 to 0.100 ml of the effluent from the DEAE chromatogram were assayed for deoxyribonuclease activity according to the spectrophotometric procedure of Kunitz (1941). The assay solution contained 0.04 mg/ml calf thymus deoxyribonucleic acid in 0.005 M

$MgSO_4$, 0.1 M sodium acetate pH 5.0. The depolymerization of the substrate was measured at 260 mμ, 25°C, in a DU spectrophotometer fitted with thermospacers. Activity unit: ΔA_{260} = 1.0/5 minutes.

Lipase

Lipase activity was determined in aliquots containing 0.100 to 0.250 ml of the effluent from the DEAE chromatograms by the tritrimetric assay of Marchis-Mouren et al. (1959). The assay system was 15 ml of olive oil emulsion containing sodium taurocholate, pH 9.0 at 37°C.

Amylase

The amylase activity in aliquots of 0.025 to 0.250 ml of the DEAE effluent was determined by the procedure described by Stein and Fischer (1958). Lintner soluble starch was used as substrate and the extent of hydrolysis was determined by the reduction of an alkaline solution of 3,5-dinitrosalicylic acid. The amount of reducing sugar was expressed as maltose equivalent. Activity units: 3 mg maltose reducing equivalents/5 minutes at 25°C.

Soybean Trypsin Inhibitor

The inhibitor was measured by inhibition of the tryptic hydrolysis of TAME. The incubation solution contained: 0.750 ml trypsin (25-70 μg/ml in 10^{-3} M HCl), 0.400 ml 0.2 M sodium phosphate, pH 7.6, and 0.050 ml of inhibitor solution. The autolysis control contained 0.050 ml phosphate buffer in place of inhibitor. After 5 minutes at room temperature, 0.400 ml aliquots of the solutions were assayed for trypsin activity in the usual manner. 1 unit = 8.4 μmoles/min inhibition of trypsin (TAME).

d. Determination of Protein

(1) Ultraviolet: A Beckman DU spectrophotometer was used to determine protein concentration by measurement of absorbancy at 280 mμ (A_{280}). The absorbancy of the effluent solutions from the chromatograms was also measured at 260 mμ to detect contamination by nucleotides. The extinction coefficients selected by Keller et al. (1958b) were used in this study. $A^{1\ cm}_{280\ m\mu}$, 1% solution: chymotrypsinogen A, 20; chymotrypsinogen B, 18; trypsinogen, 13.9; soybean trypsin inhibitor, 10.5; procarboxypeptidase A, 19.

(2) Lowry Method: Protein concentration was also determined according to the procedure of Lowry (1951). Bovine serum albumin was used as a standard to check reproducibility, but all measurements are reported in terms of the absorbancy at 755 mμ (A_{755}). A_{755} is defined by the following volume relationships: an aliquot containing 0.025 to 0.100 ml of the effluent from the chromatograms was added to 5.0 ml of 0.1 N NaOH containing 2% sodium carbonate, 0.02% sodium tartrate and 0.01% copper sulfate. After 10 minutes, 0.5 ml of Folin-Ciocalteau reagent, diluted 1:1 with H_2O, was added with vigorous mixing. After one hour, the absorbancy of the solution was determined at 755 mμ in a Beckman DU spectrophotometer.

3. Results and Discussion

a. Chromatographic Separation of Pancreatic Proteins

(1) Anionic Proteins

Figures 3 and 4 illustrate the elution diagrams obtained when zymogen granule extracts and pancreatic juice were chromatographed on diethylamino ethyl cellulose (DEAE). After the unretarded peak (cationic fraction) was collected the chromatogram was developed with a linear concentration gradient of potassium phosphate (pH 8.0). Enzyme activity (after activation, if required) was quantitatively determined across the entire envelope of the peaks. The exact correspondence in chromatographic behavior (phosphate concentration in effluent) of the enzymes (zymogens) and the similarities of the protein elution diagrams obtained from granule preparations and pancreatic juice will be demonstrated. A quantitative comparison of the proteins present in these mixtures will be presented in part 3b.

In each figure the profile of the eluted protein measured by the Lowry procedure (filled circles) and the phosphate concentration in the effluent (open circles) are presented in diagram A. The broken line marks the position of the cationic fraction which was collected in bulk and rechromatographed on IRC-50. At tube 50 (arrow) a linear phosphate gradient (potassium phosphate pH 8.0, 0.113 M/100 tubes) was applied to the top of the column.

Chymotrypsinogen B and Procarboxypeptidase B: These proteins were not separated in the present experiments but were eluted together in a relatively broad band, tubes 106-126, (0.055-0.080 M potassium phosphate in

Fig. 3. Chromatography of pH 8-soluble, non-dialyzable components of bovine zymogen granules on 1.0 x 70 cm columns of DEAE-cellulose at pH 8 and 4°C. A-Protein concentration in effluent measured by procedure of Lowry. Linear gradient of potassium phosphate applied to top of column at tube 50 (arrow). B, C - Enzyme activity in effluent (after trypsin activation if required).

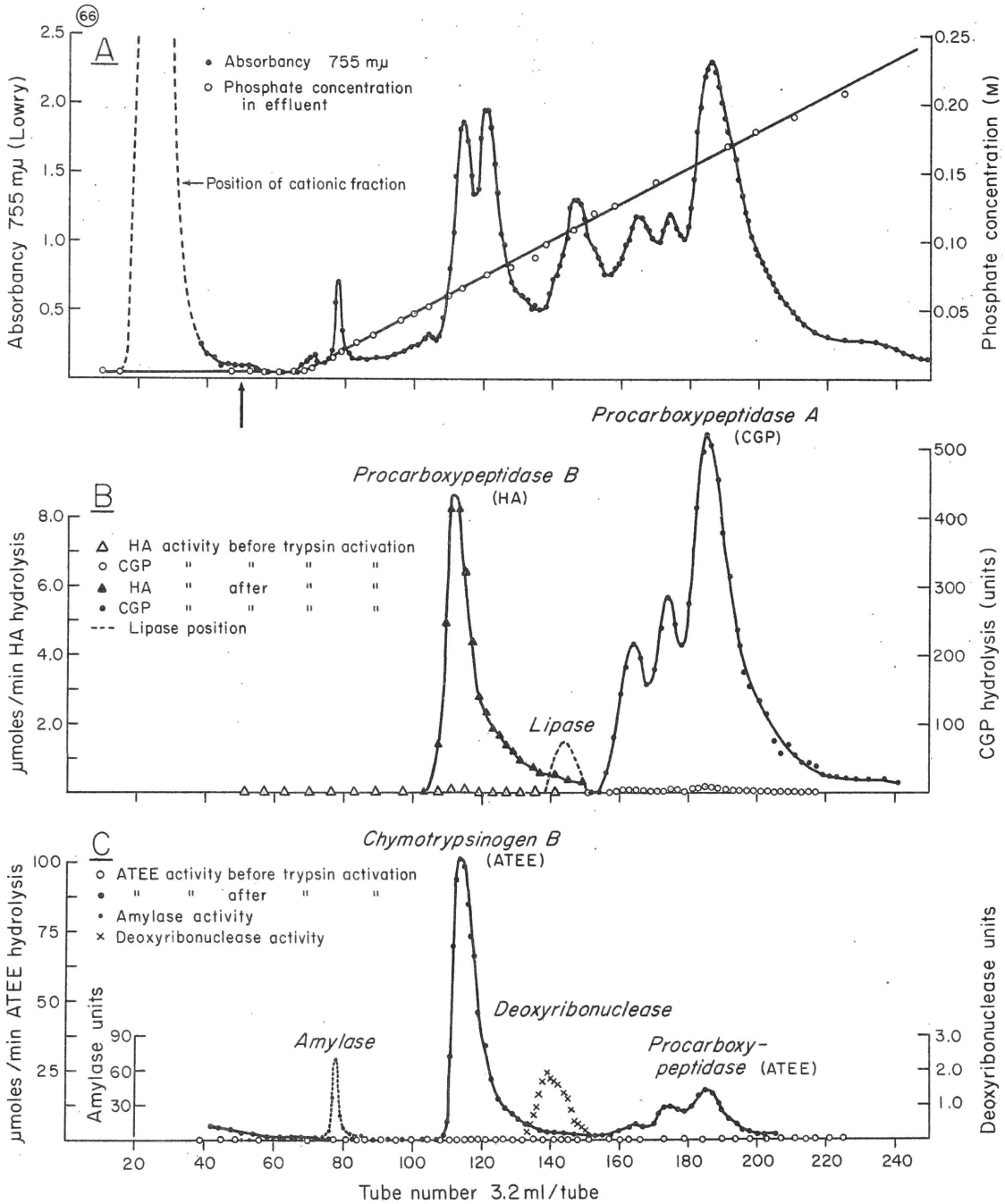
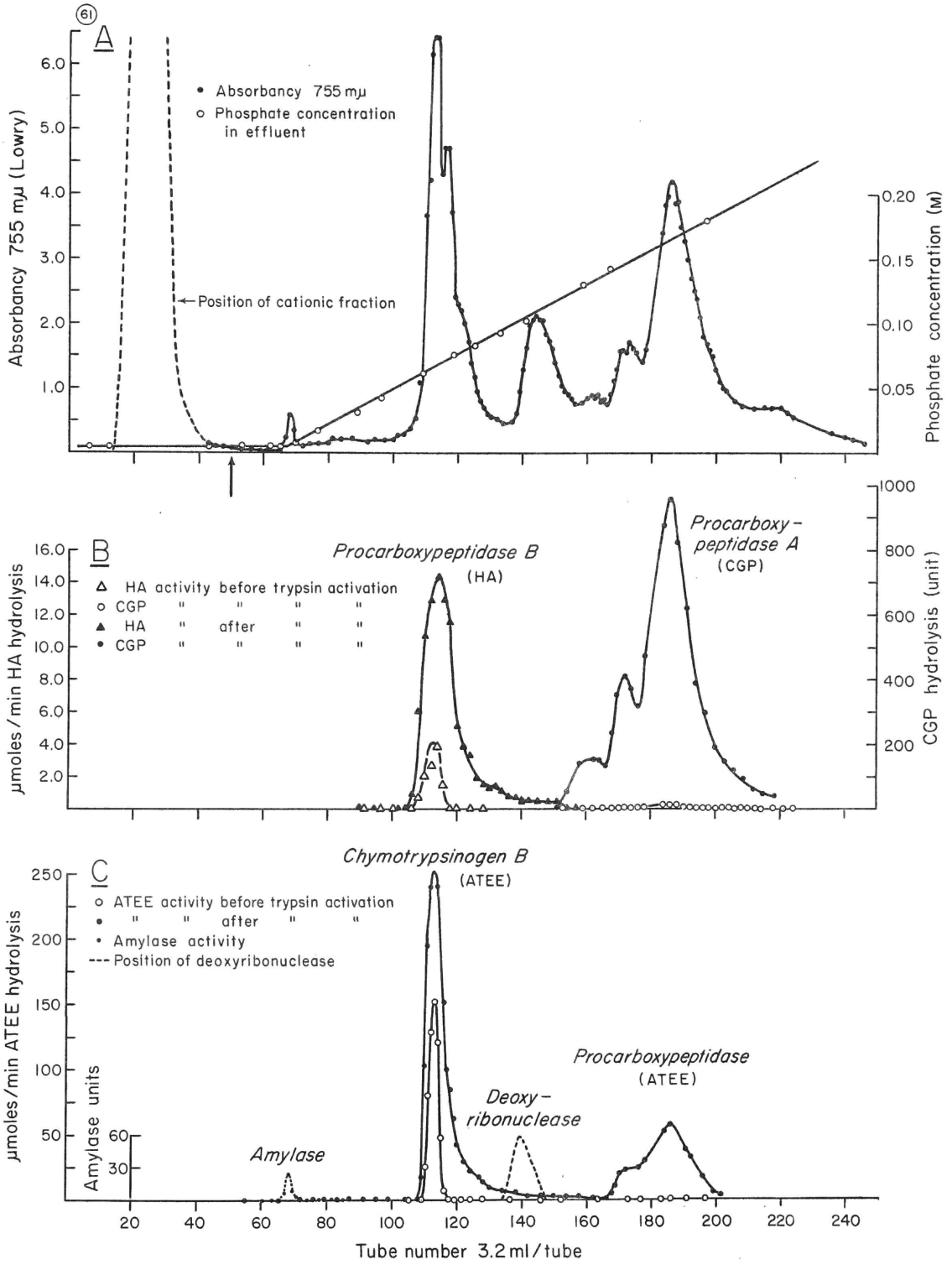

66
A
Absorbancy 755 mμ
Phosphate concentration in effluent
Position of cationic fraction
Absorbancy 755 mμ (Lowry)
Phosphate concentration (M)
Procarboxypeptidase A
(CGP)
Procarboxypeptidase B
(HA)
B
HA activity before trypsin activation
CGP " " " "
HA " after " "
CGP " " " "
Lipase position
Lipase
μmoles/min HA hydrolysis
CGP hydrolysis (units)
Chymotrypsinogen B
(ATEE)
C
ATEE activity before trypsin activation
" " after " "
Amylase activity
Deoxyribonuclease activity
Deoxyribonuclease
Amylase
Procarboxy-
peptidase (ATEE)
μmoles/min ATEE hydrolysis
Amylase units
Deoxyribonuclease units
Tube number 3.2 ml/tube

Fig. 4. Chromatography of pH 8-soluble, non-dialyzable components of bovine pancreatic juice. Conditions same as given in Fig. 3. For comparison with Fig. 3 note that ordinates in Fig. 3 are one-half scale of those in Fig. 4.


61
A
Absorbancy 755 mμ (Lowry)
Absorbancy 755 mμ
Phosphate concentration in effluent
Position of cationic fraction
Phosphate concentration (M)
B
μmoles/min HA hydrolysis
HA activity before trypsin activation
CGP " " " "
HA " after " "
CGP " " " "
Procarboxypeptidase B
(HA)
Procarboxy-peptidase A
(CGP)
CGP hydrolysis (unit)
C
μmoles/min ATEE hydrolysis
ATEE activity before trypsin activation
" " after " "
Amylase activity
Position of deoxyribonuclease
Chymotrypsinogen B
(ATEE)
Procarboxypeptidase
(ATEE)
Deoxy-ribonuclease
Amylase
Amylase units
Tube number 3.2 ml/tube

effluent) which resolved partially (Fig. 3) or appeared as a single peak with a shoulder (Fig. 4). The proteins were in the inactive form in pancreatic juice and zymogen granule extracts and required activation with trypsin for assay. Carboxypeptidase B (diagram B) was determined using hippuryl-L-arginine (HA) as substrate before (open triangles) and after (filled triangles) activation. Chymotrypsinogen B was located using acetyl-L-tyrosine ethyl ester (ATEE) before (open circles) and after (filled circles) activation.

In some experiments with both granule extracts and pancreatic juice, carboxypeptidase B and chymotrypsin B activity was found before trypsin activation (cf. Fig. 4). This phenomenon was studied in detail and it was concluded that the activity was the result of activation in the tubes after the chromatogram had been collected, and that therefore solely the zymogen forms, chymotrypsinogen B and procarboxypeptidase B, exist in granules and juice. The evidence for this conclusion is presented in the next part (a(2)).

In spite of the fact that the zymogens were not resolved chromatographically, quantitative activation and enzyme assay across the entire envelope permitted their location and the demonstration that they were chromatographically identical in both granule extracts and pancreatic juice. Visual comparison of the position of the enzyme activities and protein, or simultaneous plots of these three results indicated that chymotrypsinogen B and procarboxypeptidase B do not account for all the protein in the region (tubes 106-126). It appears as if these zymogens are present in the fast side of the protein peak(s) but that the slow (retarded) side of the envelope (second peak; tube 122 in Fig. 3 and shoulder, tube 122, Fig. 4) contains additional protein(s). To date this observation has not been pursued further.

Although separation of chymotrypsinogen B and procarboxypeptidase B was not achieved in the present experiments, these proteins have been resolved on DEAE cellulose with a reduced gradient (Pechère et al, 1958). (However, in the present work the active enzymes were separated (Fig. 6)). Since aliquots of the effluent contained both enzymes after activation, results of the enzyme determinations must be considered in terms of cross reactivity. Figure 6 demonstrates that each enzyme does not act on the

substrate of the other. This is consistent with the known specificities of the enzymes and in particular with the report of Folk et al. (1960), that carboxypeptidase B (porcine) does not act on ATEE (chymotrypsin B substrate).

When diisopropylphosphofluoridate (DFP) was added to the HA assay (carboxypeptidase B substrate) there was no effect on the rate of hydrolysis, in confirmation of the reports that carboxypeptidase B is not sensitive to DFP. Thus the measured activity against HA was not due to chymotrypsin B, which is inactivated by DFP. It may be concluded from our experiments and the reports in the literature that the synthetic substrates ATEE and HA may be used to determine these enzymes quantitatively in the presence of one another.

Procarboxypeptidase A: After activation with trypsin (3 hours, 37°C, 10-50% trypsin w/w) the proteins eluted from the column in 0.125-0.19 M potassium phosphate (tubes 155-210) catalyzed the hydrolysis of carbobenzoxy-glycyl-L-phenyalanine, CGP (filled circles in diagram B). The absence of activity before treatment with trypsin (open circles) indicates that these enzymes were in zymogen form. When different aliquots of the same solution were activated under mild conditions (24 hrs, 0°C, about 1% trypsin w/w) an endopeptidase activity demonstrable by ATEE hydrolysis was observed in all three protein peaks (cf. diagram C). The multiple forms of procarboxy-peptidase A, also showing endopeptidase activity under certain conditions of activation, have been observed in both zymogen granule extracts and pancreatic juice. On some occasions, in preparations from both sources the protein corresponding to the second of these peaks has not been observed.

Bovine procarboxypeptidase A was isolated by Keller et al. (1956,1958a) from acetone powders of pancreas and found to be a homogeneous protein possessing a molecular weight of 96,000. Trypsin activation converts the protein into several smaller components, the best characterized of which is carboxypeptidase A, of molecular weight 34,000. Moreover, after activation at low temperature with low concentrations of trypsin, procarboxy-peptidase A exhibited an endopeptidase activity. These authors also demonstrated that a protein possessing similar properties was present in bovine pancreatic juice (Keller, 1958b). In 1961, Brown et al. found that procarboxypeptidase A could be dissociated into three proteins upon treatment

at pH 10.2 at room temperature for 24 hours. The results of amino acid analyses of these components after chromatographic separation are consistent with the view that they represent the subunits of procarboxypeptidase A. One of these proteins was a trypsin activatable pro-endopeptidase with potential activity against ATEE. A second, inactive protein was assumed to be an immediate precursor of carboxypeptidase A because of the similarity of the amino acid analysis and sedimentation coefficient.

The main component in our preparations eluted in 0.16 M potassium phosphate (tube 185) was found to have a molecular weight of 92,000, determined by a high speed equilibrium ultracentrifugation method (Yphantis, 1961) using an assumed partial specific volume of 0.74*. Its specific activity against CGP was 1000 units/mg procarboxypeptidase A, which is comparable to that reported by Keller et al. (1958a) and, under appropriate activation conditions, the endopeptidase activity was demonstrable. On the basis of these chromatographic, physical, and enzymatic properties, the main component appears to be the procarboxypeptidase A described by Keller et al.

When a sample of the first component(at 0.13 M phosphate (tube 168)) was examined in the ultracentrifuge it was found to possess a molecular weight of 68,000 and a specific activity of 1300 units/mg. This protein is therefore a new procarboxypepetidase A which differs from the classical procarboxypeptidase A in molecular weight and chromatographic behavior. We propose to designate the new protein procarboxypeptidase A'.

This finding is of significance to several problems. It demonstrates that at least two precursors of carboxypeptidase A are elaborated by the pancreas, and shows that a part of the complex with a molecular weight 92,000 is not essential for the generation of carboxypeptidase activity upon exposure to trypsin. The existence of procarboxypeptidase A' suggests that the dissociation of the procarboxypeptidase A complex observed by Brown et al. (loc. cit.) may involve the artificial reversal of its formation in vivo from the subunits. It is also quite probable that a study of

* Molecular weight determinations were performed by Dr. D. A. Yphantis and Mr. Owen Griffith.

the activation of procarboxypeptidase A' would be simpler than an investigation of the same process for procarboxypeptidase A. Finally a study of procarboxypeptidase A' would undoubtedly be of interest in gaining an understanding of the forces responsible for the aggregation of the subunits of procarboxypeptidase A.

Amylase, Deoxyribonuclease and Lipase: It was observed that these enzymes are inactivated in the collection tubes when kept at 4°C. Because the estimates of Keller et al.(1958b) indicated that pancreatic juice mixtures contain small amounts of these enzymes, less than 2% amylase and 1.4% deoxyribonuclease, our study was restricted to the comparison of the chromatographic properties of the enzymes, with the expectation that their quantitative estimation in pancreatic juice and zymogen granules would be performed in the future.

Amylase was not strongly adsorbed to DEAE cellulose and was usually eluted from the column soon after the break-through of the salt gradient (tubes 65-70). Sometimes (Fig. 4, for example) it was retarded by 4 or 5 tubes after the break in the effluent salt concentration and examples of double-fronting and trailing of activity as far as tube 100 were observed. The chromatographic behavior of amylase was the same whether the enzyme was isolated from zymogen granules or pancreatic juice.

Deoxyribonuclease from zymogen granule and pancreatic juice preparations was eluted from the column at 0.1 M potassium phosphate (tube 140).

Lipase was located enzymatically at a position corresponding to deoxyribonuclease in the chromatogram from one sample of zymogen granules with the aid of an olive oil emulsion as substrate. Confirmation of the location by isolation and purification is necessary because of the small amounts of activity observed. Both lipase and deoxyribonuclease chromatographed in the same region as a significant amount (about 10%) of protein which has not yet been identified.

Unidentified Protein: This unidentified protein was eluted from the column at 0.10-0.13 M potassium phosphate (tubes 140-160). Although it contains small amounts of deoxyribonuclease and lipase, it is probable that other proteins are present. The protein from this region of the chromatogram had no action on the following substrates (either before or after tryptic activation): acetyl-L-tyrosine ethyl ester, p-toluenesulfonyl-

L-arginine methyl ester, hippuryl-L-arginine, and carbobenzoxy-glycyl-L-phenylalanine.

(2) Activation in Chymotrypsinogen B Region

The presence of active proteolytic enzymes in some preparations of pancreatic juice and zymogen granules has been noted. This partial activation was observed only on DEAE cellulose chromatograms in the region which contained chymotrypsinogen B and procarboxypeptidase B.

Two characteristics of the activation phenomenon are illustrated in Figure 5. In the absence of added trypsin the amount of detectable enzyme activity increased with time after collection. This is shown for chymotrypsin B in the top diagram of Figure 5, but was also observed for carboxypeptidase B. The second result illustrated in these diagrams is that the area of partial activation is restricted to the fast side of the precursor peak.

In order to determine the chromatographic behavior of the active enzymes, a portion of the region (tubes 112-118) shown in Figure 6 (top) was activated by incubation with 5% trypsin (w/w) overnight at 4°C. Figure 6 (bottom) shows the result obtained when an aliquot of the activation mixture containing chymotrypsin B and carboxypeptidase B was rechromatographed. The enzyme activity was located in two peaks, carboxypeptidase B (tubes 72-92) and chymotrypsin B (tubes 122-142). Forty percent of the recovered absorbancy at 280 mμ was associated with the chymotrypsin B activity and 16% was in the carboxypeptidase B region. The material in tubes 110-122 had no enzyme activity even after resubmission to the action of trypsin. The remaining ultraviolet adsorbing material eluted from the column has not been investigated. This experiment suggests that the activation products possessing enzyme activity do not have the same chromatographic properties as the precursors.

Since trypsin is the only enzyme known to activate the zymogens chymotrypsinogen B and procarboxypeptidase B, an explanation for the presence of the cationic protein in this region of the DEAE chromatogram of the anionic proteins was sought.

Figure 7 (top) demonstrates the chromatographic properties of the commercial sample of crystalline soybean trypsin inhibitor (STI) used in

Fig. 5. Observed activity in chymotrypsinogen B region from pancreatic juice samples. Chromatographic procedures same as given in Fig. 3.

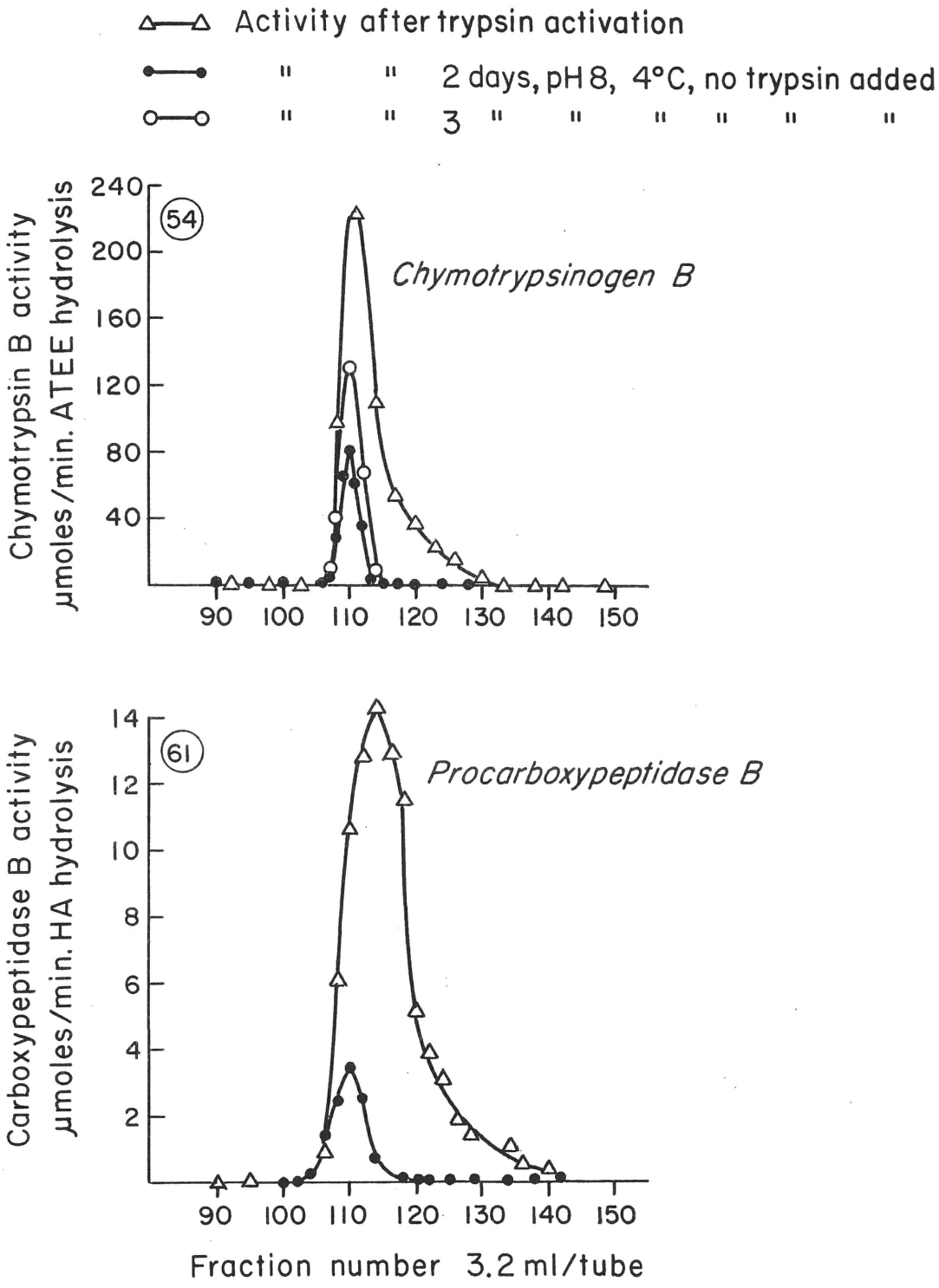

Activity after trypsin activation
" " 2 days, pH 8, 4°C, no trypsin added
" " 3 " " " " " "
Chymotrypsin B activity
µmoles/min. ATEE hydrolysis
240
200
160
120
80
40
54
Chymotrypsinogen B
90 100 110 120 130 140 150
Carboxypeptidase B activity
µmoles/min. HA hydrolysis
14
12
10
8
6
4
2
61
Procarboxypeptidase B
90 100 110 120 130 140 150
Fraction number 3.2 ml/tube

Fig. 6. Chromatography of products obtained after activation of protein from chymotrypsinogen B region of pancreatic juice. Demonstration of chromatographic behavior of chymotrypsin B and carboxypeptidase B. Chromatographic conditions were the same as those given in Fig. 3. Top: Data of Fig. 4 replotted. The abscissa of the lower diagram has been displaced by four tubes towards the origin to bring both elution curves to the same relative displacement from the break-out of the salt gradient.

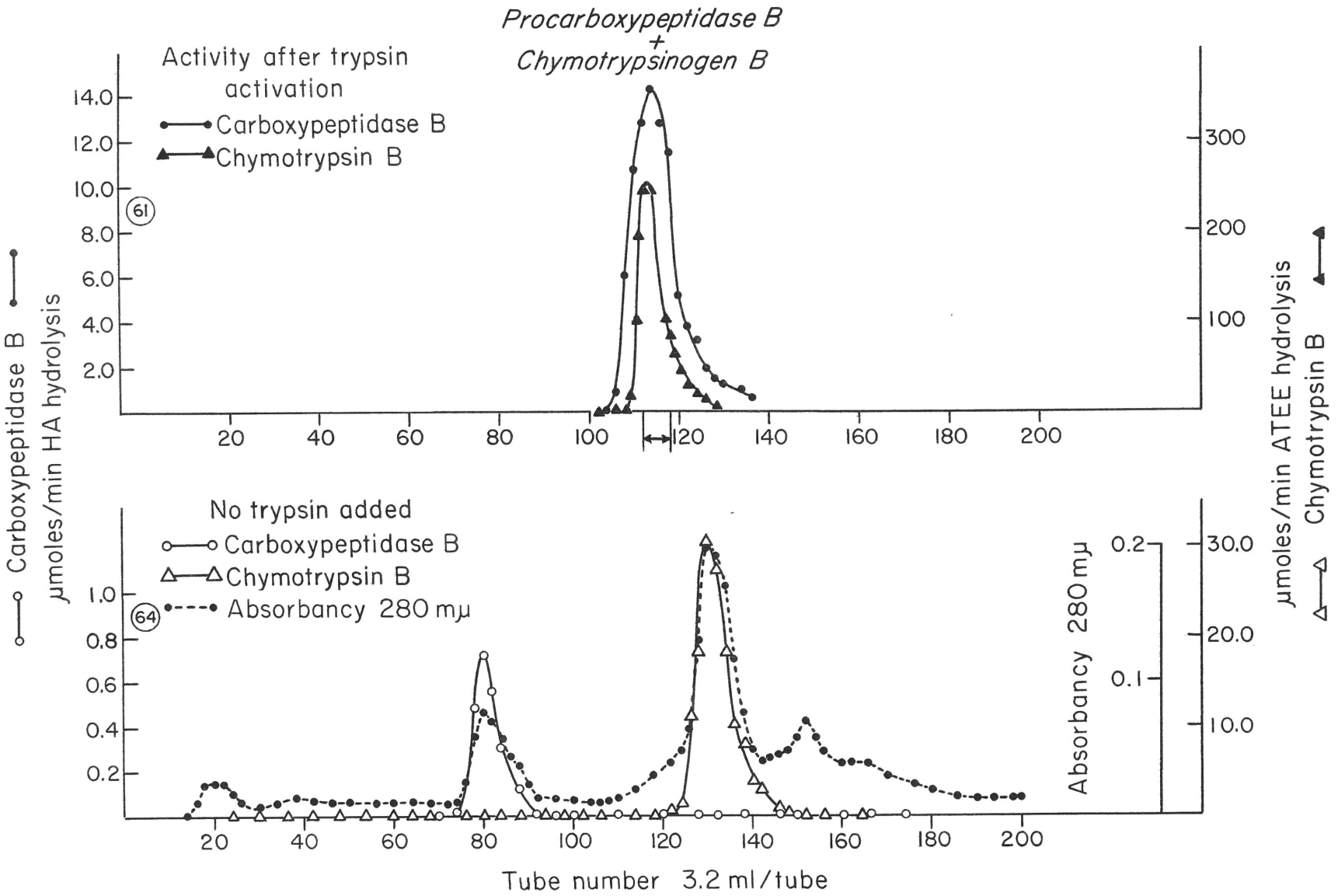
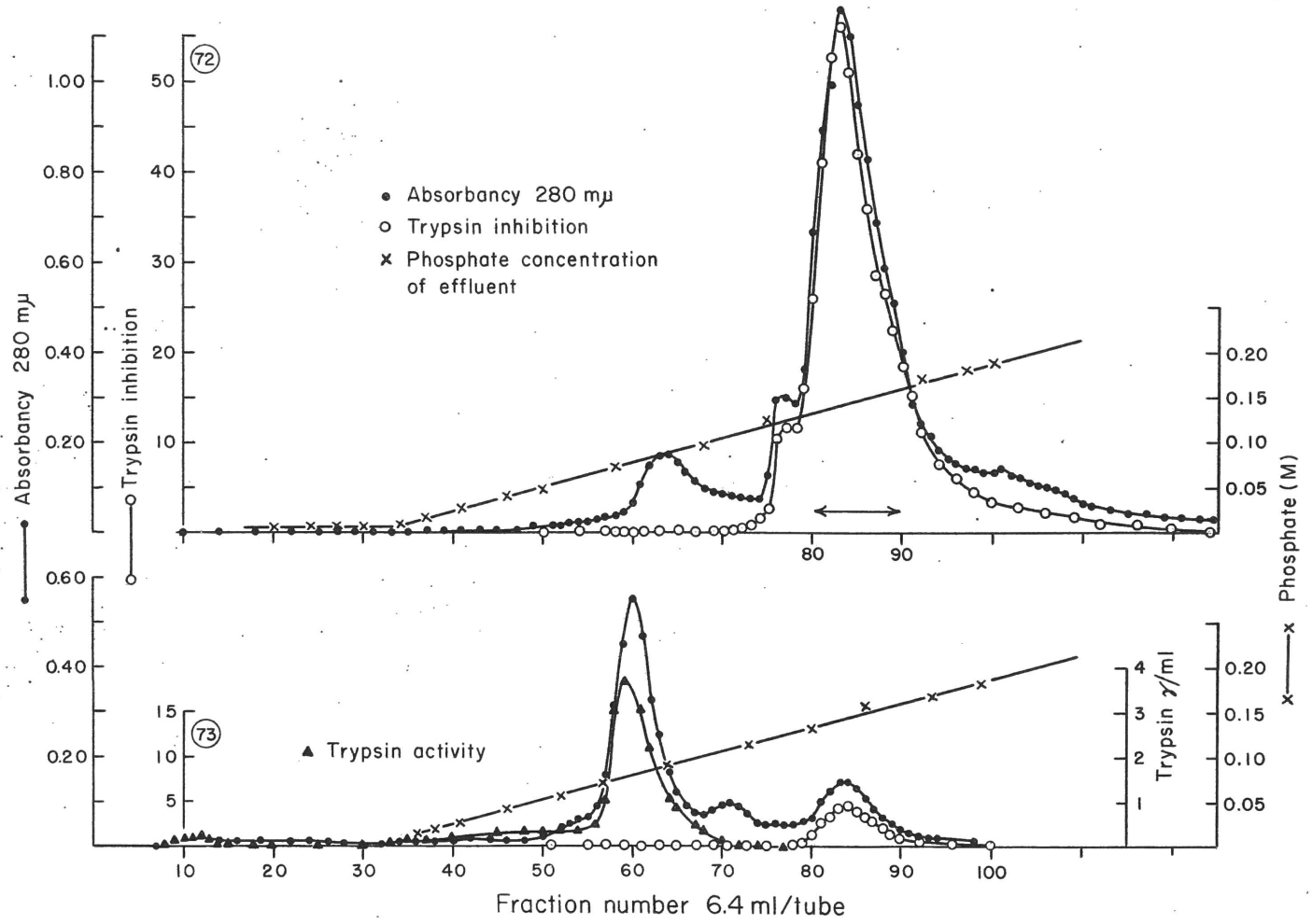

Procarboxypeptidase B
+
Chymotrypsinogen B
Activity after trypsin activation
Carboxypeptidase B
Chymotrypsin B
61
14.0
12.0
10.0
8.0
6.0
4.0
2.0
300
200
100
20
40
60
80
100
120
140
160
180
200
Carboxypeptidase B
μmoles/min HA hydrolysis
μmoles/min ATEE hydrolysis
Chymotrypsin B
No trypsin added
Carboxypeptidase B
Chymotrypsin B
Absorbancy 280 mμ
64
1.0
0.8
0.6
0.4
0.2
0.2
0.1
30.0
20.0
10.0
Absorbancy 280 mμ
Tube number 3.2 ml/tube

Fig. 7. Top: Chromatography of crystalline soybean trypsin inhibitor under identical conditions as described in Fig. 3. Bottom: Chromatography of a mixture of commercial trypsin and a sample of soybean trypsin inhibitor derived from fractions 80-90 of the chromatogram illustrated in the upper part of the figure. Conditions of chromatography were the same as given in Fig. 3.


72
Absorbancy 280 mμ
Trypsin inhibition
Phosphate concentration of effluent
Absorbancy 280 mμ
Trypsin inhibition
Phosphate (M)
1.00
0.80
0.60
0.40
0.20
50
40
30
20
10
0.20
0.15
0.10
0.05
80
90
73
Trypsin activity
0.60
0.40
0.20
15
10
5
4
3
2
1
Trypsin γ/ml
10
20
30
40
50
60
70
80
90
100
Fraction number 6.4 ml/tube

these studies and (bottom) the result obtained when material corresponding to the center of the peak (tubes 80-90) is rechromatographed in the presence of a crystalline trypsin preparation. The inhibitor was assayed by its ability to inhibit trypsin activity. The peak of absorbancy at 280 mμ at fraction 64 did not possess inhibitory activity and had a ratio of absorbancies at 280 and 260 mμ of 1.4, indicating that a small amount of non-protein ultraviolet absorbing material was present. When trypsin was rechromatographed in the presence of excess inhibitor (indicated by inhibition activity in fractions 80-90), a new peak (fraction 60) was observed. This major peak (0.08 M potassium phosphate), which corresponds to soybean trypsin inhibitor-trypsin complex, appears in a position corresponding to tubes 122-125 in Figures 3 and 4, or immediately after the chymotrypsinogen B region. The chromatographic behavior of the STI-trypsin complex on DEAE cellulose is consistent with the isoelectric point of 5.0 determined by cataphoresis (Kunitz, 1947). As Figure 7 shows, there was no detectable inhibitor activity in this region of the chromatogram but a low level of trypsin activity was observed (filled triangles). Trypsin activity was detectable in the effluent at the salt break-through (fraction 35) and extended to fraction 70; the actual amount in the peak region represents 0.5% of the trypsin in the complex. The behavior of this model system suggests that the inhibitor may act as a carrier for the adsorption of trypsin on to the DEAE cellulose column. There are at least two possible explanations for the measured trypsin activity. The inhibitor in the complex may slowly be digested by trypsin. This phenomenon, called temporary inhibition, has been demonstrated for trypsin complexes with ovomucoid inhibitor, and a "small but significant amount of trypsin was liberated" from the STI-trypsin complex in the experiments of Laskowski and Wu (1953) after two weeks at 37°C. However, these authors suggest that the denaturation of STI at this elevated temperature may have complicated the result. A second explanation is that the STI-trypsin complex may be resolved during chromatography. This is consistent with the low level of trypsin activity found immediately after the break-through of the salt gradient and the activation which favors the fast side of the zymogen peak (Fig. 5).

The experiments summarized in Figures 5-6 demonstrate that activation proceeds in the collection tubes without added trypsin, and that the

chromatographic behavior of the activated enzymes is different from that of the precursor proteins. Moreover some samples of both granule and juice protein did not exhibit activity in this region up to 4 days after collection. It has been concluded from these observations that the activity of chymotrypsin B and carboxypeptidase B in their zymogen region is not due to the presence of these active enzymes as components of the exocrine secretion but rather to activation in the tubes after collection. It is tentatively concluded that the trypsin responsible for this activation was adsorbed to DEAE cellulose as part of the STI-trypsin complex.

(3) Cationic Proteins

The cationic protein fraction from the DEAE cellulose chromatograms (Figs. 3 and 4) was rechromatographed on IRC-50 at pH 6.04 and pH 6.49. Representative effluent curves for the zymogen granule and pancreatic juice preparations have been illustrated simultaneously to facilitate comparison (cf. Figs. 8-11). The outstanding feature of these results is the identity in chromatographic behavior of the cationic proteins isolated from zymogen granules and pancreatic juice.

Ribonuclease (Figs. 8-10): Figure 8 demonstrates the chromatographic behavior and relative distribution of ribonuclease activity (open circles) present in the cationic fraction of both preparations. Ribonuclease A, tubes number 40 to 48, is the major component possessing enzyme activity. In 6 preparations (3 pancreatic juice and 3 zymogen granule) ribonuclease A represented 82-85% of the recovered enzyme activity. In these experiments 88 to 93% of the enzyme activity and 99 to 115% of the protein (Lowry) was recovered after chromatography. The specific activity of ribonuclease A was the same, within experimental error, for the enzyme isolated by the chromatographic methods from pancreatic juice, zymogen granules or acid extracts of the gland (cf. Table 8).

All the samples contained similar proportions (7 to 10%) of a second component, moving at tube 34. Martin and Porter (1951) and Hirs et al. (1953) found that when acid extracts of pancreas are chromatographed in this way two peaks of enzyme activity are demonstrable. Since we have employed the chromatographic procedure of Hirs et al., and the second component appears at the same effluent volume as described by these authors,

we have followed their nomenclature and assigned the name ribonuclease B to this component. This result stands in contradiction to the report of Keller et al. (1958b) who were unable to detect ribonuclease B in pancreatic juice.* Ribonuclease A from pancreatic juice is identical chemically to ribonuclease A from acid extracts of the gland.** Whether in fact the ribonuclease B from zymogen granules and pancreatic juice and that from acid extracts are identical is not yet known. However these experiments show that ribonuclease B is present in zymogen granules and therefore must be considered a component of the exocrine secretion.

The relative amount of ribonuclease A in the cationic fraction is obscured by the expanded scale employed for Figure 8. The elution diagrams for the protein (Lowry) of the same experiments are presented in Figure 9. In addition to the similarity of the distribution of the enzymes already discussed, it is apparent that there is identity in the protein complement of these samples and quantitative data based on protein concentration (Lowry) and activity are presented in the next part.

The poorly resolved proteins in tubes 20-33 are the two proteolytic zymogens (trypsinogen and chymotrypsinogen A) and the soybean trypsin inhibitor which was added to prevent activation. Better resolution of these proteins and of the ribonuclease activity present in this region may be achieved when chromatography is carried out in 0.2 M sodium phosphate at pH 6.04 and when the length of the column is increased (cf. Fig. 10). The distribution of protein (filled circles) indicates that trypsinogen (32-42) is well separated from chymotrypsinogen A (56-68). Furthermore, the ribonuclease activity (open circles), which appeared as three peaks at pH 6.49, is resolved into at least three zones plus ribonuclease B (90-110) and ribonuclease A (tubes 190-230).

With the improved resolution obtained by this technique an exact correspondence in chromatographic position between ribonuclease activity in zymogen granules and pancreatic juice is evident. If the proteins responsible for the enzymatic activity in tubes 30-90 have the same specific

* As Fig. 8 shows, in the absence of enzymatic measurements ribonuclease B would not be detected by the spectrophotometric methods employed by Dr. Keller (P. J. Keller, personal communication, and Keller and Cohen, 1961).

** T. H. Plummer, personal communication.

activity as ribonuclease A, which represents 2 to 3% of the total protein in zymogen granules and pancreatic juice, the correspondence between the preparations becomes even more striking when the fact is considered that these minor components account for only 0.1 to 0.2% of the total protein of these preparations.

The heterogeneity of the ribonuclease present in acid extracts of bovine pancreas or in crystalline preparations of ribonuclease prepared from these extracts has been demonstrated by partition chromatography (Martin and Porter, 1951), ion exchange chromatography (Hirs et al., 1953; Taborsky, 1959), countercurrent distribution (King and Craig, 1958) and by zone electrophoresis on starch (Raacke and Li, 1954). In addition to showing that ribonuclease B was present in the gland Martin and Porter (1951) and Hirs et al. (1953) demonstrated that ribonuclease A was not transformed into ribonuclease B as the result of sulfuric acid treatment, autolysis of the gland, or heating at 100°C for 15 minutes.

In the only study in which secondary proteins possessing ribonuclease activity have been characterized, two proteins have been demonstrated chemically to be related to ribonuclease A. Eaker (1962) isolated des-lysyl glutamyl ribonuclease and des-lysyl pyroglutamyl ribonuclease from a crystalline preparation of ribonuclease.* These proteins differ from ribonuclease A only in the loss of the N-terminal residue. The first possesses an N-terminal glutamic acid residue, whereas in the second protein the glutamic acid is in the pyrollidone form.

A second possible explanation for the multiplicity of ribonuclease components is that the gland synthesizes a second enzyme, which, like ribonuclease, hydrolyzes yeast ribonucleic acid, but possesses a different specificity. The presence of all four 3'-mononucleotides in ribonucleic acid digests after the action of pancreatic extracts (Jones and Perkins, 1923; Schmidt et al. 1951) and crystalline ribonuclease (Loring and Carpenter, 1943) cannot be explained in terms of the specificity of ribonuclease A. The enzyme does not hydrolyze all the bonds between the component nucleotides of ribonucleic acid but only bonds between the 3'-pyrimidine

* This particular preparation contained only 46% ribonuclease A. There is great variation in the composition of crystalline ribonuclease preparations.

Fig. 8. Chromatography of cationic fraction on IRC-50. Column, 0.9 x 30 cm, equilibrated with 0.2 M sodium phosphate at pH 6.49, 4°.

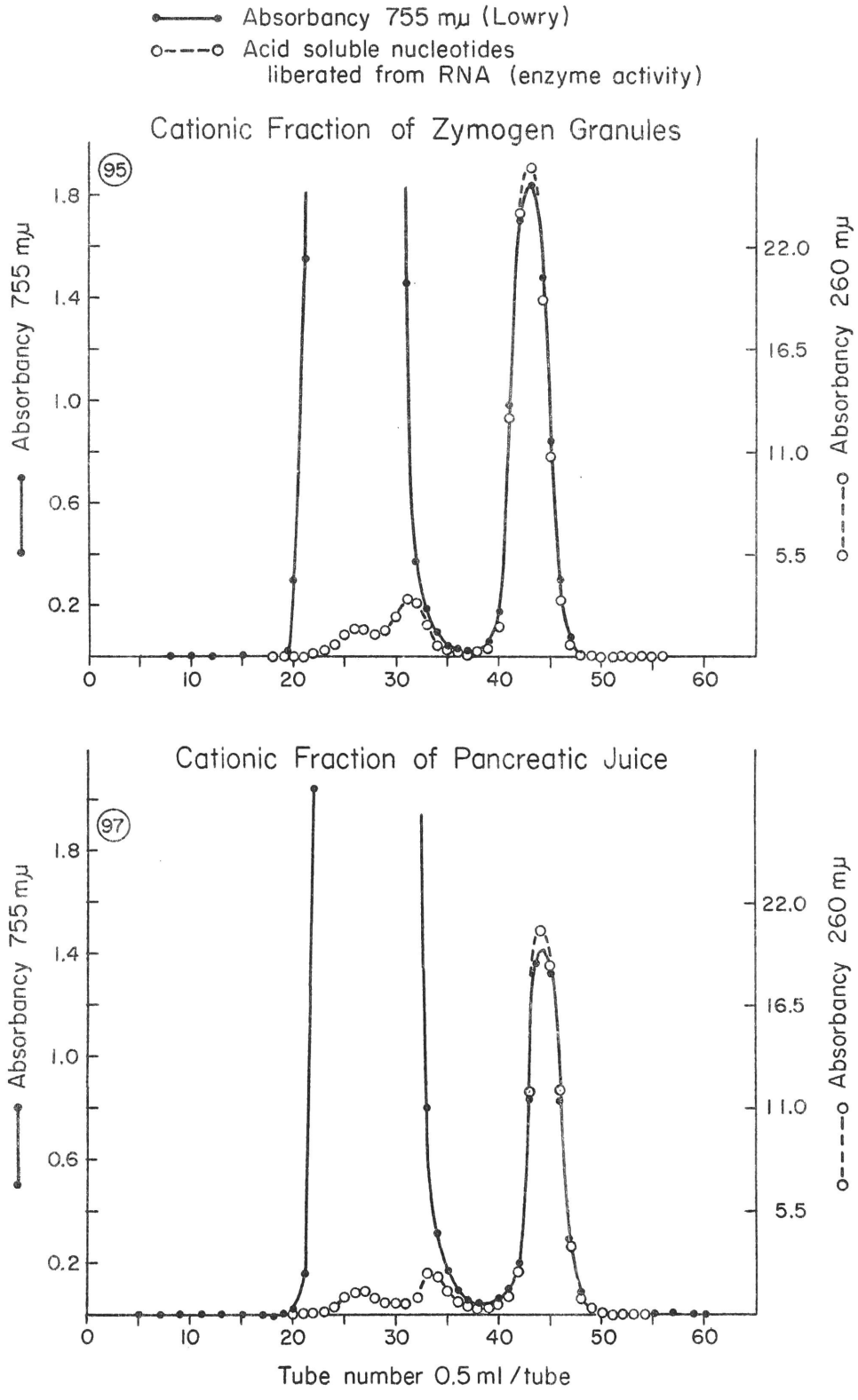
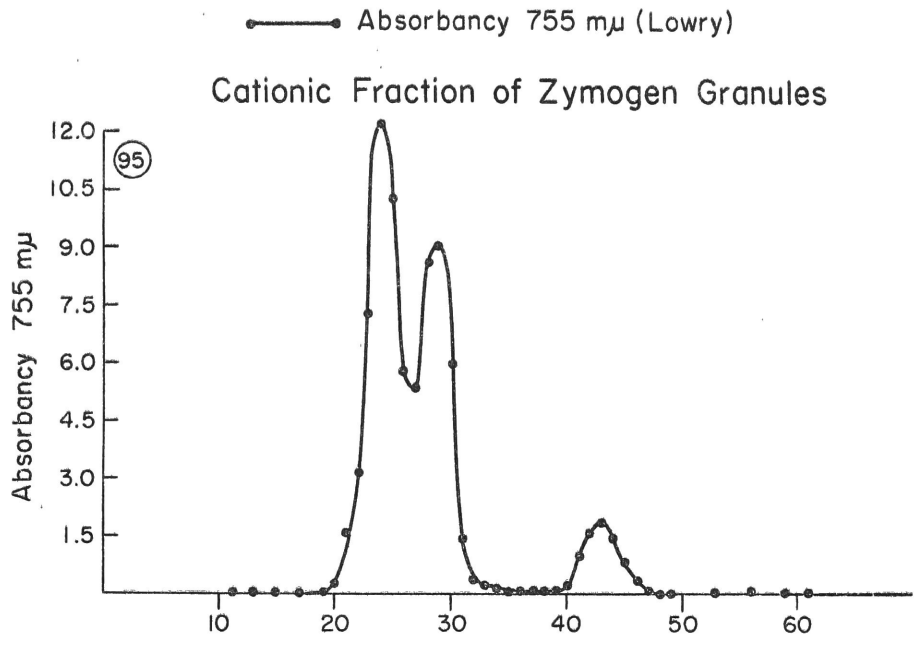

Absorbancy 755 mμ (Lowry)
Acid soluble nucleotides liberated from RNA (enzyme activity)
Cationic Fraction of Zymogen Granules
95
Absorbancy 755 mμ
Absorbancy 260 mμ
1.8
1.4
1.0
0.6
0.2
22.0
16.5
11.0
5.5
0
10
20
30
40
50
60
Cationic Fraction of Pancreatic Juice
97
Absorbancy 755 mμ
Absorbancy 260 mμ
Tube number 0.5 ml / tube

Fig. 9. Chromatography of cationic fraction on IRC-50 at pH 6.49. The results shown in Fig. 8 have been replotted to demonstrate the complete elution profile.


Absorbancy 755 mμ (Lowry)
Cationic Fraction of Zymogen Granules
95
Absorbancy 755 mμ
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
10
20
30
40
50
60

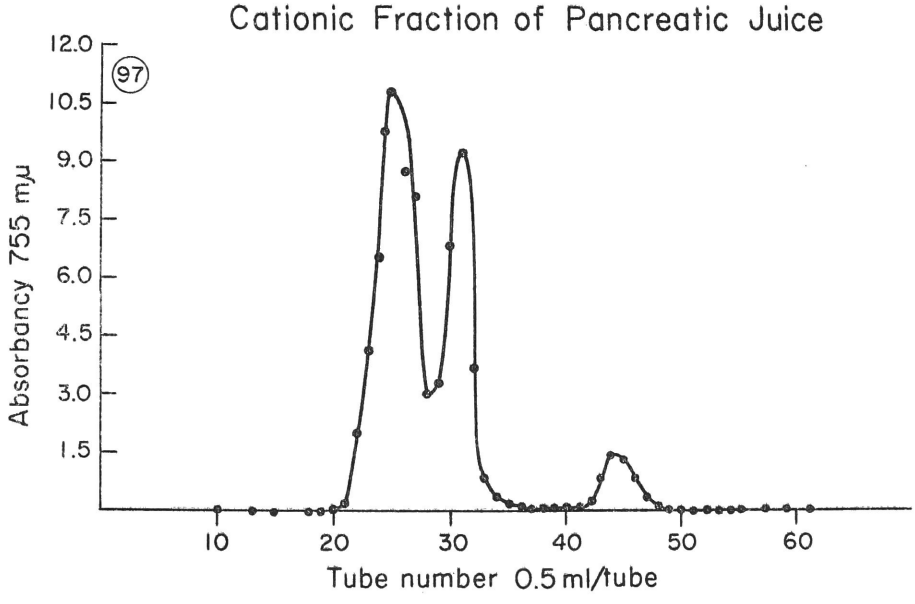
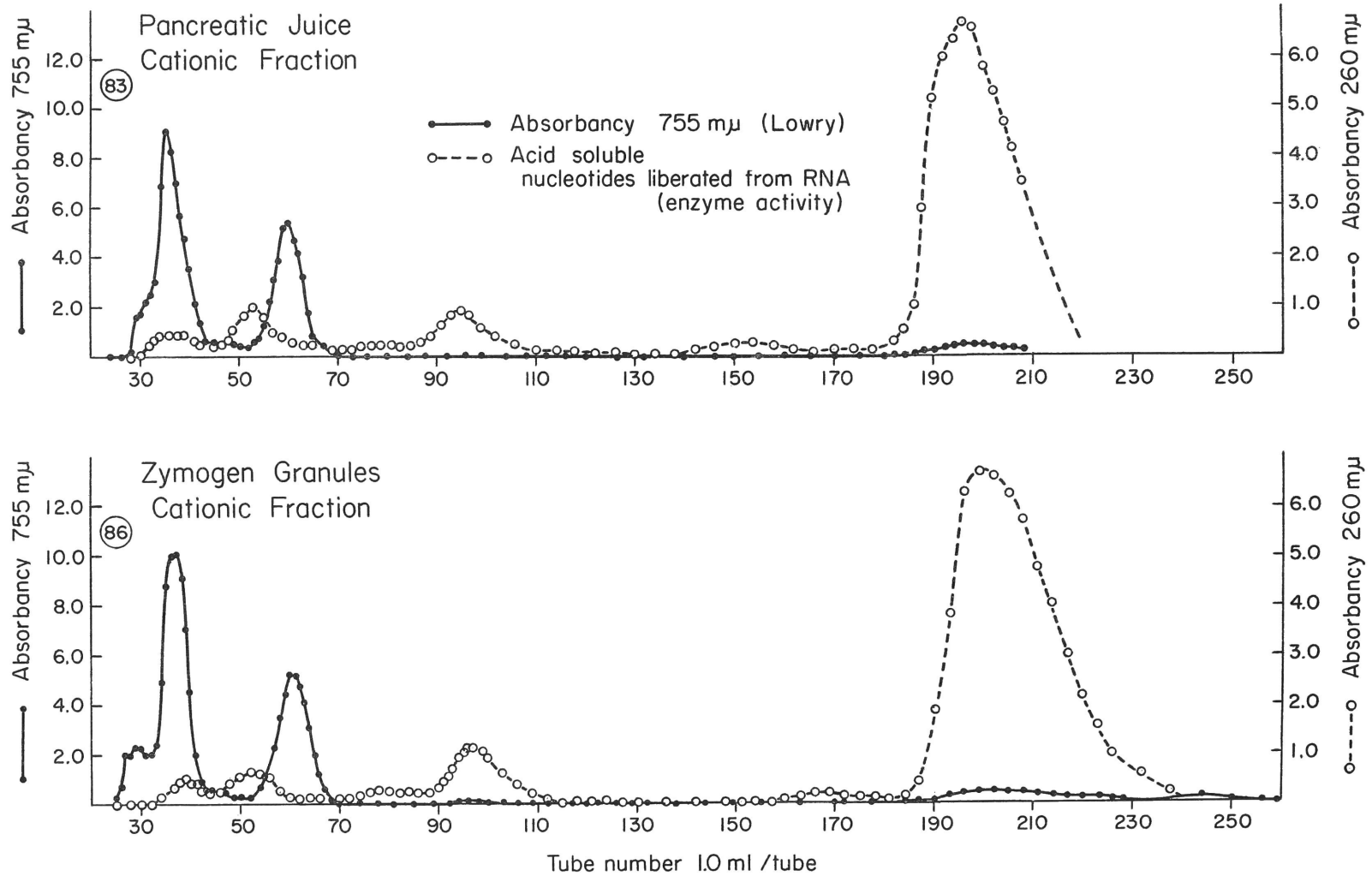

Cationic Fraction of Pancreatic Juice
97
Absorbancy 755 mμ
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
10
20
30
40
50
60
Tube number 0.5 ml/tube

Fig. 10. Chromatography of cationic fraction on IRC-50. Column 0.9 x 70 cm, equilibrated with 0.2 M sodium phosphate at pH 6.04, 4°.


Pancreatic Juice
Cationic Fraction
83
Absorbancy 755 mμ (Lowry)
Acid soluble
nucleotides liberated from RNA
(enzyme activity)
Absorbancy 755 mμ
Absorbancy 260 mμ
Zymogen Granules
Cationic Fraction
86
Tube number 1.0 ml /tube

nucleotide phosphoryl groups and the adjacent purine or pyrimidine nucleotides. In consequence the liberation of pyrimidine 3'-mononucleotides but not purine 3'-mononucleotides ensues. It is therefore possible that the pancreas may contain a ribonuclease which acts at purine nucleotides. A specific phosphodiesterase fraction isolated from bovine pancreas (Davis and Allen, 1956) hydrolyzes both purine and pyrimidine nucleoside 2',3'-phosphates to the corresponding 2'-nucleotides but does not act on yeast ribonucleic acid to any appreciable extent.

Our results have a bearing on these problems. There is the possibility that the multiple forms of ribonuclease are artifacts of preparation. Although only one of the forms, ribonuclease B, has been identified, in terms of its only characterized property - chromatography on IRC-50 at pH 6.47 - several other forms of ribonuclease can be demonstrated in the cationic fraction of pancreatic juice, and a direct chromatographic correlation can be made with extracts of zymogen granules. The procedures did not involve acid extraction and the solutions were at pH 5.8-8.0 at all times. Direct chromatography of pancreatic juice on IRC-50 demonstrates that the multiple forms of ribonuclease activity are not artifacts of the DEAE filtration*. Therefore it may be concluded that the multiple forms of ribonuclease are normal constituents of the exocrine secretion. The isolation of these proteins from pancreatic juice will permit in the future a detailed examination of their ribonuclease activity. It is possible that a nuclease of different specificity from ribonuclease A exists, and will be recognized by its activity against chemically defined synthetic substrates.

Trypsinogen and Chymotrypsinogen (Fig. 11): The elution curves showing protein concentration and enzyme activity of the proteolytic zymogens present in the cationic fractions of pancreatic juice and zymogen granules are presented in Figure 11. Identification was based on chromatographic position and enzyme activity toward synthetic substrates.

Both zymogens were completely inactive immediately after collection and required activation. Trypsinogen was activated by an acid protease

* Dr. T. H. Plummer, personal communication.

isolated from aspergillus, and trypsin was used to activate chymotrypsinogen A. Trypsin activity was measured by the hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME), and chymotrypsin was assayed using acetyl-L-tyrosine ethyl ester (ATEE). The best (highest) specific activities of trypsinogen and chymotrypsinogen A, for the center 50-60% of the peaks, taken from six experiments were chosen to normalize these curves. The tube by tube correspondence between protein concentration and activity measurements in the granule and juice preparations indicate the overall homogeneity of the cationic proteins and their exact correspondence in chromatographic properties and potential specific activity*. The potential specific activity data for individual experiments are presented in Table 8. It is clear that the values obtained for trypsinogen and chymotrypsinogen A compare excellently with the literature value for trypsin and with an internal standard used for chymotrypsinogen respectively**. The consistency of the results within the experiments and the close approach to the expected specific activity indicate that reproducible and nearly complete activation was achieved.

Both the trypsinogen and the protein component between the major peaks, tubes 45-55, catalyzed the hydrolysis of ATEE, the substrate usually employed for chymotrypsin.

The amount of cross reactivity of trypsin with the chymotrypsin substrates is small when compared on a weight basis, but is significant because these enzymes are used for the determination of the primary structures of proteins. Inagami and Sturtevant (1960) demonstrated by kinetic experiments using the specific inhibitors indole and soybean trypsin inhibitor that the observed "cross reactivity" (hydrolysis of benzoyl-L-arginine methyl ester by chymotrypsin and hydrolysis of acetyl-L-tyrosine ethyl ester by trypsin) was not the result of contamination. Commercial samples of trypsin (Cole and Kinkade, 1961; Marous et al., 1962) and trypsinogen (Liener, 1960) purified by chromatography have been shown to contain ATEE activity corresponding to from 0.5 to 2.0% contamination by chymotrypsin. In the present experiments the acetyl-L-tyrosine ethyl ester

* The specific activities reported for the proteolytic zymogens are in fact "Potential Specific Activities".

** An internal standard was used for chymotrypsinogen A because several active forms may be produced during activation. An end group study would be required to determine which form or forms are present in the activation mixture.

hydrolysis was 7 μmoles/min/mg trypsinogen, which corresponds to 1.6% of the rate obtained with chymotrypsinogen A*. Our result confirms the previous observations and extends them to trypsinogen isolated from zymogen granule extracts and pancreatic juice.

The identification of the protein associated with small amounts of ATEEase activity demonstrated between the major peaks is uncertain. Since activation is required, the protein does not represent active chymotrypsin, which is eluted from the column more rapidly than chymotrypsinogen**. The identification of this trypsin activatable zymogen requires further research.

The peak moving closest to the break-through of the column contains soybean trypsin inhibitor. The content of soybean trypsin inhibitor was determined by the inhibition of trypsin. Small amounts of other proteins may be present in this region. The specific activity of the inhibitor was only 50 to 75% of that found for the chromatographically purified material; however, the sample of crystalline soybean trypsin inhibitor used in these experiments contained about 20% of inactive material that absorbed at 280 mμ (cf. Fig. 7).

Independent experiments indicated that the soybean trypsin inhibitor-trypsin complex is not retarded by the column and chromatographs in the same position as STI (tubes 26-33). When the STI region was assayed for potential trypsin activity (STI-trypsin complex) no activity was found. The assay is based on the fact that the STI-trypsin complex is dissociated in acid and the trypsin present in such a solution may be determined using p-toluenesulfonyl-L-arginine methyl ester at pH 8 before the complex is reconstituted (Green, 1953). It has been concluded that the cationic fraction did not contain trypsin during collection nor did it activate during dialysis at pH 5.8 during chromatography in the presence of STI.

The chromatographic behavior of trypsinogen, chymotrypsinogen and ribonuclease A was the same for commercially available samples prepared from acid extracts of the gland and for their counterparts isolated from

* This estimate may be high because of the partial contribution made by the ATEEase activity present between the major peaks.

** Dr. C. H. W. Hirs, personal communication.

Fig. 11. Chromatography of cationic fraction on IRC-50 at pH 6.04. The chromatographic conditions are the same as those used in the experiments in Fig. 10.

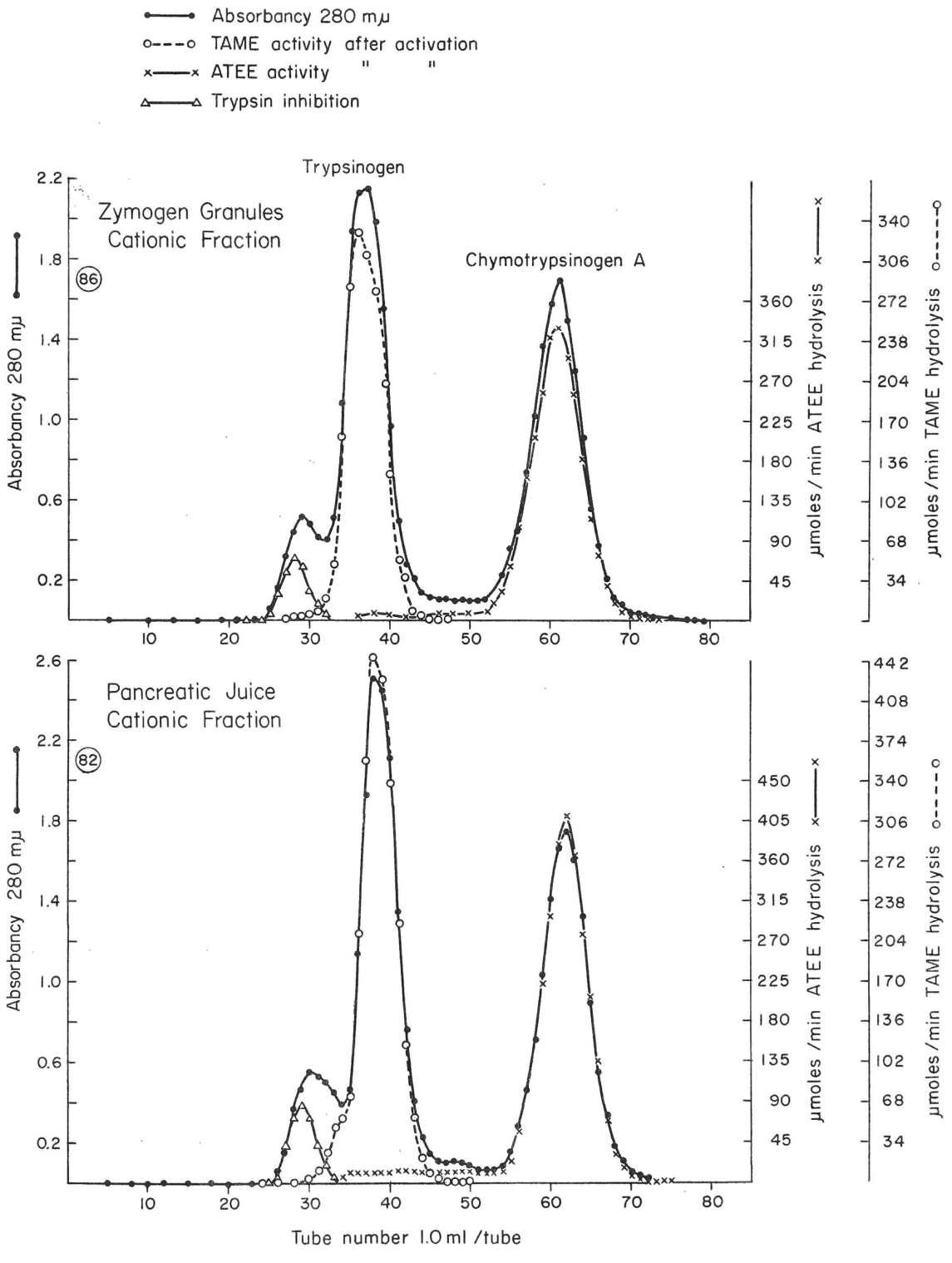

Absorbancy 280 mμ
TAME activity after activation
ATEE activity " "
Trypsin inhibition
Trypsinogen
Chymotrypsinogen A
Zymogen Granules
Cationic Fraction
86
Pancreatic Juice
Cationic Fraction
82
Absorbancy 280 mμ
μmoles / min ATEE hydrolysis
μmoles /min TAME hydrolysis
Tube number 1.0 ml /tube

TABLE 8

SPECIFIC ACTIVITY ASSIGNMENT

TRYPSINOGEN

Exp. No.	% of Peak Used in calculation	Potential Specific Activity (TAME) μmoles/min/mg Trypsinogen[1,2]	
82	59	250	Best
83	50	229	
84	65	240	
85	57	236	Average
86	50	204	
87	75	217	

Average 229 ± 17; Best 239 ± 9; Trypsinogen[3] 280.

CHYMOTRYPSINOGEN

Exp. No.	% of Peak Used in calculation	Potential Specific Activitie (ATEE) μmoles/min/mg Chymotrypsinogen[1,2]	
82	60	442	Best
84	50	442	
85	72	454	
87	63	454	Average
83	50	418	
86	58	381	

Average 432 ± 26; Best 448 ± 7; Chymotrypsinogen A 400. (same activation contions)[4]

RIBONUCLEASE A

Exp. No.	% of Peak Used in calculation	Specific Activity (RNA) Units/mg Ribonuclease A[1]	
91	88	94	Best
95	83	94	
97	95	95	Average
93	80	85	
94	65	82	

Average 90 ± 6; Best 94; Ribonuclease A 86.

[1] Protein concentration determined spectrophotometrically.

$A^{1\ cm.}_{280\ m\mu}$ (1%)

13.9 Trypsinogen
20.0 Chmyotrypsinogen A
7.0 Ribonuclease A

[2] Activation conditions given in text.

[3] Cole and Kinkade (1961)

[4] Commercial sample which had been prepared by chromatography on IRC-50

zymogen granule extracts and pancreatic juice.

b. Quantitative Distribution of Protein in Chromatograms: Protein was determined by two procedures: spectrophotometry at 280 mμ and by the Lowry procedure. The complete experimental results are furnished by Table 9. For clarity in presentation these results have been represented as a series of bar graphs, which will be used in the subsequent discussion.

The distribution of recovered protein as measured by absorbancy at 280 mμ in the elution diagrams of pancreatic juice is illustrated in Figure 12. 89-100 percent of the protein applied to the columns was recovered in these experiments. Because the anionic proteins were not completely resolved by the chromatographic procedures, it was necessary to divide the DEAE cellulose elution diagrams into six regions denoted by the numbers under the elution profile. The amount of protein recovered in these regions and in the peaks on the IRC-50 elution diagrams is represented by the bars as percent of recovered protein. Experiment numbers appear directly under the bars. It will be seen that the agreement between individual experiments is satisfactory. The juice samples analyzed were obtained over a period of several weeks from a single animal, and it is therefore evident that the protein composition remained essentially invariant.

It was necessary to compare the protein compositions of zymogen granule and juice preparations on the basis of determinations by the Lowry method. Figure 13 shows a replot of Figure 3-A with additional results obtained by measurements at 280 mμ. A plot of the ratio of the absorbancies of the effluent fractions measured at 280 and 260 mμ is also included. Particularly evident deviations from the average ratio are to be seen at tubes 105, 135 and 185. Comparison of the results obtained by direct spectrophotometry and the Lowry method show that these regions exhibit artifactual peaks (cf. arrow) on the curve obtained by measurements at 280 mμ. These peaks are most probably occasioned by the presence of nucleotide material in the zymogen granule extract (cf. Table 7).

The results in Figure 14 are self-explanatory and demonstrate for the preparations analyzed a satisfactory coincidence of the relative distribution of the proteins as measured by the Lowry procedure.

Fig. 12. Relative distribution of recovered protein in pancreatic juice expressed in terms of absorbancy measurements at 280 mμ. The chromatographic patterns have been divided into nine separate regions: 1, trypsinogen; 2, chymotrypsinogen A; 3, ribonuclease; 4, amylase, and other proteins moving in the region after the emergence of the cationic fraction and extending to 0.055 M potassium phosphate; 5, chymotrypsinogen B, procarboxypeptidase B, and unidentified protein(s); 6, deoxyribonuclease, lipase and unidentified protein(s); 7, procarboxypeptidase A'; 8, procarboxypeptidase A; 9, trailing zone containing procarboxypeptidase A. Individual experiment numbers given below bars permit correlation with Table 9.

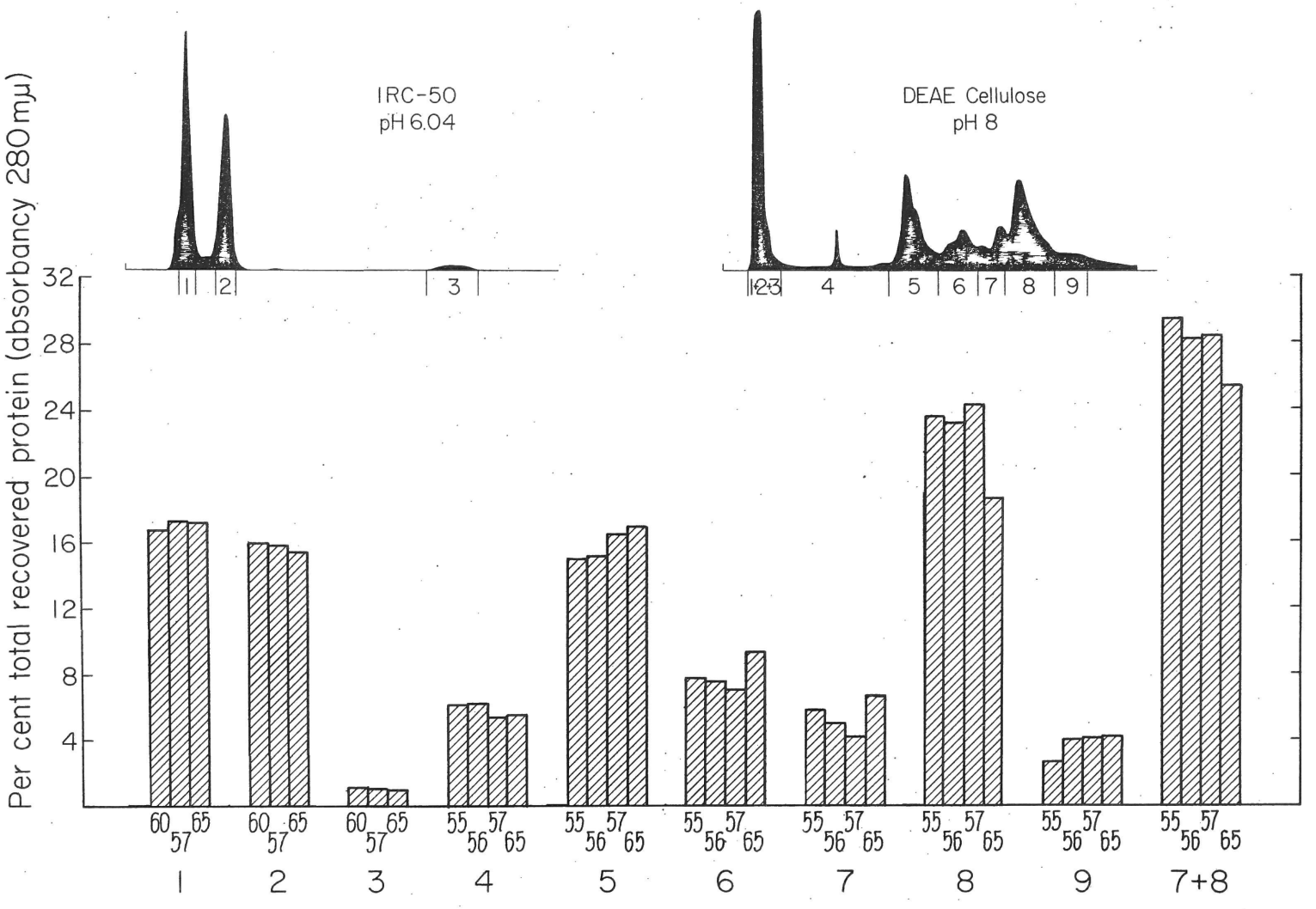

IRC-50
pH 6.04
DEAE Cellulose
pH 8
1
2
3
1
2
3
4
5
6
7
8
9
Per cent total recovered protein (absorbancy 280mμ)
32
28
24
20
16
12
8
4
60 57 65
55 56 57 65
1
2
3
4
5
6
7
8
9
7+8

Fig. 13. Chromatography of zymogen granule extract. Absorbancy measurements at 280 mμ and results of determinations by the Lowry method on the effluent of chromatogram presented in Fig. 3.

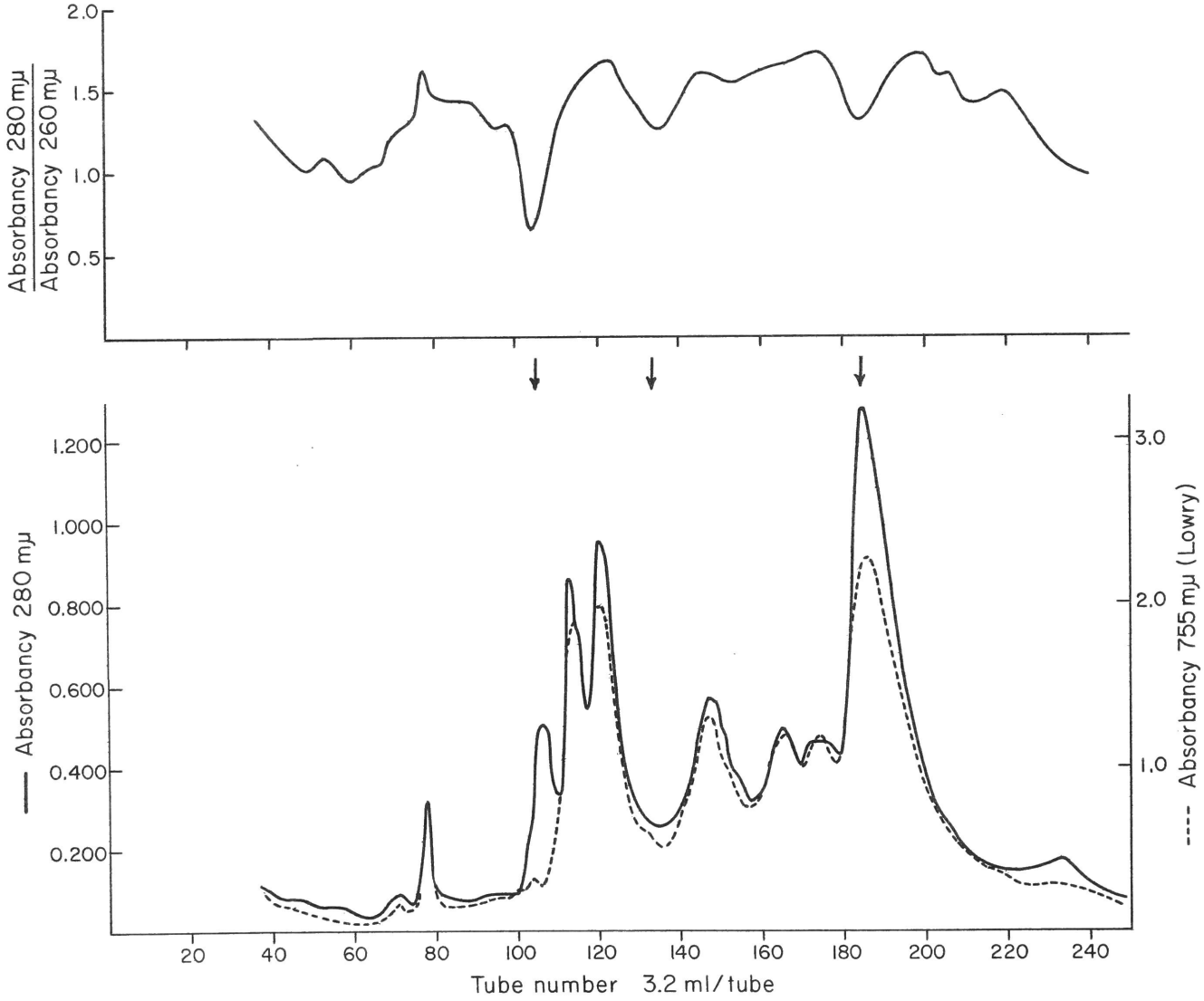

Absorbancy 280mμ / Absorbancy 260mμ
2.0
1.5
1.0
0.5
Absorbancy 280 mμ
1.200
1.000
0.800
0.600
0.400
0.200
Absorbancy 755 mμ (Lowry)
3.0
2.0
1.0
20
40
60
80
100
120
140
160
180
200
220
240
Tube number 3.2 ml/tube

Fig. 14. Relative distribution of recovered protein in zymogen granules and pancreatic juice expressed in terms of protein determinations by the Lowry method.

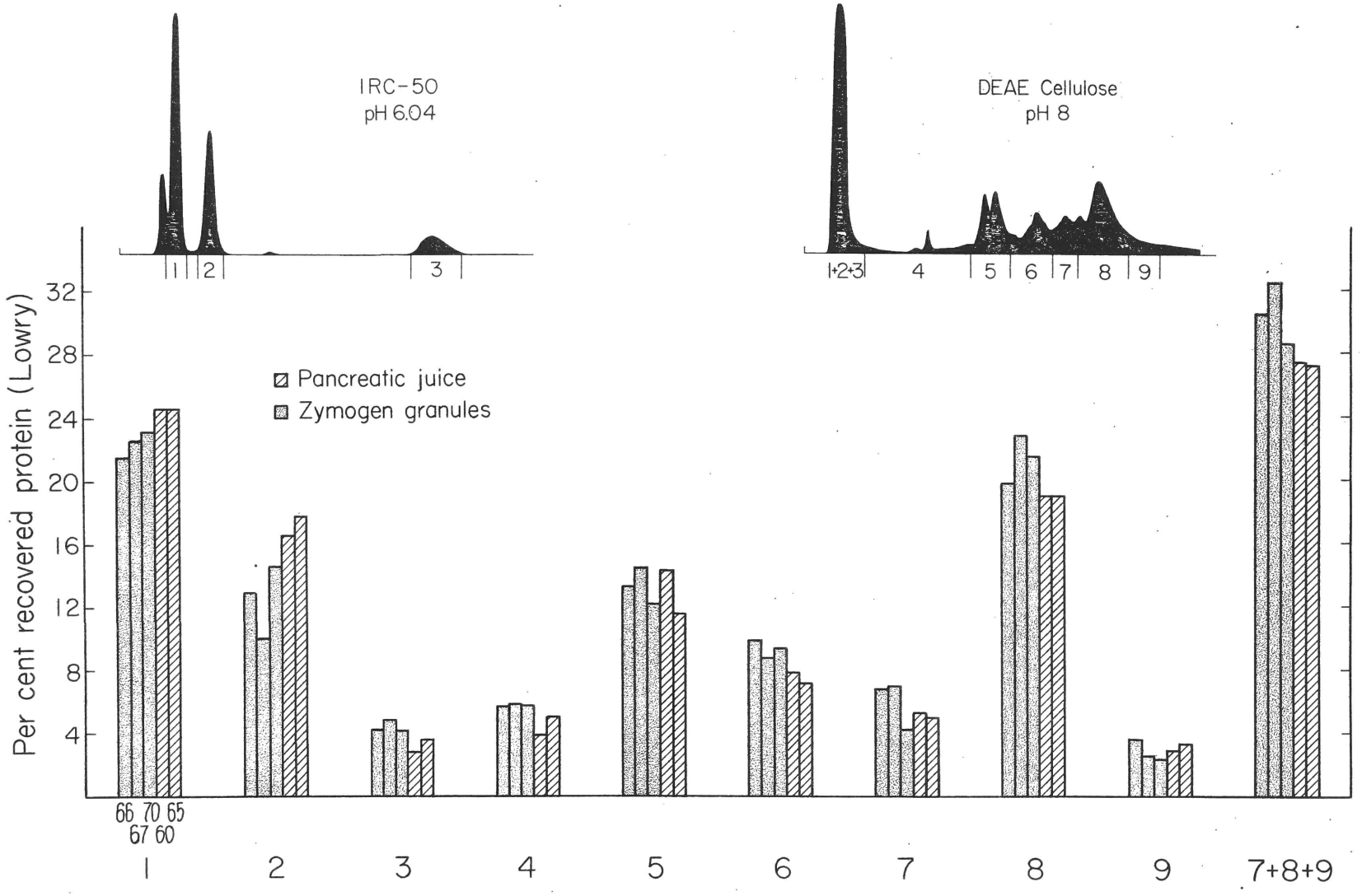

IRC-50
pH 6.04
1
2
3
DEAE Cellulose
pH 8
1+2+3
4
5
6
7
8
9
Per cent recovered protein (Lowry)
32
28
24
20
16
12
8
4
Pancreatic juice
Zymogen granules
66 70 65
67 60
1
2
3
4
5
6
7
8
9
7+8+9

TABLE 9

DISTRIBUTION OF RECOVERED PROTEIN

The chromatographic effluent patterns have been divided into nine regions. The values given are as per cent of recovered protein determined by absorbancy at 280 mμ (B) or by the procedure of Lowry, absorbancy at 755 mμ (A). These data are presented in the form of bar graphs in Figs. 12 and 14. The secretory proteins present in these regions have been identified in the legend to Fig. 12.

A	Region Number									% recovery DEAE	% recovery IRC-50
Exp.	1	2	3	4	5	6	7	8	9	cellulose	
66-G	21.6	12.1	4.3	5.7	13.3	9.9	6.9	19.9	3.6	85	100
67-G	22.5	10.0	4.8	5.9	14.4	8.9	7.0	22.9	2.6	86	103
70-G	23.2	14.6	4.2	5.8	12.1	9.4	4.4	21.5	2.4	80	109
60-J	24.6	16.7	3.2	3.7	14.2	8.0	5.4	19.0	2.9	-	104
65-J	24.6	18.1	3.6	4.9	12.8	7.2	5.0	19.1	3.3	-	103
B	Region Number									% recovery DEAE	% recovery IRC-50
Exp.	1	2	3	4	5	6	7	8	9	cellulose	
55-J				6.2	15.1	7.8	5.9	23.6	2.7	94	-
56-J				6.3	15.3	7.6	5.0	23.1	4.0	100	-
57-J	17.4	15.8	1.2	5.4	16.5	7.1	4.1	24.3	4.2	89	98
60-J	16.8	16.0	1.2							(90)	95
65-J	17.4	15.5	1.1	5.7	17.1	9.5	6.7	18.5	4.3	93	98

c. Quantitative Determination of Enzyme Activity

(1) Relative Proportions of Enzymes

The total number of enzyme activity units measured for the five proteolytic enzymes after activation, and ribonuclease A has been tabulated in column I of Table 10. The results in Table 10 have been used to derive the bar graph presented in Figure 15. In experiment 66, for instance, the potential chymotrypsinogen B activity (as units measured from the chromatogram) was used as a quotient for the potential procarboxypeptidase A and B activity to afford relative quantities in terms of ratios of enzyme units. The quantity of chymotrypsinogen B was set at 100%. The average value of these ratios (units procarboxypeptidase A)/(units chymotrypsinogen B) and (units procarboxypeptidase B)/(units chymotrypsinogen B)was calculated in all the granule preparations (experiments 66, 67, 70 and 71). Two proportionality factors were derived to make the calculated average values of these ratios for procarboxypeptidase A and procarboxypeptidase B equal to 100%. Thus the bars in Figure 15 for the granule preparations demonstrate the variation of the values of the individual ratios from the average, but does not show the magnitude of the ratio.

In dealing with the results obtained with pancreatic juice, on the other hand, the ratios were calculated in the same way for individual experiments, but in deriving the columns for procarboxypeptidase A and procarboxypeptidase B the propotionality factors found for the average of the granule preparations were used instead of the factors determined from the experiments with the juice preparations themselves. Under these circumstances the coincidence of the heavy lines (the average values) in Figure 15 demonstrates a more critical criterion for the evaluation of the extent to which the relative proportions of the proteins in the samples from the two sources are identical.

Figure 16 (black bars) demonstrates a similar comparison for the cationic proteins, which have been related to recovered chymotrypsinogen A. In view of the complete chromatographic resolution attained, coupled with the well established spectrophotometric and enzymatic characteristics of these proteins, a more extensive comparison may be achieved.

Fig. 15. Comparison of the relative distribution of anionic components of zymogen granule and pancreatic juice extracts from activity measurements. The results of Table 10 have been recalculated in this bar graph. The relative proportions of the zymogens have been derived solely from the results of enzyme activity measurements following activation. The quantities are given in arbitrary units and calculated in a form designed to facilitate direct comparison of the relative proportions of the proteins. The calculations are described in the text, as is the significance of the heavy lines over the columns for procarboxypeptidase A and procarboxypeptidase B.

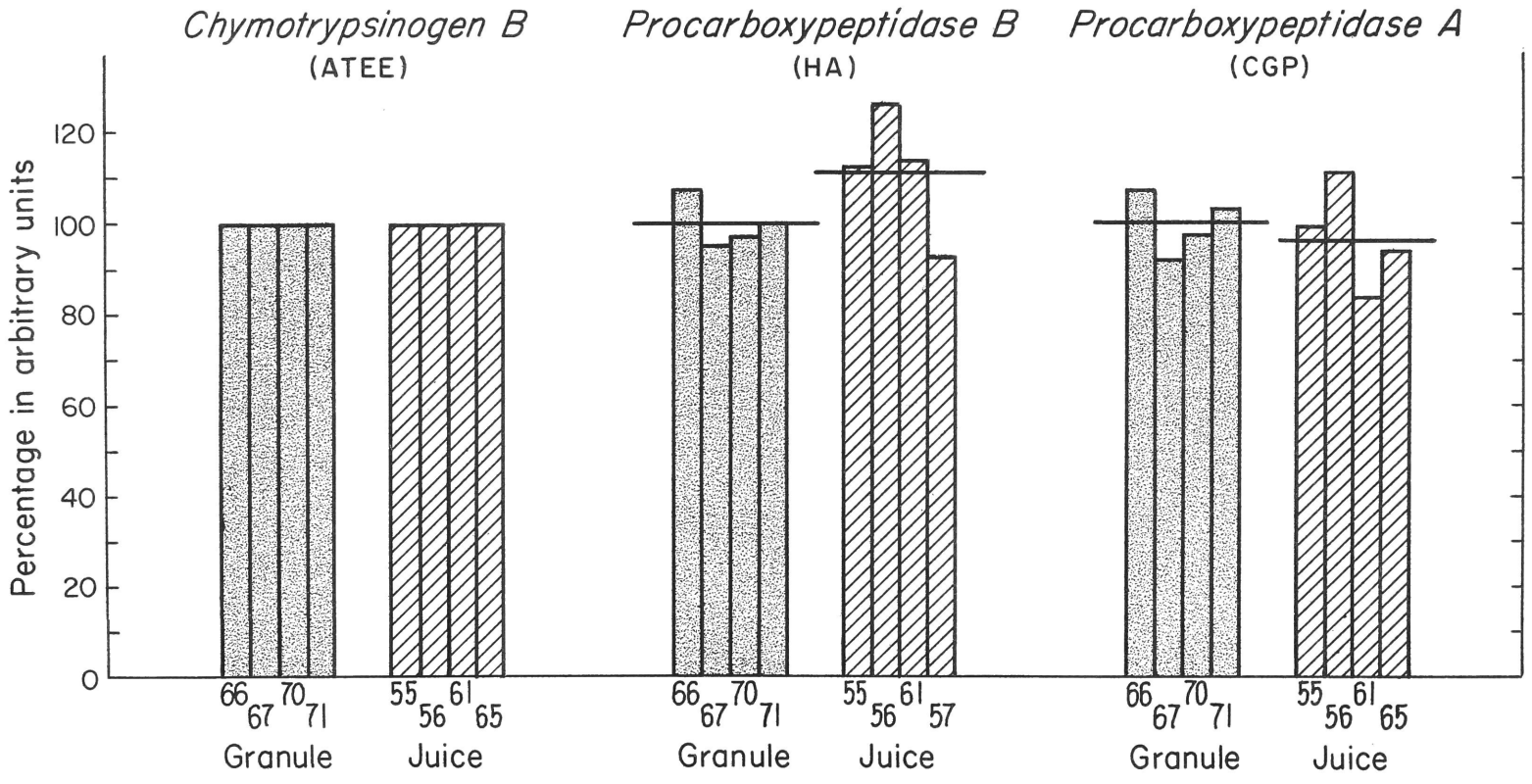

Chymotrypsinogen B
(ATEE)
Procarboxypeptidase B
(HA)
Procarboxypeptidase A
(CGP)
Percentage in arbitrary units
0
20
40
60
80
100
120
66 67 70 71
55 56 61 65
66 67 70 71
55 56 61 57
66 67 70 71
55 56 61 65
Granule
Juice
Granule
Juice
Granule
Juice

Fig. 16. Comparison of relative distribution of cationic components in zymogen granule and pancreatic juice extracts on a weight basis. Two series of experiments are summarized. The black bars represent determinations by activity (cf. Table 10); the grey bars represent the results of spetrophotometric measurements of the areas under the peaks on the effluent curves of IRC-50 chromatograms. To convert activity measurements and spetrophotometric determinations to a weight basis, the specific activities and extinction coefficients given in Table 8 were used. The values for chymotrypsinogen A have been arbitrarily set at 1.0.

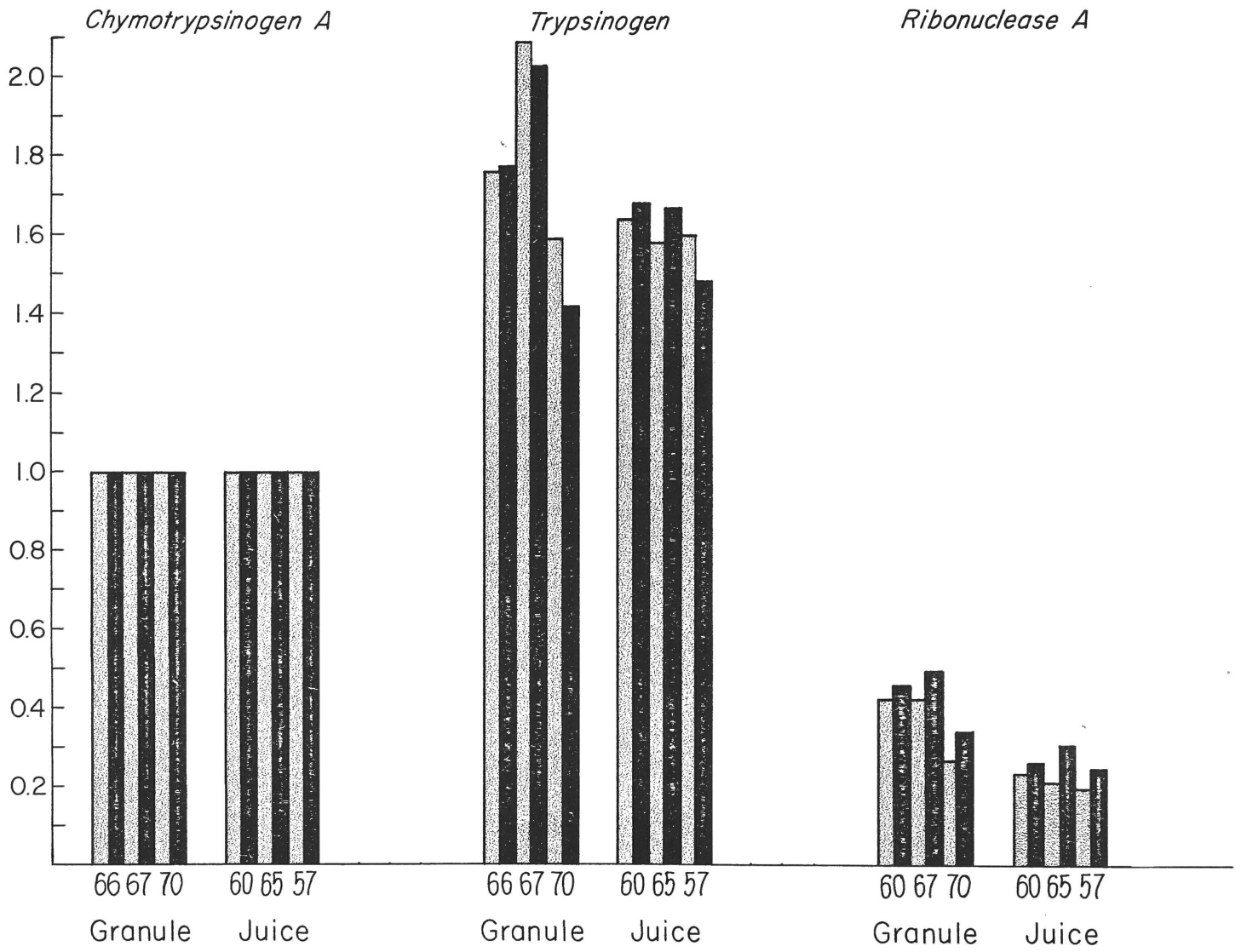
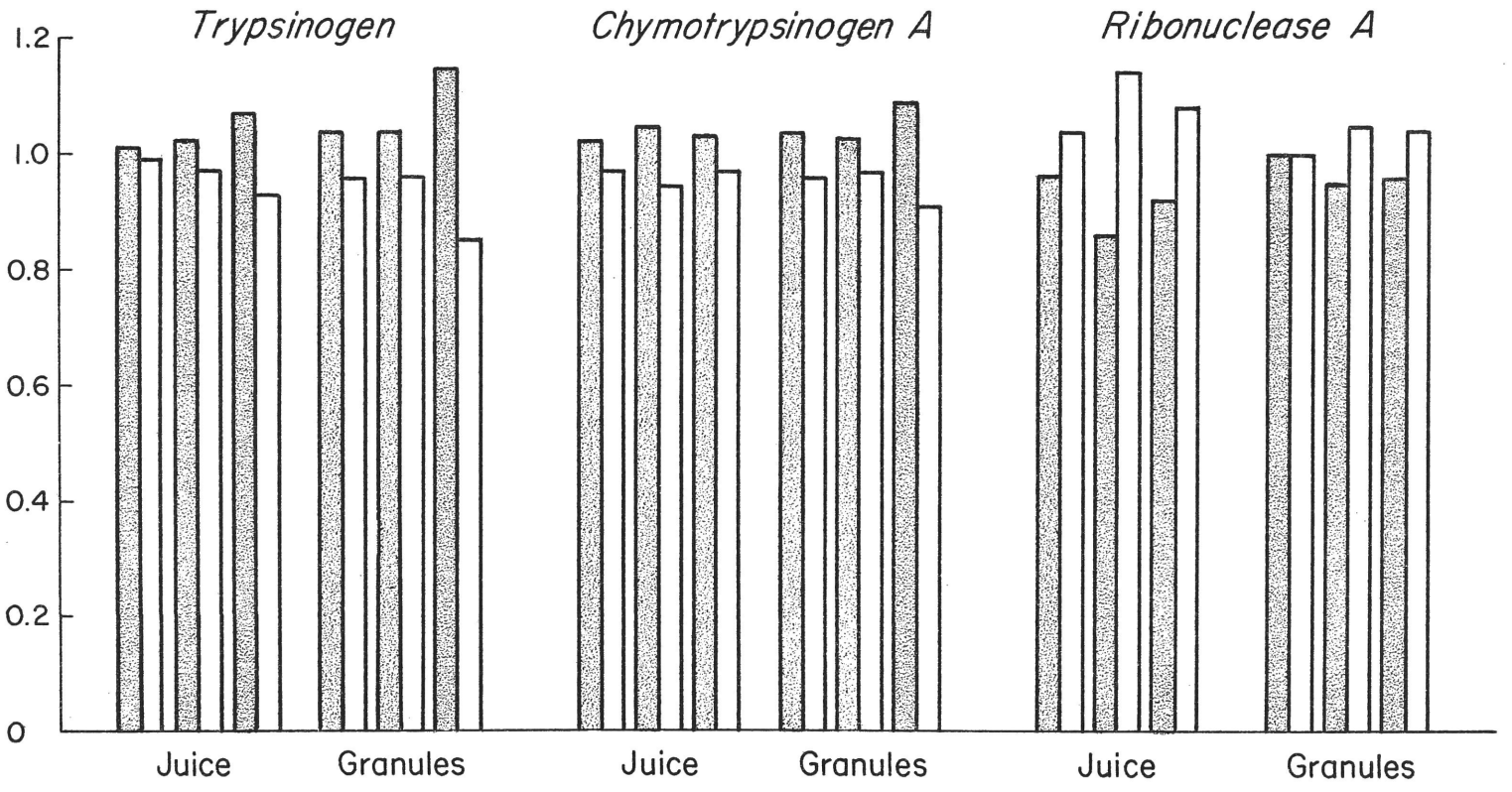

Chymotrypsinogen A
Trypsinogen
Ribonuclease A
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
66 67 70
60 65 57
66 67 70
60 65 57
60 67 70
60 65 57
Granule
Juice
Granule
Juice
Granule
Juice

Fig. 17. Comparison of spectrophotometric and enzymatic quantitation of principal components of cationic fraction of zymogen granules and pancreatic juice. The values were obtained by application of the specific activities and extinction coefficients shown in Table 8. The paired values represent spectrophotometric (grey) and activity (open) determinations on individual chromatograms. The results obtained by both procedures were used to calculate an average value for each component; this average has been set equal to 1.0 throughout.


Trypsinogen
Chymotrypsinogen A
Ribonuclease A
1.2
1.0
0.8
0.6
0.4
0.2
0
Juice
Granules
Juice
Granules
Juice
Granules

The best (highest) specific activities (cf. Table 8) determined on the center 50-60% of the peaks have been applied to the total recovered activity in order to express the result on a weight basis. Because one set of specific activities was used for all samples the relative distribution, but not the weight distribution shown in Figure 16 (black bars) is independent of the specific activity coefficients used. It was possible to check this result for the cationic proteins by determining the peak areas in terms of absorbancy at 280 mμ. The relative proportions of cationic proteins determined by the spectrophotometric measurements are presented as gray bars in Figure 16, and on this basis there is good agreement between the juice and granule preparations. Again, the relative proportions but not the weight proportions are independent of the extinction coefficients. The reliability of the result depends on the complete chromatographic resolution of the proteins, and the fact that they have been obtained previously in sufficiently pure form that their extinction coefficients are known. These requirements appear to have been fulfilled for the cationic proteins, but, as demonstrated previously, the anionic proteins have not been resolved, nor are all the extinction coefficients known.

A direct comparison of the activity and spectrophotometric procedures is illustrated in Figure 17. In this case the values are not internally related to chymotrypsinogen A, but to the weight of protein determined in eack peak for each experiment. The average value has been set at 1.0 and the results obtained by spectrophotometric determinations are represented by gray bars whereas the enzyme activity measurements are represented by the open bars.

(2) Specific Activity

Figures 15 and 16 have illustrated that the relative proportions of the proteins determined by quantitative enzyme assays are the same in zymogen granule extracts and pancreatic juice. However, the results have been restricted to a description of concentrations relative to chymotrypsinogen A and chymotrypsinogen B.

Concentration may be expressed in other ways besides recovered enzyme activity. For example, it may be expressed relative to weight or volume of water. The water content of granules is unknown but

would be important for the purpose of comparison with other vacuoles containing protein, such as red blood cells. A more meaningful way to represent concentration of enzyme activity for the comparison of juice and granules is in terms of the total protein in the mixture. For example, if the granule preparation contained an inert protein (or the juice contained a mucoprotein secreted by the ducts but not present in the granules), the relative proportions of zymogens would be the same but their dilution by inert protein would not show in the calculation (based on enzymatic activity) illustrated by Figures 15-16*. The concentration of enzyme activity may be conveniently expressed as a quotient, with the amount of total protein in the denominator. This quotient (specific activity, activity per unit of protein) can be determined at each stage of purification, for each component, for each mixture. If the composition and the concentrations in both mixtures were identical at the outset, the determined specific activities should be identical at each stage of purification.

The data for protein concentration determined by the procedure of Lowry and represented as absorbancy at 755 mμ are presented in columns II, III and IV of Table 10, and the quotients obtained by dividing the enzyme activity in column I by protein concentrations are also tabulated in Table 10. Figure 18 illustrates the purification progression for procarboxypeptidase A and chymotrypsinogen A in granule preparations (gray bars) and pancreatic juice (striped bars). The specific activities are identical to each other at each step within the errors inherent in the methods used. This argues in favor of identical concentrations of these zymogens. The complete graphical representation of the specific activities for all the individual enzymes at the level of total protein are presented in Figure 19 (column V, Table 10); and at the level of protein in the fraction, anionic or cationic, in Figure 20 (column VI, Table 10). Column VII of the same table contains specific activities relative to protein present in each chromatographic peak. The lack of separation of chymotrypsinogen B and procarboxypeptidase B, and the existence of multiple forms of procarboxypeptidase A make the absolute values of these specific activities meaningless because more than one protein is present. However, the consistency of the values indicates identity between juice and granules.

* If different inert proteins were present in both samples to the same extent they would not be distinguished on the basis of enzyme concentration relative to the protein, but could be recognized under favorable circumstances in the chromatographic effluent patterns.

TABLE 10

SPECIFIC ACTIVITY OF SECRETORY PROTEIN

DURING CHROMATOGRAPHIC FRACTIONATION

The total number of enzyme activity units recovered for each experiment is tabulated in Column I. The recovered protein determined by the procedure of Lowry et al (1951), given in absorbancy units ($A_{755m\mu}$), is recorded for total protein (Column II), fraction protein, total cationic or total anionic protein (Column III), and protein in chromatographic region (Column IV). The specific activities for each level of purification for zymogen granule extracts (G) and pancreatic juice (J) are presented in Columns V, VI and VII.

		I	II	III	IV	V	VI	VII
ENZYME	SAMPLE	ENZYME ACTIVITY UNITS*	A_{755} TOTAL	A_{755} CATIONIC	A_{755} CHROMATOGRAPHY REGION**	S.A. I/II	S.A. I/III	S.A. I/IV
Tg.	66-G	5700	750	290	160[1]	7.6	20	36
	67-G	3400	450	170	100	7.5	20	34
	70-G	12200	1870	800	430	6.5	15	28
	65-J	10100	1150	550	280	8.8	18	36
	60-J	13800	1500	670	370	9.2	20	37
	57-J	10400	-	570	300	-	18	35
Chg. A	66-G	6000	750	290	91[2]	8.0	21	66
	67-G	3150	450	170	45	7.0	19	70
	70-G	16200	1870	800	270	8.7	20	60
	65-J	11400	1150	550	210	9.9	21	55
	60-J	15500	1500	670	250	10.4	23	62
	57-J	13200	-	570	200	-	23	66
RNAase	66-G	510	750	290	31[3]	0.68	1.8	17
	67-G	290	450	170	22	0.65	1.7	13
	70-G	1000	1870	800	79	0.53	1.3	13
	65-J	650	1150	550	42	0.57	1.2	15
	60-J	770	1500	670	48	0.51	1.2	16
	57-J	600	-	570	44	-	1.1	14

TABLE 10 Continued

SPECIFIC ACTIVITY OF SECRETORY PROTEIN

DURING CHROMATOGRAPHIC FRACTIONATION

		I	II	III	IV	V	VI	VII
ENZYME	SAMPLE	ENZYME ACTIVITY UNITS*	A_{755} TOTAL	A_{755} ANIONIC	A_{755} CHROMATOGRAPHY REGION**	S.A. I/II	S.A. I/III	S.A. I/IV
Chg.B	66-G	2900	750	460	100^{5}	3.9	6.3	29
	67-G	2100	450	280	65	4.6	7.4	32
	70-G	6900	1870	1070	227	3.7	6.5	30
	61-J	6300	-	747	215	-	8.4	29
	65-J	4480	1150	600	148	3.9	7.5	30
Pro.B	66-G	310	750	460	100^{5}	0.42	0.68	3.1
	67-G	200	450	280	65	0.44	0.71	3.1
	70-G	670	1870	1070	227	0.36	0.63	3.0
	61-J	590	-	747	215	-	0.79	2.8
	65-J	-	1150	600	148	-	-	-
Pro.A	66-G	40500	750	460	230^{7+8+9}	54	88	176
	67-G	25200	450	280	150	56	89	171
	70-G	87000	1870	1070	530	47	82	165
	61-J	69100	-	750	350	-	92	200
	65-J	55100	1150	600	320	48	92	173

* Activity Units

Trypsinogen:	μmoles/min.	TAME hydrolysis
Chymotrypsinogen A:	μmoles/min.	ATEE hydrolysis
Ribonuclease A:	units	Yeast RNA hydrolysis
Chymotrypsinogen B:	μmoles/min.	ATEE hydrolysis
Procarboxypeptidase B:	μmoles/min.	HA hydrolysis
Procarboxypeptidase A:	μmoles/min.	CGP hydrolysis

**Superscript number refers chromatographic region as defined in Figure 14.

Fig. 18. Average potential specific activity of procarboxypeptidase A and chymotrypsinogen A at successive stages of fractionation from zymogen granules (gray) and pancreatic juice (striped). The specific activities are expressed in arbitrary units, with the maximum specific activity measured at the stage of chromatographic isolation set at 100. The average values used are calculated from Table 10.

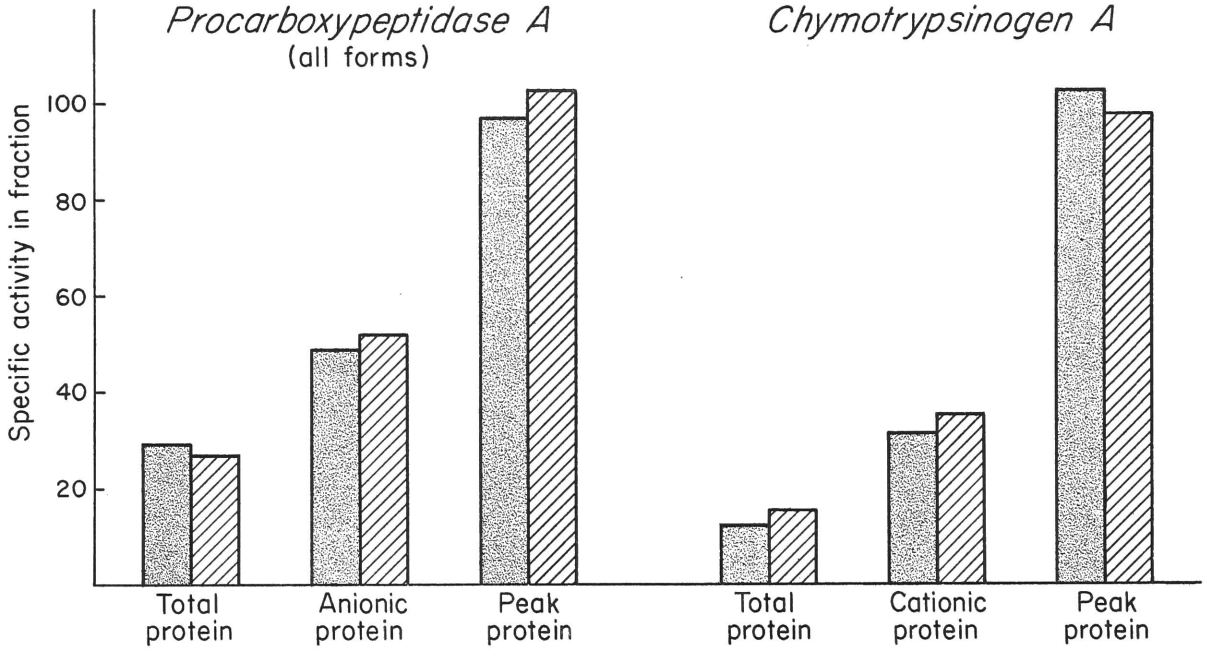

Procarboxypeptidase A
(all forms)
Chymotrypsinogen A
Specific activity in fraction
100
80
60
40
20
Total protein
Anionic protein
Peak protein
Total protein
Cationic protein
Peak protein

Fig. 19. Potential specific activity related to total recovered protein (Lowry) obtained in individual experiments with extracts of zymogen granules and bovine pancreatic juice. Average granule value has been set at 100. Differences from the average granule value in the determinations for pancreatic juice components (striped) may be read directly. The values are taken from Table 10, column V.

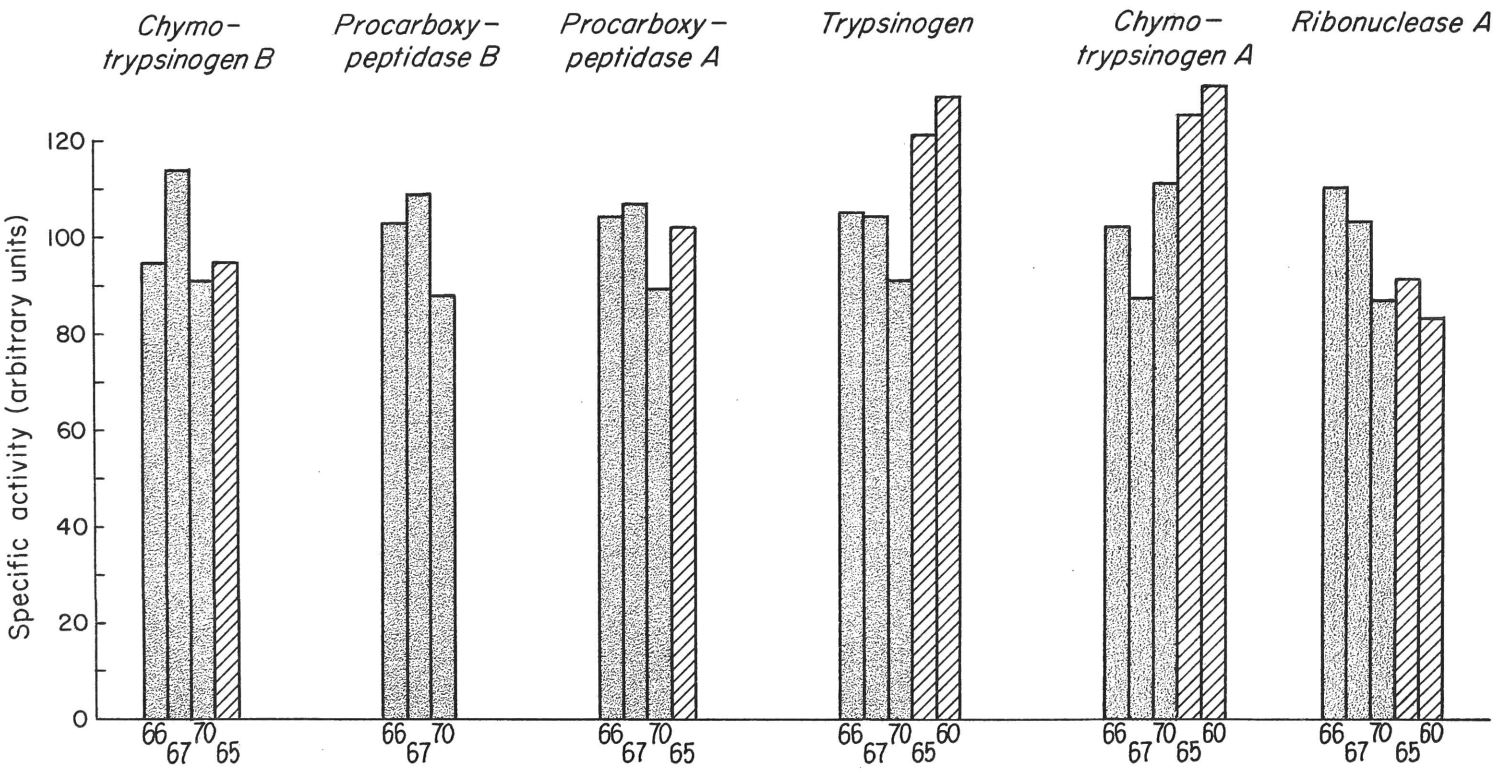
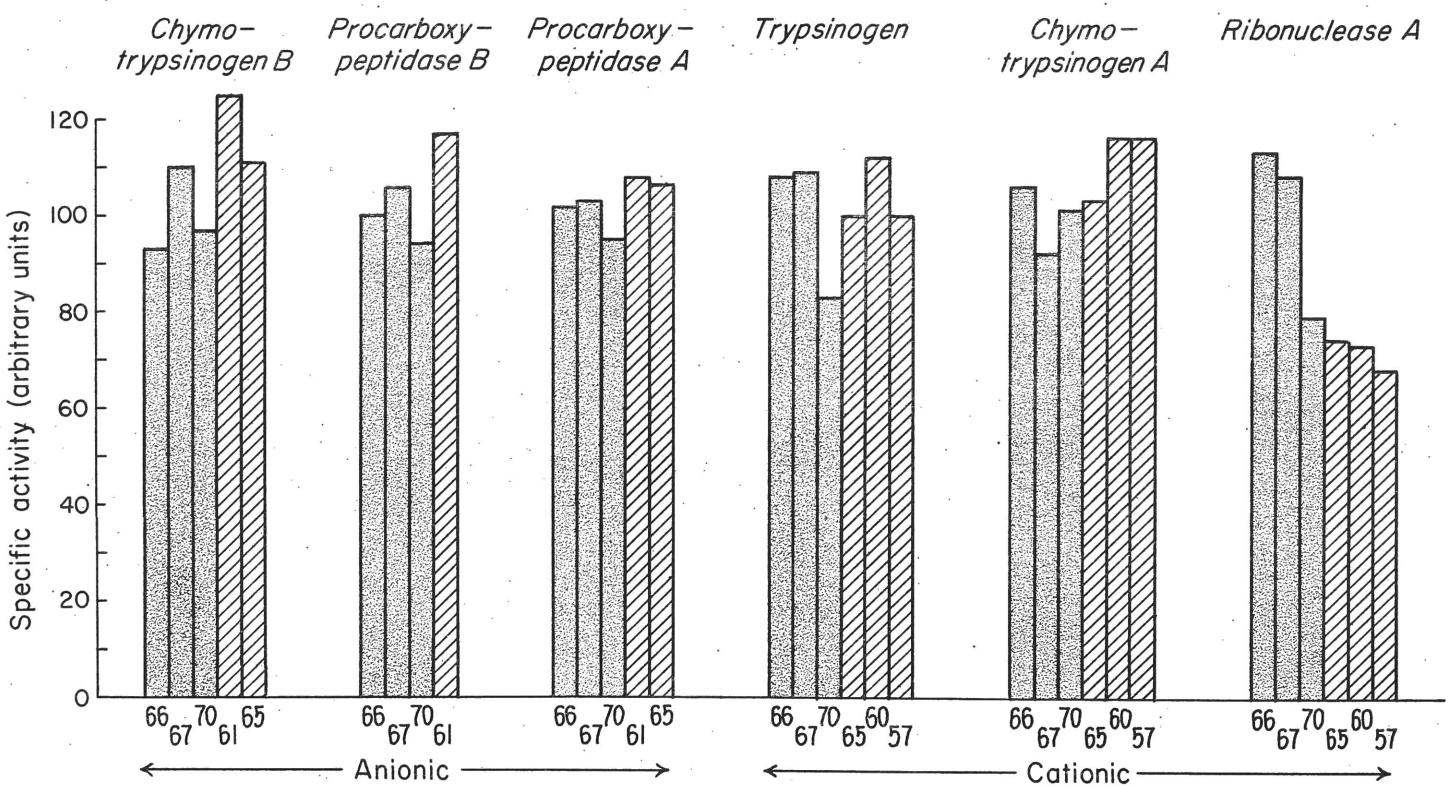

Chymo-trypsinogen B
Procarboxy-peptidase B
Procarboxy-peptidase A
Trypsinogen
Chymo-trypsinogen A
Ribonuclease A
Specific activity (arbitrary units)
0
20
40
60
80
100
120
66 67 70 65
66 67 70
66 67 70 65
66 67 70 65 60
66 67 70 65 60
66 67 70 65 60

Fig. 20. Potential specific activity related to protein (Lowry) recovered in either the anionic or cationic fraction obtained in individual experiments with extracts of bovine zymogen granules and pancreatic juice. Average granule value has been set at 100. Differences from the average granule value in the determination for pancreatic juice components (striped) may be read directly. The values are taken from Table 10, column VI.


Chymo–trypsinogen B
Procarboxy–peptidase B
Procarboxy–peptidase A
Trypsinogen
Chymo–trypsinogen A
Ribonuclease A
Specific activity (arbitrary units)
0
20
40
60
80
100
120
66 67 70 61 65
66 67 70 61
66 67 70 61 65
66 67 70 65 60 57
66 67 70 65 60 57
66 67 70 65 60 57
Anionic
Cationic

d. Composition of the Pancreatic Secretion

The spectrophotometric measurements at 280 mμ demonstrated that virtually complete recovery (average 95%) of the material absorbing at this wave length in pancreatic juice extracts was achieved. This makes it likely that complete recovery of protein is attained in the fractionation scheme for pancreatic juice extracts. The difficulties brought on by the presence of nucleotide-like material in the granule extracts necessitated the use of the Lowry procedure for the determination of proteins. The recovery of protein on the basis of determinations by this method from the granule extracts was on the average 85%. Thus it is possible that approximately 10% of the protein in the granule extracts was not recovered. The electron micrographs (cf. Section II) of the granule preparation before lysis and solubilization of the protein revealed the presence of contamination that could have contributed proteins not secreted in the juice. However, since the results presented in Tables 9 and 10 and Figures 12-20 demonstrated the equivalence of the granules and pancreatic juice extracts in terms of effluent pattern, relative amounts of enzyme or potential enzyme activity, and specific activity, the conclusion may be reached that the proteins accounted for in the zymogen granule extracts are present in concentrations identical to those found in the pancreatic juice.

As a final exercise a table of composition (Table 11) may be constructed on the basis of the spectrophotometric results. This table defines the protein composition of the pancreatic juice in terms of the analytical procedures most convenient for its determination. To convert such spectrophotometric results to a weight accounting would require complete resolution of all proteins and knowledge of the extinction coefficients or color yields in the Lowry procedure of all the proteins present. The conversion to a weight basis has been attempted in Table 11 with the assumption that the most prevalent proteins will determine the effective extinction coefficient of the mixture. The extinction coefficients used by Keller et al. for the total mixture and for individual proteins have been used for this purpose in order to facilitate comparison of the present results with those in Table 4.

It should however be re-emphasized that the use of these extinction coefficients in regions of the chromatograms where the resolution was not

complete is fraught with considerable hazards. For example, region 5 would appear on this basis to constitute 16% of the total protein in the form of chymotrypsinogen B and procarboxypeptidase B. In actuality the activation measurements demonstrate that these proteins represent at the most 12% of the total protein when a reasonable assumption of their potential specific activity is made.

TABLE 11

COMPOSITION OF PANCREATIC SECRETION

Based on Spectrophotometric Determinations

Region	Protein	% A 280 Recovered	Extinction Coefficient	Weight Percent	Reliable Weight Percent
1	Trypsinogen	17.4	13.9[b]	22.5	23
2	Chymotrypsinogen A	15.7	20.0[b]	14.2	14
3	Ribonuclease A	1.2	7.0[b]	3.1	3
4	Amylase[a]	5.9	19.2[c]	5.5	-
5	Chymotrypsinogen B[a] Procarboxypeptidase B	15.5	18[b]	15.5	(6)[d] (6)[d]
6	Unknown	8.0	19.2[c]	7.5	
7	Procarboxypeptidase A'	5.3			
8	Procarboxypeptidase A	22.4	19[b,c]	30.0	29[e]
9	Procarboxypeptidase A[a]	3.8			
	Mixture		18[b]		
	Total	95			69(81)

[a] Other proteins present in significant amounts

[b] Determined on isolated highly purified proteins (cf. Keller et al., 1958b)

[c] Estimated values (P. J. Keller, personal communication)

[d] From activity measurements and assumed potential specific activities 450 μmoles ATEE hydrolysis/min./mg. chymotrypsinogen B and 46 μmoles HA hydrolysis/min./mg. procarboxypeptidase B.

[e] Corrected for soybean trypsin inhibitor

SECTION V

DISCUSSION

The zymogen granule fraction prepared in the course of this work from bovine pancreas is of sufficient purity to permit the isolation of the constitutive proteins and a study of their detailed chemical and enzymatic properties. The quality of the preparation has been ascertained in morphological and chemical terms.

On the basis of the results obtained by the Schneider partition, our zymogen granule fraction contains less than 1% nucleic acid, about 5% phospholipid and about 94% protein (Table 7). Comparison with preparations described in the literature indicates that this fraction is more homogeneous than those previously obtained by Van Lancker and Holtzer (1959) from mouse and by Siekevitz and Palade (1958a) from guinea pig pancreas. In these cases a complete fractionation of the gland was attempted and apparently purity of the zymogen granule fraction was sacrificed in order to obtain complete recovery. In the present work a more homogeneous preparation was possible because of the large amounts of glandular tissue available as a starting material, and because high yield was of secondary importance. In this respect our conditions compare with those of Hokin (1955) who isolated from dog pancreas a zymogen granule fraction the gross chemistry of which is similar to our preparation.

Systematic electron microscopic examination of our zymogen granule fractions has shown that they are contaminated by small but clearly recognizable amounts of microsomes, mitochondria and chromatin fragments which could account for the nucleic acid and part of the phospholipid content of the fractions. A perusal of the microscopic evidence shown by Siekevitz and Palade shows that in terms of morphologically recognizable contaminants our fractions compare favorably with their best preparations obtained by centrifugation in a density gradient. The zymogen granule fraction isolated from bovine pancreas by Keller and Cohen (1961) was not defined in terms of morphology or gross chemistry and as such cannot be compared with ours. It may be assumed, though, that it was as heterogeneous as Siekevitz and Palade's preparation since it was isolated by using their procedures. It appears therefore that our preparation compares favorably with those

described in the literature, with the possible exception of Hokin's, and that it has a limited (tolerable) amount of contamination by other cellular components. As such it can be used for a meaningful comparative study of zymogen granule proteins and pancreatic juice.

The membrane fraction, isolated by centrifugation after the lysis of zymogen granules and solubilization of 95% of the protein content, represents a potentially significant starting material for future biochemical and morphological studies of cellular membranes. Most of these smooth membranes represent in all probability the membranes seen in electron micrographs around isolated or intracellular zymogen granules. A similar membrane fraction was recently derived from amylase containing parotic granules by Schramm and Danon (1961) who demonstrated the membranes in electron micrographs of shadowed preparations.

Some of the biochemical events associated with the secretory process, which include the filling of the Golgi vacuoles and membrane relocation connected with the process of extrusion from the cell, may be approached by means of this preparation. As has been pointed out in the introduction, recent evidence suggests that the zymogen granules are formed by the concentration of secretory protein within the smooth membrane limited vacuoles of the Golgi zone. Moreover, the extrusion of the contents of the zymogen granules after fusion of the granule membrane to the plasma membrane at the apical pole of the cell suggests that the three types of membranes involved (Golgi - zymogen granule - and plasma membrane) are related to each other. The membrane fraction obtained may prove useful in the localization of the increased phospholipid turnover shown by Hokin to occur concomitantly with increased protein secretion (cf. Redman and Hokin, 1959).

The main object of this study has been to compare the protein composition of zymogen granules and pancreatic juice. This has been achieved using techniques for chromatography, enzyme activation, and assay already in the literature. The quantitative data presented indicated an exact correspondence between proteins extracted from the granules and those present in the pancreatic juice. The data obtained by integration of the areas under the protein peaks in the chromatograms has been supplemented by independent and more reliable quantitative enzymatic determinations. For reasons already discussed, both methods must be used together to prove identity in the absence of complete resolution. The results of Keller and

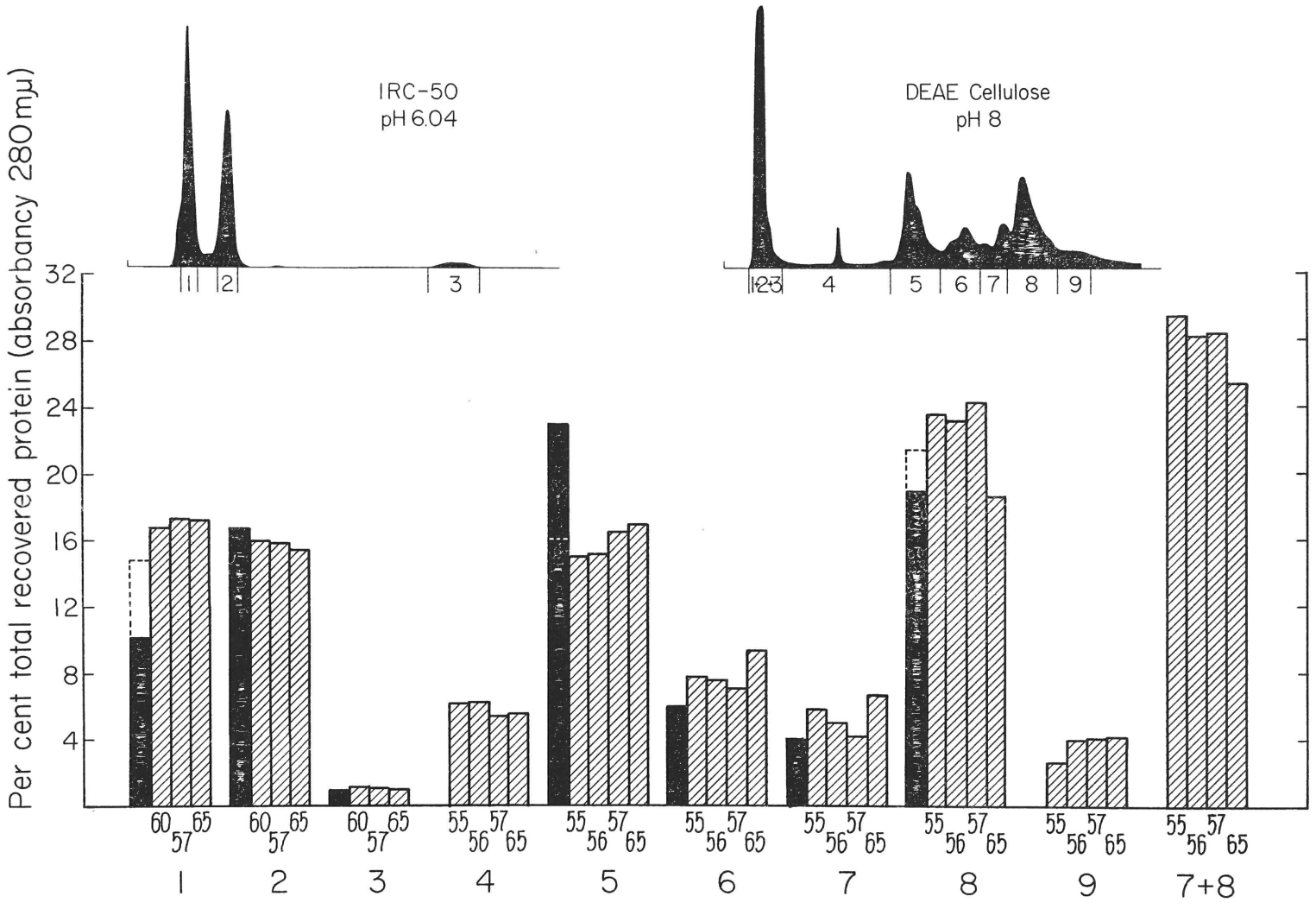
Cohen should be discussed in this context. They showed only similarities in the distribution of recovered protein in the elution diagrams. The enzyme activity was qualitatively established and protein identification was based primarily on position. Our studies demonstrate by quantitative assays over the entire envelope of the eluted peaks that eight secretory enzyme or potential enzyme activities, which chromatographed as 13 distinct chromatographic peaks, were identical in both mixtures.

The composition derived by Keller et al. from spectrophotometric measurements (cf. Fig. 21) agrees satisfactorily with that obtained in the present work with the notable and important exception of the value for trypsinogen (region 1) which was significantly smaller in the Seattle experiments. The interpretation of the discrepancies is facilitated by a second difference noted by Keller et al.; active enzyme was recognized in the procarboxypeptidase B region, a result which now may be ascribed to the presence of soybean trypsin inhibitor-trypsin complex. The area of the peaks in the procarboxypeptidase B region (region 5, Fig. 21) was about 30% greater than in the present work, demonstrating the presence of significant quantities of the complex; by contrast, the trypsinogen region in the experiments of Keller et al. was lower by approximately the amount to be expected from the excess present in the procarboxypeptidase B region.

Quantitative enzyme assay permitted the discovery of procarboxypeptidase A', a previously unidentified component; the demonstration that components previously believed to be homogeneous were in reality mixtures of proteins; and conclusive proof that ribonuclease B and other multiple forms of ribonuclease are present in pancreatic juice and zymogen granules and therefore should be considered normal constituents of the exocrine secretion.

The clear-cut demonstration of the identity in protein composition of extracts of zymogen granules and pancreatic juice provides strong evidence for the existence of a direct relationship in the secretory process between the granules and the pancreatic juice, and therefore constitutes a practically complete validation of the Heidenhain hypothesis. The completion of the present work represents the furthest point yet attained in the analysis of the secretory process by the application of analytical methods to isolates derived from pooled specimens. Further refinement of the approach described in this thesis is undoubtedly possible: the protein fractionation

Fig. 21. Relative distribution of recovered protein in pancreatic juice expressed in terms of absorbancy measurements at 280 mμ. The bar graph is the same as that presented in Fig. 12 except that the results of Keller et al. (1958b), recalculated in terms of the units used in Fig. 12, and based on the same division of the effluent patterns, have been added (black areas). If the area above the dashed line in region 5 is assumed to represent soybean trypsin inhibitor-trypsin complex, the components of that complex may be reassigned to region 1 (trypsinogen) and 8 (soybean trypsin inhibitor).


IRC-50
pH 6.04
DEAE Cellulose
pH 8
1
2
3
1+2
3
4
5
6
7
8
9
Per cent total recovered protein (absorbancy 280 mμ)
32
28
24
20
16
12
8
4
60 57 65
55 56 57 65
1
2
3
4
5
6
7
8
9
7+8

techniques employed are capable of greater resolution under appropriate conditions, the use of techniques such as gel filtration and electrophoresis has not as yet been fully explored. In time it may be anticipated that a complete description of all the proteins in the secretion will be attained. The demonstration of a direct correspondence between the granules and juice even among the minor components, partially foreshadowed in the present experiments by the analyses for ribonuclease B and multiple ribonucleases, will eventually complete this analytical approach.

In a wider sense, however, the results in this thesis will complement kinetic studies on the biosynthesis and extrusion of the secretory proteins. A combination of kinetic analysis coupled with precise information on the composition of the secretion will eventually be able to prove that the only source of the secretion is the zymogen granule. This problem remains an active area of research, particularly in view of the possibility (discussed by Redman and Hokin) that certain enzymes in the secretion (e.g. amylase) derive directly from the cytoplasmic matrix or the endoplasmic reticulum without passage through the granules. The use of protein labeled with radioisotopes and the technique described in this thesis suggest that a definitive experiment, involving the measurement of protein specific activity in the cytoplasm, the granules and the pancreatic juice, can now be contemplated to determine the extent, if any, of the non-zymogen granule shunt.

SECTION VI - BIBLIOGRAPHY

Alm, R. S., Williams, R. J. P. and Tiselius, A. (1952) Acta Chem. Scand., 6, 826.

Anfinsen, C. B. and White, F. H., Jr. (1961) in Boyer, P. D., Lardy, H. and Myrbäck, K., eds., The enzymes, ed. 2, New York, Academic Press, v. 5, p.95.

Anson, M.L. (1937) J. Gen. Physiol., 20, 663.

Babkin, B. P. (1950) Secretory mechanism of the digestive glands, ed. 2, New York, Hoeber-Harper.

Bernard, C. (1849) Arch. Gén. Méd., ser. 4, 19, 60 (Engl. trans., Med. Classics, 1938, 3, 600.)

Bernard, C. (1856) Compt. Rend. Acad. Sci., 43, Supp., 379.

Boardman, N. K. and Partridge, S. M. (1955) Biochem. J., 59, 543.

Bouchardat, A. and Sandras, C.-M.-S. (1845) Compt. Rend. Acad. Sci., 20, 1085.

Brown, J. R., Cox, D. J., Greenshilds, R. N., Walsh, K. A., Yamasaki, M., and Neurath, H. (1961) Proc. Nat. Acad. Sci., 47, 1554.

Butler, H. C., Brinkman, D. C. and Klavano, P. A. (1960) Am. J. Vet. Res., 21, 205.

Byrne, G. M., Phinney, J. I., Schachter, M. and Young, E. (1951) J. Biol. Chem., 192, 683.

Caro, L. G. and Palade, G. E. (1961) Compt. Rend. Soc. Biol., 155, 1750.

Claude, A. (1943) Biol. Symp., 10, 111.

Cole, R. D. and Kinkade, J. M. (1961) J. Biol. Chem., 236, 2443.

Craig, L. C. and King, T. P. (1955) J. Am. Chem. Soc., 77, 6620.

Daly, M. M. and Mirsky, A.E. (1952) J. Gen. Physiol., 36, 243.

Davie, E. W. and Neurath, H. (1955) J. Biol. Chem., 212, 515.

Davis, F. F. and Allen, F. W. (1956) Biochim. et Biophys. Acta, 21, 14.

de Duve, C. and Berthet, J. (1954) Internat. Rev. Cytol., 3, 225.

Delcourt, A. and Delcourt, R. (1953) Compt. Rend. Soc. Biol., 147, 1104.

Desnuelle, P. (1961) Adv. Enzymol., 23, 129.

Desnuelle, P. and Rovery, M. (1961) Adv. Protein Chem., 16, 139.

Dickman, S. R. and Morrill, G. A. (1959) Ann. New York Acad. Sci., 81, 585.

Dische, Z. (1930) Mikrochemie, 8, 4.

Eaker, D. L. (1962) Structural and enzymatic studies with des-lysyl forms of bovine pancreatic ribonuclease, Doctoral dissertation, The Rockefeller Institute, New York.

Fischer, E. H. and Bernfeld, P. (1948) Helvet. Chim. Acta, 31, 1831.

Fischer, E. H. and Stein, E. A. (1960) in Boyer, P. D., Lardy, H. and Myrbäck, K., eds., The enzymes, ed. 2, New York, Academic Press, v. 4, p. 313.

Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem., 66, 375.

Folk, J. E. and Gladner, J. A. (1958) J. Biol. Chem., 231, 379.

Folk, J. E. and Gladner, J. A. (1961) Biochim. et Biophys. Acta, 48, 139.

Folk, J. E., Piez, K. A., Carroll, W. R. and Gladner, J. A. (1960) J. Biol. Chem., 235, 2272.

Gabeloteau, C. and Desnuelle, P. (1960) Biochim. et Biophys. Acta, 42, 230.

Gage, S. H. (1945) Trans. Am. Microscop. Soc., 64, 151.

Goodsir, J. (1844) Trans. Roy. Soc. Edinburgh, 15, 295.

Grant, N. H. and Robbins, K. C. (1955) Proc. Soc. Exp. Biol. and Med., 90, 264.

Grant, N. H. and Robbins, K. C. (1956) J. Am. Chem. Soc., 78, 5888.

Green, N. M. (1953) J. Biol. Chem., 205, 535.

Green, N. M. and Neurath, H. (1954) in Neurath, H. and Bailey, K., eds., The proteins, New York, Academic Press, v. 2, pt. B, p. 1057.

Grossberg, A. L., Komarov, S. A. and Shay, H. (1952) Am. J. Physiol., 168, 269.

Hamilton, P. B. (1958) Anal. Chem., 30, 914.

Hansson, E. (1959) Acta Physiol. Scand., 46, Supp. 161.

Harfenist, E. J. and Craig, L. C. (1952) J. Am. Chem. Soc., 74, 3083.

Hartley, B. S. (in press) International Congress of Biochemistry, 5th, Moscow, 1961, Proceedings.

Heidenhain, R. (1875) Arch. ges. Physiol., 10, 557.

Heidenhain, R. (1883) in Hermann, L., ed., Handbuch der Physiologie, Leipzig, F. C. W. Vogel, v. 5, p. 173.

Hirs, C. H. W. (1953) J. Biol. Chem., 205, 93.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1953) J. Biol. Chem., 200, 493.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1960) J. Biol. Chem., 235, 633.

Hofmann, T. (1960) Bull. Soc. Chim. Biol., 42, 1279.

Hokin, L. E. (1955) Biochim. et Biophys. Acta, 18, 379.

Hutchison, W. C. and Munro, H. N. (1961) Analyst, 86, 768.

Inagami, T. and Sturtevant, J. M. (1960) J. Biol. Chem., 235, 1019.

Jones, W. and Perkins, M. E. (1923) J. Biol. Chem., 55, 557.

Junqueira, L. C. U. and Hirsch, G. C. (1956) Internat. Rev. Cytol., 5, 323.

Kalnitsky, G., Hummel, G. P., Resnick, H., Carter, J. R., Barnett, L. B. and Dierks, C. (1959) Ann. New York Acad. Sci., 81, 542.

Kazal, L. A., Spicer, D. S. and Brahinsky, R. A. (1948) J. Am. Chem. Soc., 70, 3034.

Keith, C. K., Kazenko, A. and Laskowski, M. (1947) J. Biol. Chem., 170, 227.

Keller, P. J. and Cohen, E. (1961) J. Biol. Chem., 236, 1407.

Keller, P. J., Cohen, E. and Neurath, H. (1956) J. Biol. Chem., 223, 457.

Keller, P. J., Cohen, E. and Neurath, H. (1958a) J. Biol. Chem., 230, 905.

Keller, P. J., Cohen, E. and Neurath, H. (1958b) J. Biol. Chem., 233, 344.

King, T. P. and Craig, L. C. (1958) J. Am. Chem. Soc., 80, 3366.

Korner, A. and Debro, J. R. (1956) Nature, 178, 1067.

Kühne, W. and Lea, A. S. (1882) Untersuch. Physiol. Inst. Univ. Heidelberg, 1872-83, 2, 448.

Kunitz, M. (1940) J. Gen. Physiol., 24, 15.

Kunitz, M. (1947) J. Gen. Physiol., 30, 311.

Kunitz, M. (1950) J. Gen. Physiol., 33, 349.

Kunitz, M. and Northrop, J. H. (1935) J. Gen. Physiol., 18, 433.

Kunitz, M. and Northrop, J. H. (1936) J. Gen. Physiol., 19, 991.

Kurosumi, K. (1961) Internat. Rev. Cytol., 11, 1.

Lacy, P. E. (1957) Diabetes, 6, 498.

Laird, A. K. and Barton, A. D. (1957) Biochim. et Biophys. Acta, 25, 56.

Lamy, F., Craig, C. P. and Tauber, S. (1961) J. Biol. Chem., 236, 86.

Laskowski, M. and Wu, F. C. (1953) J. Biol. Chem., 204, 797.

Laskowski, M., Sr. (1961) in Boyer, P. D., Lardy, H. and Myrbäck, K., eds. The enzymes, ed. 2, New York, Academic Press, v. 5, p. 123.

Le Bron, E., Mocoroa, F. and Stulz, E. (1931) Compt. Rend. Acad. Sci., 193, 79.

Liener, I. E. (1960) Arch. Biochem. and Biophys., 88, 216.

Light, A. and Simpson, M. V. (1956) Biochim. et Biophys. Acta, 20, 251.

Loring, H. S. and Carpenter, F. H. (1943) J. Biol. Chem., 150, 381.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem., 193, 265.

McDonald, M. R. and Kunitz, M. (1941) J. Gen. Physiol., 25, 53.

Marchis-Mouren, G., Sarda, L. and Desnuelle, P. (1959) Arch. Biochem. and Biophys., 83, 309.

Maroux, S., Rovery, M. and Desnuelle, P. (1962) Biochim. et Biophys. Acta, 56, 202.

Marshall, J. M., Jr. (1954) Exp. Cell Res., 6, 240.

Martin, A. J. P. and Porter, R. R. (1951) Biochem. J., 49, 215.

Mejbaum, W. (1939) Z. Physiol. Chem., 258, 117.

Moore, S. and Stein, W. H. (1954) J. Biol. Chem., 211, 907.

Munro, M. P. and Thomas, J. E. (1945) Am. J. Physiol., 145, 140.

Nakanishi, K. (1959) J. Biochem., 46, 1553.

Neurath, H. (1960) in Boyer, P. D., Lardy, H. and Myrbäck, K., eds., The enzymes, ed. 2, New York, Academic Press, v. 4, p. 11.

Newman, S. B., Borysko, E. and Swerdlow, M. (1949) J. Res. Nat. Bur. Stand., 43, 183.

Northrop, J. H., Kunitz, M. and Herriott, R. M. (1948) Crystalline enzymes, ed. 2, New York, Columbia University Press.

Oglivie, R. F. (1933) J. Path. and Bact., 37, 473.

Oppenheimer, K. (1925) Die Fermente und ihre Wirkungen, ed. 5, Leipzig, G. Thieme, v. 2, p. 894.

Palade, G. E. (1952) J. Exp. Med., 95, 285.

Palade, G. E. (1956) J. Biophys. and Biochem. Cytol., 2, 417.

Palade, G. E. and Siekevitz, P. (1956) J. Biophys. and Biochem. Cytol., 2, 171.

Palade, G. E., Siekevitz, P. and Caro, L. G. (in press) in Ciba Foundation Symposium on the Normal and Abnormal Functions of the Endocrine Cell of the Pancreas, London, 1961.

Pechère, J.-F., Dixon, G. H., Maybury, R. H. and Neurath, H. (1958) J. Biol. Chem., 233, 1364.

Pechère, J.-F., and Neurath, H. (1957) J. Biol. Chem., 229, 389.

Petermann, M. L. and Pavlovec, A. (1961) J. Biol. Chem., 236, 3235.

Peterson, E. A. and Sober, H. A. (1956) J. Am. Chem. Soc., 78, 751.

Ponder, E. (1948) Hemolysis and related phenomena, New York, Grune & Stratton, p. 120.

Raacke, I. D. and Li, C. H. (1954) Biochim. et Biophys. Acta, 14, 290.

Redman, C. M. and Hokin, L. E. (1959) J. Biophys. and Biochem. Cytol., 6, 207.

Rimon, A. and Shapiro, B. (1959) Biochem. J., 71, 620.

Rothschild, H.A. and Junqueira, L. C. U. (1956) Nature, 178, 258.

Rovery, M., Fabre, C. and Desnuelle, P. (1953) Biochim. et Biophys. Acta, 12, 547.

Rovery, M., Poilroux, M., Yoshida, A. and Desnuelle, P. (1957) Biochim. et Biophys. Acta, 23, 608.

Sarda, L. and Desnuelle, P. (1958) Biochim. et Biophys. Acta, 30, 513.

Schmidt, G. (1955) in Chargaff, E. and Davidson, J. N., eds., The nucleic acids, New York, Academic Press, v. 1, p. 555.

Schmidt, G., Cubiles, R. and Thannhauser, S. J. (1951) J. Cell. and Comp. Physiol., 38, Supp. 1, 61.

Schneider, W. C. (1957) in Colowick, S.P. and Kaplan, N. O., eds., Methods in enzymology, New York, Academic Press, v. 3, p. 680.

Schramm, M., and Danon, D. (1961) Biochim. et Biophys. Acta, 50, 102.

Shapiro, B. (1953) Biochem. J., 53, 663.

Siekevitz, P. and Palade, G. E. (1958a) J. Biophys. and Biochem. Cytol., 4, 203.

Siekevitz, P. and Palade, G. E. (1958b) J. Biophys. and Biochem. Cytol., 4, 309.

Siekevitz, P. and Palade, G. E. (1960) J. Biophys. and Biochem. Cytol., 7, 619.

Sjöstrand, F. S. and Hanzon, V. (1954) Exp. Cell Res., 7, 415.

Soejima, M. and Shimura, K. (1958) Nature, 182, 49.

Stein, E. A. and Fischer, E. H. (1958) J. Biol. Chem., 232, 867.

Stoeckenius, W. and Kracht, J. (1958) Endokrinologie, 36, 135.

Swell, L., Dailey, R. E., Field, H., Jr. and Treadwell, C. R. (1955) Arch. Biochem. and Biophys., 59, 393.

Taborsky, G. (1959) J. Biol. Chem., 234, 2652.

Tallan, H. H. (1958) Biochim. et Biophys. Acta, 27, 407.

Thomas, J. E. (1950) The external secretion of the pancreas, Springfield, Ill., C. C. Thomas.

Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1945) Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, Minn., Burgess.

Vallee, B. L. (1961) Fed. Proc., 20, Supp. 10, 71.

Van Lancker, J. L. and Holtzer, R. L. (1959) J. Biol. Chem., 234, 2359.

Vernon, H. M. (1913) J. Physiol., 47, 325.

Waldschmidt-Leitz, E. and Purr, A. (1929) Ber. Deut. Chem. Ges., 62, 2217.

Walsh, K. A., Kauffman, D. L. and Neurath, H. (1961) Fed. Proc., 20, 385.

Watson, M. L. (1958) J. Biophys. and Biochem. Cytol., 4, 727.

Yamashina, I. (1956) Biochim. et Biophys. Acta, 20, 433.

Yphantis, D. A. (1962) American Chemical Society, 141st meeting, Washington, D.C., 1962, Abstracts of papers, p. 16B.

End