1973

Studies on the Secretory Process of Rabbit Parotid

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STUDIES ON THE SECRETORY PROCESS

OF

THE RABBIT PAROTID

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

by

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Approved for publication,

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December 1, 1973

The Rockefeller University

New York

ACKNOWLEDGMENT

I wish to express my appreciation to Dr. Detlev W. Bronk for extending to me the opportunity to study at The Rockefeller University. I also acknowledge the valuable advice and assistance of a number of scientists in other laboratories at Rockefeller: Dr. Donald Elliot, Dr. Bruce Cunningham, Dr. Owen Griffith, Dr. William Bowers, Dr. Ann Hubbard, Dr. Ralph Steinman, and Dr. Stanford Moore.

I am deeply appreciative of the hospitality extended by Dr. Sidney Udenfriend to use the facilities of his laboratory at the Roche Institute of Molecular Biology in Nutley, New Jersey, and I am further indebted to Dr. Udenfriend's collaborators, Dr. Stanley Stein and Dr. Peter Böhlen, for their advice and assistance in using their amino acid analyzer equipped with the fluorescamine detection system.

In giving me technical assistance at many stages of my graduate research I am most grateful to my wife, Louise E. Castle, and in advising and preparing the illustrations for this thesis I express my sincere thanks to Frank and Ann Hubbard, Roland Blitchke, and Philip Male.

Finally, to my advisors, Dr. George E. Palade and Dr. James D. Jamieson, I am exceedingly grateful for their most stimulating guidance and encouragement. I hope that the thoroughness, preciseness of interpretation, and enthusiasm displayed by Dr. Palade and the members of his laboratory will remain as the most permanent features of my scientific education.

SUMMARY

An *in vitro* incubation system which allows the labeling and isolation of secretion granules — and their subsequent resolution into membrane and content subfractions — has been developed by using dissected lobules of rabbit parotid glands. The system can be used to study a series of aspects related to the last phases of the secretory cycle (concentration, storage, discharge). The work reported in this thesis characterizes the system and presents data concerning the relative rates of synthesis of membrane and content proteins.

The dissected lobules can be maintained *in vitro* in a tissue culture medium for 9 to 10 hr during which period their acinar cells retain their organization, synthesize proteins at a constant rate, and discharge their secretory proteins by exocytosis upon adrenergic stimulation. Autoradiographic experiments have shown that the acinar cells of the system transport newly synthesized secretory proteins along an intracellular pathway (rough ER → Golgi complex → (condensing vacuole) → secretion granules) similar to that already described for the pancreatic exocrine cells (Jamieson and Palade, 1967a,b) but at a slower rate, especially for the last steps of the operation. Under these conditions it was possible to recognize by morphological and kinetic criteria an immature secretion granule stage preceding the final storage stage (secretion granule). These experiments indicate the conditions needed to label the content of the secretion granules.

A reasonably pure secretion granule fraction has been isolated from parotid tissue homogenates by a new procedure which prevents granule aggregation by avoiding pelleting and by using EDTA in the suspension media. Purification of the fraction is obtained by flotation of membranous debris and sedimentation of the granules in a discontinuous sucrose density gradient. The fraction has been shown to be morphologically and cytochemically homogeneous as assessed by

electron microscopy and enzymatic assays. Since it accounts for ~15% of the amylase activity of the original homogenate, it is considered representative of the total granule population of the gland.

Secretion granules were lysed by gradual exposure to hypotonic media, and the lysate subsequently was resolved into content and membrane subfractions by centrifugation. The latter subfraction appeared as a homogeneous population of flattened and infolded vesicles. When assessed for residual granule content by enzymatic assays, the membrane subfraction was found to contain low, but assayable, levels of amylase activity (~0.03% of the total granule complement). Contamination by the entire spectrum of secretory proteins, especially by those species having estimated molecular weights between 95,000 and 130,000 daltons was detected when gel electropherograms of membrane subfractions were compared to gel electropherograms of: 1) mixtures of membrane and labeled content subfractions, and 2) secretory proteins released from granules by the physiologic process of exocytosis.

The membrane and content subfractions obtained from secretion granules of lobules that had been labeled with a mixture of [^{14}C]-amino acids during *in vitro* incubation, have been used to compare biosynthetic rates (assumed to be proportional to specific radioactivities) of polypeptides of granule membranes and content at two different levels: 1) specific radioactivities of unresolved mixtures, and 2) specific radioactivities of individual gel bands of the subfractions. The first level proved unreliable due to the magnitude and variability of content adsorption to the membranes. The results of the comparison at the more refined, second level have shown that all membrane polypeptides analyzed, with the exception of a majority component of estimated molecular weight 40,000 daltons, have specific radioactivities substantially (two- to tenfold) lower than those of content polypeptides destined for secretion. This has been taken as indicative that polypeptides of the granule membrane are not synthesized *pari passu* with those of the secretory content, but rather predate the packaged exportable products. The major membrane polypeptide

having a specific radioactivity comparable to that of the content polypeptides represents one of the most intriguing findings of this thesis. It may represent a modifier required to convert a Golgi membrane into a secretion granule membrane, i.e., a membrane competent for exocytosis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Fig. No.

I. INTRODUCTION

INTRODUCTION

Among the various cell types of mammalian organisms that are specialized for the secretion of macromolecules, the exocrine glands that supply enzymes to the gastrointestinal tract have been particularly useful for studying and understanding the mechanisms by which cells synthesize and process proteins and glycoproteins for export. The exocrine pancreas and to a lesser extent the parotid salivary gland have been extensively investigated because they offer large, compact cellular populations characterized by the preponderance of a single secretory cell type (85%-90% of the cellular volume of the gland) and because each acinar cell itself is so highly specialized for the export of protein. Thus, in addition to their interest as primary contributors to the mammalian digestive process, these glands have the advantage of possessing a more homogeneous cell population than other tissues (adenohypophysis, liver); moreover, their cells are functionally less diverse than in other glands (liver, adrenal). Hence, the pancreas and parotid provide model systems particularly well-suited for the investigation of general aspects of the synthesis and subsequent processing of secretory proteins.

For the exocrine pancreas, in particular, the combined morphological and biochemical studies of Siekevitz and Palade (1956-1960; Palade *et al.*, 1962; Caro and Palade, 1964) and Jamieson and Palade (Jamieson, 1972) have focused on the exportable products, and have characterized the pathway and the timetable according to which secretory proteins newly synthesized on polyribosomes attached to the endoplasmic reticulum are segregated, transported, packaged, stored and released by specific intracellular compartments. The studies of Jamieson and Palade (1967a,b) have further emphasized the advantage of investigating model secretory systems *in vitro* where: a) the composition of the extracellular environment can be both flexibly and precisely controlled, b) the

discharged secretory products are easily collected for analysis, and c) the tissue of a single animal can support an experiment of multiple timepoints or other variables.

In comparison to the substantial body of information that has been acquired concerning the nature and processing of secretory proteins, especially those of the exocrine pancreas (Keller et al., 1963; Greene et al., 1963; Jamieson, 1972; Tartakoff, 1973), relatively little is known regarding the interactions of intracellular membranes involved in the overall secretion process. Biochemical and autoradiographic studies have identified two sites where these interactions are of critical importance. The first site is between the rough endoplasmic reticulum, the site of synthesis and segregation of secretory protein, and the Golgi complex where proteins are concentrated and packaged for storage and eventual discharge. Available evidence (Jamieson and Palade, 1967a,b; Meldolesi et al., 1971a,b,c) suggests that a shuttle mechanism involving smooth membrane vesicles functions in the transport of exportable proteins between the two distinct compartments by pinching off from one compartment and fusing with the other. More recent evidence obtained using liver (Ehrenreich et al., 1973; Bergeron et al., 1973) indicates that during these interactions the membranes retain their individuality; there is no randomization of their enzymatic components. The second site where a highly specific interaction is involved, is between the membrane of the secretion granule and the membrane on the cellular discharge front (membrane defining the lumen in exocrine glands). Morphologically, it is quite clear in the pancreas (Palade, unpublished observations) and in other cells (Lagunoff, 1973; Rölich et al., 1971) that the granule membrane fuses with the interacting cell membrane and that the fused membranes undergo an ordered, stepwise reduction of their layers until the two membranes become continuous, thus creating complete communication between the intragranular compartment and the extracellular space. Very little is known about the molecular basis of this specific membrane interaction except that it is dependent definitely on cellular energy (Hokin and Hokin, 1962; Schramm, 1967; Jamieson and Palade, 1971a), and presumably

on calcium (Heisler et al., 1972; Poisner, 1970; Rasmussen and Tenenhouse, 1968), and that in many cases, it involves a mediation by cyclic nucleotides (Malamud, 1972; Schramm and Naim, 1970; Rasmussen and Tenenhouse, 1968; Kuo and Greengard, 1969).

The fusion, however, leads to a rather intriguing and important question in cellular organelle economy. The membrane used to package, store, and effect release of secretory products is retained as a part of the cell during the discharge process. In fact, as a result of induction of massive exocytosis, the cell membrane becomes greatly expanded by accommodating the newly introduced granule membranes. The subsequent return of the expanded apical surface area to its normal proportions suggests that the cell is somehow capable of achieving a reverse in the direction of membrane translocation. Reinternalization of excess "luminal" membrane is morphologically well-documented, but the subsequent fate of the recovered membrane is still in question. It has been postulated that this membrane which is specialized in packaging and effecting release of secretory protein is recycled intact (Palade, 1959), or disassembled with reutilization of its macromolecular components (Fawcett, 1962), or completely degraded and resynthesized pari passu with the secretory proteins (Amsterdam et al., 1971).

The experiments reported in this thesis were carried out to provide data necessary for choosing between these alternatives. For the study at hand and related studies of the specific interactions of intracellular membranes, the exocrine pancreas did not appear to be the system of choice. As shown by Meldolesi et al. (1971), endogenous lipase activity in the guinea pig pancreas has demonstrable effects on the structure and chemistry of native cellular membranes, including those of zymogen granules. In addition, there is a substantial potential for degradation of membrane proteins by activated protease zymogens, especially during experiments covering extended periods of time. To avoid artifacts induced by membrane alteration or partial degradation, attention has been shifted to the parotid salivary gland in which no lipolytic and proteo-

lytic activities were expected on account of what is known about the nature of its secretion.

The parotid salivary gland of the rat has been studied by Schramm and his collaborators (Schramm, 1967), but since the information obtained is less extensive than that available for the exocrine pancreas, it was necessary to: I. develop a satisfactory system for incubation of the tissue *in vitro* in which the composition of the medium bathing the tissue can be easily manipulated and in which the tissue maintains structural integrity, a constant rate of protein biosynthesis, and sensitivity to stimulation by secretatogues; II. study by autoradiography the pathway and rate of intracellular transport and packaging of newly synthesized secretory protein in order to determine incubation times which insure processing of incorporated protein radioactivity to the secretion granule stage; III. develop techniques for fractionating the tissue to obtain a secretion granule fraction of high purity; IV. subfractionate the granules to separate the membranes from the secretory content; V. assess these fractions and subfractions for polypeptide composition, and, to a limited extent, enzymatic composition; and finally, VI. investigate the rates of synthesis of the polypeptides of secretion granule membranes relative to those of the secretory content destined for discharge. The data thus obtained should provide the premises needed for choosing among the three alternative fates of granule membrane post-exocytosis (recycling as structural units, disassembly, or degradation). If membrane polypeptide components are synthesized at rates comparable to those of content polypeptides, then the alternatives entailing conservation of membranes or their components (reutilization, disassembly) are unlikely. On the other hand, if rates of synthesis are very different then, by definition, *pari passu* synthesis cannot apply, and subsequent experiments should determine whether recycling or disassembly obtains. It should be kept in mind, however, that the results may reveal a more complex situation unanticipated by any one of the hypotheses mentioned and implying greatly different rates of synthesis for individual membrane polypeptides presumably related to their different functions.

II. METHODS AND MATERIALS

METHODS AND MATERIALS

Animal Used and Tissue Preparation

The rabbit was selected as experimental animal on the basis of gland size and a relatively high amylase concentration (see Table I). Furthermore, the well-defined connective tissue capsule of the rabbit parotid facilitated surgical removal with a minimum of contaminating adipose tissue. 3-5 lb. male New Zealand white rabbits were either fed ad libitum, in the case of experiments designed to characterize the in vitro incubation system and explore intracellular transport by autoradiography, or fasted overnight for a period of 12-16 hr in hopes of standardizing the tissue prior to cellular fractionation (granule isolation) experiments. They were sacrificed with an overdose of sodium pentobarbital injected into the ear vein. Both parotid glands were removed and placed immediately in chilled incubation medium at 4° C.

Dissected lobules of parotid tissue replaced the thin (0.5 mm in thickness) tissue slices used by Jamieson (1966) and Jamieson and Palade (1967a) for in vitro incubation. The excised glands were distended by injecting incubation medium into their stroma, and tiny individual lobules (1 x 2 x 3 mm) rendered visible by the edema were dissected, taking care to cut through duct and connective tissue, thus minimizing damage to acini.

Incubation Procedures

Incubation conditions differed with the design of the experiment as follows:

Continuous Incorporation of Labeled Leucine. Dissected lobules were placed in 25 ml Erlenmeyer flasks (7-9 lobules per flask) containing 5 ml of Nutrient Mixture F12 (referred to subsequently as F12)

Table I

Size and Amylase Content of Parotid Glands of Various Mammals

Animal	Body Weight g	Wet Weight (Pair of Parotids) g	Specific Activity of Amylase in Glands units*/mg protein	units*/mg DNA
Rat	160	0.8	1.85×10^2	2.41×10^4
Guinea Pig	270	0.3	0.47×10^2	0.84×10^3
Hamster	100	0.2	0.95×10^2	0.43×10^4
Rabbit	~2300	2-2.5	0.65×10^2	1.68×10^4

Parotids were quickly removed from sacrificed animals and placed in 0.9% NaCl. After excess connective tissue, lymph nodes, and fat were dissected away, the glands were weighed and then diced and homogenized extensively in distilled water (2.0 ml per 0.3 g wet weight of gland). Aliquots were taken for assays of amylase activity, total protein, and DNA.

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*1 unit of amylase activity = 1 μmole maltose released per min at 30°.

a chemically defined tissue culture medium (Ham, 1965) supplemented with L-[4, 5-^{3}H]leucine at 5 μCi/ml. Flasks containing lobules and medium were gassed with 95% O_2 + 5% CO_2 (pH 7.2) and kept on ice for 10 min. At the onset of incubation the flasks were transferred to a 37° C water bath and agitated at 70 cycles/min. The O_2 + CO_2 atmosphere was maintained either by continuous gas flow or by intermittent gassing at 20-30 min intervals. For long-term incubation 50-100 units/ml penicillin and 0.05 mg/ml streptomycin were added to the medium to prevent bacterial growth. At specified timepoints lobules were removed, washed with isotonic saline, and homogenized in 2.0 ml distilled water to provide samples for chemical and radioactivity assays.

Morphology. To study the fine structure of the in vitro incubated parotid, two to three lobules were incubated as described above, except that the label was omitted. At selected timepoints the tissue was placed directly into hypertonic aldehyde fixative and processed for electron microscopy.

Autoradiography. To introduce a short well-defined pulse of radioactivity into secretory protein with [^{3}H]leucine, it was necessary to reduce the tissue's endogenous pool of unlabeled leucine before and during pulse incubation. Since commercial F12 contains 0.1 mM L-leucine, it was replaced by Krebs-Ringer-bicarbonate (KRB) medium (Krebs, 1950) supplemented with all amino acids (except leucine) at concentrations found in Eagle's Minimal Essential Medium (MEM) (Eagle, 1959; Jamieson, 1966); during the pulse, L-[4, 5-^{3}H]leucine (specific activity: 54 Ci/mmole) was present at a concentration of 300 μCi/ml (5.4 μM) previously determined to be sufficient for EM autoradiography (Jamieson and Palade, 1967a). Dissected lobules that had been incubated 10 min at 0° C in leucine-free medium before the pulse were transferred to 4 ml of gassed pulse medium in a 10 ml Erlenmeyer flask. After 5-10 min equilibration at 0° C the pulse flask was warmed to 37° C for 4 min. Lobules were then immediately washed in a large volume (~200 ml) of F12 supplemented with ^{1}H-leucine to a concentration of 4 mM, and subjected to chase incubation (37° C and 70 cycles/min) in 20 ml of this same me-

dium in 125 ml Erlenmeyer flasks. At specified timepoints tissue was removed and immersed in hypertonic, phosphate-buffered formaldehyde. The ratio of leucine concentration in the chase medium to leucine concentration in the pulse medium was ~750, while the volume dilution from pulse to chase was five-fold.

Stimulated Discharge of Secretory Product. To investigate the discharge of secretion from parotid lobules, isoproterenol was chosen as a secretagogue since it is known to be a powerful sympathomimetic stimulant effective for salivary glands (Amsterdam et al., 1969; Simson, 1969; Barka, 1971; and Byrt, 1966). Dose-response studies to determine optimal isoproterenol concentration were performed by initially pulse-labeling lobules with [^{3}H]leucine and then chase incubating in the absence (control) or presence of isoproterenol concentrations covering five orders of magnitude: 1×10^{-8}M - 1×10^{-4}M. At specified times aliquots of incubation medium were removed for assay of released protein-associated radioactivity for eventual comparison to radioactivity remaining in the tissue post-incubation exactly in the manner indicated in the following paragraph which describes techniques used to estimate relative proportions of label incorporated into secretory proteins and proteins retained for intracellular use.

To estimate the partitioning of incorporated [^{3}H]leucine between exportable and non-exportable proteins, parotid lobules were pulse labeled and then chased for the time required (3 hr) to bring labeled secretory proteins into secretion granules. At that time isoproterenol was added to a final concentration of 1 μM (determined by the dose-response experiments described above to be the optimal concentration for the in vitro lobule system), and the incubation was continued for 4 hr. Percentage release of protein radioactivity and amylase into the medium was determined using discharge assays previously reported by Jamieson and Palade (1971). At selected timepoints aliquots of incubation media were removed for assay and replaced by fresh medium, which (where appropriate) contained stimulant to compensate for that which had been depleted by both oxidation and medium removal. Following the

last timepoint the tissue was homogenized and assayed to estimate amylase and incorporated radioactivity still associated with the tissue.

Synthesis of Granule Secretory Content and Membranes. Estimation of the rates of synthesis of polypeptides of granule membranes versus polypeptides of granule content requires the incorporation of sufficient labeled amino acid to provide accurate measurements of specific radioactivity in both granule subfractions. To this intent the tissue of four parotid glands (an amount sufficient for the ultimate isolation of assayable quantities of granule membrane) was dissected into lobules and pulse-labeled for 30 min using a mixture of fifteen ^{14}C-labeled amino acids. Pulse medium consisted of 10 ml of KRB supplemented with unlabeled amino acids to one-half the final concentration found in Eagle's MEM and containing in addition 25 or 100 μCi/ml of reconstituted [^{14}C]algal protein hydrolysate having the composition given in Table II. At the end of the pulse, lobules were washed with ~100 ml F12 at 37° C and were then chase incubated in 100 ml F12 (4 hr with change of medium at 2 hr) to allow the bulk of the incorporated radioactivity to be transported to secretory granules. The tissue in its entirety was then fractionated to obtain secretion granules.

Processing of Tissue and Subcellular Fractions for Microscopy

Tissue, excluding lobules for autoradiography, was initially fixed for 4-20 hr at room temperature in either 4% glutaraldehyde or a mixture of 2% formaldehyde and 2% glutaraldehyde buffered to pH 7.2 with hypertonic potassium phosphate (0.12 - 0.2 M) or with sodium cacodylate (0.15 - 0.2 M) (Graham and Karnovsky, 1966). The hypertonic conditions were necessary because preliminary work indicated that in their absence the tissue is damaged by swelling, explosion, and extraction of secretory granules. Tissue was then post-fixed 3-4 hr at 0.4° C in 1% osmium tetroxide buffered to pH 7.2 as described above, washed with two or three changes of 0.17 M NaCl, stained in-block for 60 min at 0° C with 0.5% magnesium uranyl acetate in 0.17 M NaCl, dehydrated in ethanol and embedded in Epon (Luft, 1961). Sections 0.5 μm thick were

Table II

The Composition of Reconstituted [^{14}C]Algal Protein Hydrolysate

Amino Acid	Specific Activity mCi/mmole	µCi Amino Acid/ mCi Algal Profile	Algal Profile Concentration(mM) in Pulse Medium at 100 µCi/ml	Pulse Medium - Total Amino Acid Concentration(mM)
Alanine	156	100	.064	.114
Arginine	210	70	.033	.333
Aspartic Acid	208	90	.043	.093
Glutamic Acid	254	125	.049	.099
Glycine	103	40	.039	.089
Histidine	312	15	.005	.105
Isoleucine	314	50	.016	.216
Leucine	314	125	.040	.240
Lysine	314	60	.019	.219
Phenylalanine	460	75	.016	.116
Proline	252	50	.020	.070
Serine	156	40	.026	.076
Threonine	208	50	.024	.224
Tyrosine	455	40	.009	.109
Valine	260	70	.027	.227

stained in 1% methylene blue, 1% sodium borate (Richardson et al., 1960), and used for surveying the material before cutting thin (silver) sections for electron microscopy. These sections were stained in alcoholic uranyl acetate and alkaline lead citrate (Venable and Coggeshall, 1965) and observed in a Siemens Elmiskop I or 101.

For autoradiography, lobules were initially fixed in 10% formaldehyde in 0.175 M potassium phosphate (pH 7.2), using five to six changes to promote extraction of unincorporated [^{3}H]leucine (Wuhr et al., 1969; Ashley and Peters, 1969). The specimens were post-fixed, dehydrated, and embedded as described above except that in-block staining with magnesium uranyl acetate was omitted.

Secretion granule fractions (collected in 1.9 M sucrose, 40 mM potassium phosphate, 1 mM EDTA) were diluted by one of two ways: a) with three volumes of a mixture of 2% glutaraldehyde, 2% formaldehyde in 0.08 M potassium phosphate pH 7.2 or b) with one volume of a mixture of 4% glutaraldehyde in 0.2 M potassium phosphate pH 7.2. Aldehyde fixation was carried out in suspension at room temperature for 8-16 hr after which samples were pelleted in 0.4 ml polypropylene conical tubes in a Beckman 152 microfuge. Subsequently, the centrifuge tubes were cut away just above the pellet surface and the pellet plus residual tube were placed in 1% OsO_4 buffered with 0.1 M potassium phosphate pH 7.2 for post-fixation at 4°. After a two hr osmium fixation, the pellets were bisected vertically and then fixed for two additional hr. Past this step processing of the fixed pellets was performed exactly as described above for regular morphological survey of tissue.

Pellets of secretion granule membranes in polyallomer centrifuge tubes were fixed, post-fixed, and stained with magnesium uranyl acetate in the tube. At the onset of dehydration the pellets plus adhering tube bottoms were cut and subsequently embedded as units taking care to orient the material so sectioning would expose the entire thickness of the pellet. In trimming the Epon block for microtomy the residual piece of centrifuge tube was easily removable, leaving behind the embedded pellet undisturbed. Sections were placed on bar grids to further

insure visualization of the entire pellet thickness.

Processing of Specimens for Autoradiography

Light microscope (LM) autoradiography was initially performed as previously described (Jamieson and Palade, 1967a) in 0.5 μm thick Epon sections affixed to glass slides to provide an estimate for length of exposure necessary to produce sufficient silver grains for electron microscope (EM) autoradiography. In general, the number of days of exposure required to yield an adequate grain count for LM autoradiography approximated the number of weeks necessary for exposure for EM autoradiography.

After satisfactory assessment of LM autoradiography, pale gold sections were cut, collected on 200 mesh copper grids (Formvar and carbon coated) and covered with Ilford L4 photographic emulsion using the loop method of Caro and van Tubergen (1962). Exposure was performed in light-tight boxes containing dessicant at 4° C. After photographic processing (developer: Microdol X) and hydrolysis of emulsion gelatin (by 20 min immersion in 0.1 N NaOH), the sections were doubly stained (Venable and Coggeshall, 1965) and micrographed at low magnification in a Siemens Elmiskop I. Grain counts on the resultant micrographs were used to quantitate the results.

Isolation of Parotid Secretion Granules

To isolate secretion granules from rabbit parotid a new procedure was devised which produces a clean preparation with satisfactory yield. Many aspects of the protocol (listed stepwise below) are discussed in detail in the Results.

Stepwise Fractionation Scheme.

1. Parotid tissue (either glands dissected free of surrounding connective tissue or incubated lobules) was divided into 0.4-0.6 g batches.
2. Each batch of tissue was thoroughly minced in a few drops of homogenization medium with a single-edge razor blade on a Teflon plate.
3. Homogenization (3 strokes at 1300-1600 rpm) was performed in 0.28 M sucrose, 40 mM potassium phosphate, 0.2 mM EDTA pH 7.2 in a Brendler

type (Brendler, 1951) tissue grinder (glass mortar, serated-tip Teflon pestle with 0.004-0.006 in clearance and 10 ml capacity) to give a 10% (w/v) tissue homogenate.

4. Debris which settled by gravity was rehomogenized (3 strokes, 1600 rpm) in 4-5 ml (precise volume depending on initial tissue weight) homogenization medium and was combined with the supernate of the initial homogenate. The combined supernates were designated: Total homogenate (5% tissue weight per volume).

5. Large debris, unbroken cells and nuclei were pelleted from the total homogenate at 600 x g_{av} for 8 min in conical glass centrifuge tubes in an International PR6000 centrifuge with #269 rotor.

6. The resulting supernate was layered over a step gradient (in 15 ml round bottom Corex tubes) consisting of a 3 ml layer of 0.42 M sucrose, 40 mM potassium phosphate, 1 mM EDTA pH 7.2 over a 2 ml cushion of 2.0 M sucrose, 40 mM potassium phosphate, 1 mM EDTA pH 7.2.

7. Centrifugation carried out at 2100 x g_{av} for 15 min in an International PR6000 centrifuge with #269 rotor yielded a crude granule fraction located at the 0.42-2.0 M sucrose interface.

8. The crude granule fraction was collected, and the sucrose concentration was adjusted to 1.7 M using 2.1 M sucrose, 40 mM potassium phosphate, 1 mM EDTA.

9. The diluted crude granule fraction was layered centrally in a sandwich gradient having as lower layers 2.1 M and 1.9 M sucrose (1.5 ml each) and an overlay of 1.6 M sucrose (3 ml). All sucrose solutions were supplemented with phosphate and EDTA.

10. By centrifugation at 160000 x g_{av} for 90 min in an International B60 ultracentrifuge equipped with an SB283 rotor, a purified granule fraction distributed in the 1.9 M sucrose band was obtained with membranous debris floating upwards from the load, through the 1.6 M overlay.

Granule yield was determined as percentage recovery of whole homogenate amylase activity, and contamination by other subcellular organelles (mitochondria, microsomes, nuclei, and lysosomes) was assessed both morphologically and biochemically on resuspended granule

pellets using the assays enumerated in the chemical and enzymatic assays section of Methods.

Lysis of Secretion Granules and Subsequent Separation of Granule Membranes and Secretory Content

Secretion granules isolated in 1.9 M sucrose, 40 mM potassium phosphate, 1 mM EDTA were lysed by reducing in a stepwise manner the tonicity of the isolation medium, using for dilution purposes 20 mM $NaHCO_3$, 0.5 mM EDTA pH 7.2 (designated Lysis Medium).

1. Isolated granules along with three volumes of lysis medium were placed in an Amicon ultrafiltration chamber outfitted with a PM10 sieving membrane (which retains molecules with molecular weight 10000 or greater). At this point sucrose and phosphate concentrations were approximately 0.5 M and 10 mM respectively.
2. The diluted granules were concentrated to one-half the initial volume under pressure (N_2 at 60 psi).
3. Lysis medium was added up to the original volume, thus reducing approximate sucrose and phosphate concentrations to 0.25 M and 5 mM respectively. At this stage partial clearing of the suspension was observed.
4. The rediluted granules were then concentrated to one-third the original volume under pressure.
5. Lysis medium was then added up to the original volume, thus reducing the approximate sucrose and phosphate concentrations to 0.08 M and 1.6 mM respectively. At this point the suspension completely cleared indicating apparently complete granule lysis.
6. The lysate was concentrated to ~15 ml and removed from the Amicon chamber. The chamber was washed with an additional 5 ml lysis medium, the wash being combined with the concentrated lysate.

The concentrated lysate was resolved into membranes and content by a two-step centrifugation presented pictorially and discussed in some detail in the Results. Specifically, this procedure involved layering granule lysate over a discontinuous sucrose gradient containing steps of 0.2 M, 0.6 M and 1.5 M sucrose supplemented with 20 mM $NaHCO_3$,

0.5 mM EDTA pH 7.2. Centrifugation using an International B60 ultracentrifuge with an SB283 rotor was carried out for 60 min at 160000 x g_{av} to separate membranes (at the 0.6-1.5 M sucrose interface) from granule content (remaining in the original load). The granule content was collected (including some of the 0.2 M layer beneath) and used according to the requirements of the experiment, either directly for enzyme assays, or concentrated for amylase precipitation (see below), or processed for use in polyacrylamide gel electrophoresis (lyophilization, resuspension in 0.1 M NH_4HCO_3, desalting over Biogel P 2, lyophilization and resuspension in water). The granule membranes were harvested and diluted five-fold with lysis medium. The diluted membranes were then layered over a 0.6 M sucrose cushion (containing 20 mM $NaHCO_3$, 0.5 mM EDTA pH 7.2) and pelleted at 160000 x g_{av} for 60 min in the same SB283 rotor. The resultant pellets were resuspended for chemical or enzymatic assay, fixed (as previously described) for morphological observation, or dissolved in sodium dodecyl sulfate for polyacrylamide gel electrophoresis.

The granule membrane fraction was assessed for contamination by other cellular organelles by characteristic enzymatic assays and by morphological survey. Degree of separation of content from granule membrane fragments was determined by two methods: 1) assay of the membrane fraction for the content enzyme amylase, and 2) identification and assessment of Coomassie Brilliant Blue stained bands which represent secretory content polypeptides in polyacrylamide gel electrophoretic patterns of granule membranes.

Collection of Parotid Saliva from an In Vivo Stimulated Animal

To obtain a sample of secretion released by the parotid glands *in vivo*, the parotid ducts of a rabbit were cannulated, and secretion was collected from the cannulae following systemic stimulation in the following manner. The rabbit was anaesthetized with sodium pentabarbital, and an incision was made along the length of the jaw exposing the masseter muscle on both sides. The parotid ducts which run across the surface of this muscle were exposed from the surrounding

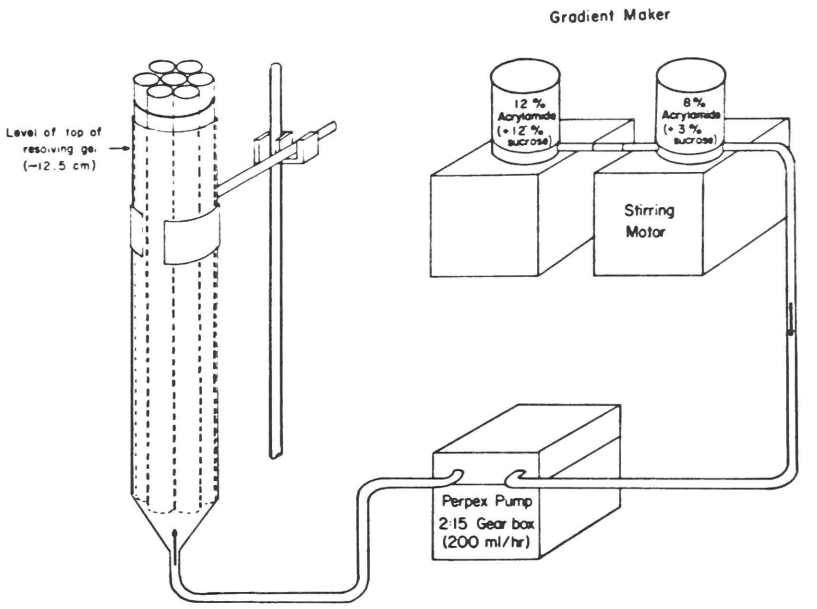
connective tissue, and small polyethylene tubes were inserted by perforating the wall of the ducts. These tubes were secured by ligature. Stimulation of parotid secretion was achieved by intraperitoneal injection of isoproterenol (4 x 1 ml of a 20 mg/ml solution) at fifteen min intervals. 1 1/2 ml secretion (highly concentrated in protein) was collected over 1 1/4 hr. The collected secretion was lyophilized, resuspended in 0.1 M NH_4HCO_3, desalted by gel filtration over Biogel P2 and subsequently lyophilized to remove NH_4HCO_3, and finally redissolved in either water or solutions directly applicable for gel electrophoresis.

Purification of Amylase by the Alcoholic Glycogen-Precipitation Procedure

Granule content (obtained by the centrifugation which resolved granule lysate into membranes and content) was concentrated in an Amicon ultrafiltration cell outfitted with a PM10 membrane. 1 ml of the final concentrate contained 200 units (μmoles maltose released per min at 30° C) amylase. The procedure of Schramm and Loyter (1966) was then followed directly, making adjustments, however, to compensate for the fact that the initial amylase concentration was below the 500 units/ml level indicated in the original reference. To 2.5 ml granule content was added dropwise 1.67 ml cold 100% ethanol. This mixture was spun at 10000 x g_{av} for 20 min. The resulting supernatant (~4.1 ml) was treated with 0.20 ml 0.2 M sodium phosphate buffer pH 8.0, 0.20 ml glycogen solution (2% w/v, clarified by initial centrifugation), and 0.20 ml 100% ethanol. The resulting precipitate was pelleted and washed twice by centrifugation, and the final pellet was drained and dissolved for electrophoresis. The intent to perform electrophoresis in dissociating solvents rendered unnecessary glycogen digestion and limit dextrin removal steps. To check the extent of amylase removal and the effect of the procedure on other proteins of the content, the supernate of the glycogen-amylase precipitate was lyophilized and the resulting residue was resuspended in solutions appropriate for gel electrophoresis.

Polyacrylamide Gel Electrophoresis of Granules and Their Subfractions

Polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS) was performed by the method of Shapiro et al. (1967). This alkaline discontinuous pH system utilizes the Tris-glycine pH 8.4 resolving gel buffer of Ornstein and Davis (1962, 1964), a pH 6.7 Tris-phosphate stacking gel, and a running pH of about 9. Tubes 5 or 6 mm diameter containing resolving gels of length 10-13 cm and stacking gels of volume 0.4 ml were used in all instances reported here and were routinely run for approximately 12 hours at a current of less than 1 ma per tube, the actual time of electrophoresis being established by migration of the tracking dye, bromphenol blue. In initial studies resolving gels were 11% acrylamide (stock acrylamide 37.5: 1, acrylamide: N,N'-methylene bis-acrylamide) made as specified by Maizel (1971). Subsequently, the following 8-12% acrylamide gel gradient method was devised to yield optimal separation and sharpness of polypeptide bands obtained from both secretion granule membranes and content. In this procedure, seven 15 cm long, 6 mm (inner diameter) glass tubes were close-packed in hexagonal arrangement using a rubber band at one end. The tube bundle was then inserted into the barrel of a 50 cc disposable (polyethylene) syringe, vertically mounted. The syringe outlet was connected to a Perpex pump and gradient maker (Fig. 1). 12% acrylamide + 12% sucrose was placed in the back reservoir and 8% acrylamide + 3% sucrose was placed in the mixing chamber of the gradient maker. The gradient was thus generated by pumping in the less dense acrylamide solution first. The purpose of the sucrose gradient was to stabilize the acrylamide gradient against convection patterns caused by heat evolved during polymerization. Pumping took 20 min, and the polymerization catalyst ammonium persulfate was reduced to 1/10 the amount recommended by Maizel (1971) to insure a polymerization time of at least 40 min. The gradients thus generated were each overlaid with isobutanol (or n-butanol) prior to polymerization as suggested by Glossmann and Neville (1971).



Generation of 8-12 % Polyacrylamide Gradients

Fig. 1. The method developed for making uniform gels with a continuous gradient of 8-12% polyacrylamide. The gradient maker chambers containing acrylamide solutions (compositions as described by Maizel (1971) but, in addition, supplemented with sucrose) are connected via a Perpex peristaltic pump (flow rate ~200 ml/hr) to the 50 ml disposable syringe cartridge containing the glass tubes (6 mm i.d.) that ultimately contain the gels. The gradient generated (lowest density first) fills the entire syringe cartridge; however, only the acrylamide solution within the tubes is overlaid with isobutanol prior to polymerization. 1-2 hr after polymerization the base of syringe was cut away, and the entire cylinder of polymerized acrylamide containing the gel tubes was removed; subsequently, individual tubes were dissected free of surrounding gel and secured in place in the electrophoresis chamber. Isobutanol was washed from the surface, and the resolving gels were overlaid with 0.4 ml spacer gel solution as described in the text.

Following electrophoresis, tracking dye fronts were marked with India ink, and the gels were either: 1) fixed in 25% isopropanol, 10% acetic acid (Steck et al., 1971) and stained simultaneously for protein with Coomassie Brilliant Blue (0.07-0.08% for 8 hr) with subsequent destaining in 10% isopropanol, 10% acetic acid followed by 5% isopropanol, 10% acetic acid or 2) fixed only (with isopropanol-acetic acid), then reacted with periodic acid and stained with Schiff reagent for PAS (periodic acid-Schiff) -positive carbohydrate according to the procedure of Steck et al. (1971).

Destained gels were photographed with a Polaroid MP3 camera and scanned in a Gilford spectrophotometer equipped with a linear transport system to obtain an optical density profile for polypeptides (wavelength 550 nm) and PAS-positive bands (wavelength 560 nm (Steck et al., 1971)).

Finally, gels containing radioactivity were sliced transversely into 1 mm-thick sections with a gel slicer provided with multiple, equally spaced cutting wires. Each slice was dissolved in 0.5 ml of 20% H_2O_2 (36-48 hr at 45° C) for scintillation counting.

Amino Acid Analysis of Polypeptides of Granule Membranes and Secretory Content

10 µl aliquots (corresponding to 8-10 µg protein) of granule content (dissolved in water or in 2% SDS) and granule membrane (dissolved in 2% SDS) or stained polypeptide bands cut out from polyacrylamide gels were placed in small glass hydrolysis ampoules and lyophilized. Constant boiling (5.7 N) HCl was added (100 µl per 10 µl of soluble sample and 200 µl (containing 1% thioglycollic acid) per mm original thickness of gel slice), and the tubes were sequentially evacuated and flushed with nitrogen five times before final evacuation and sealing. Hydrolysis was performed usually for 24-27 hr at 110° C. Upon termination of hydrolysis, the hydrolysate and 200 µl of doubly deionized water used to wash the hydrolysis ampoule were transferred to a 1 ml chamber and evacuated to dryness on a flash evaporator at 40° C. An additional

100 µl of water was added to the 1 ml evacuation chamber and re-evaporation performed. The dried residue was finally dissolved in 100-200 µl of 0.2 N sodium citrate, pH 2.2, the loading buffer for amino acid analyzer columns.

Amino acid analyses on 50 µl aliquots of reconstituted hydrolysates were performed according to the procedure of Stein et al. (1973), using a single long column (Felix and Terkelson, 1973) filled with Durrum DC-4A resin and maintained at 53° C. The classic colorimetric ninhydrin detection system for the column effluent of Spackman et al. (1958) is replaced in this procedure by a fluorometric detection system using fluorescamine which reacts with free primary amines (Udenfriend, 1972a,b). The fluorophors have a 390 nm excitation maximum and 475 nm emission maximum. Areas under recorded peaks were determined by an attached Autolab System IV Integrator (Sterling N.J.). Where desired, fractionated amino acid residues (already reacted with fluorescamine) were recovered (after passing through the microphotofluorimeter) for concentration to dryness and scintillation counting.

In all analyses norleucine (250 picomoles per 50 µl) was included as internal standard. The use of the internal standard allows compensation for small errors in loading volume and is essential for quantitation of the number of amino acids applied to the column -- used ultimately for determination of total protein and thus calculation of specific radioactivity.

Radioactivity, Chemical, and Enzymatic Assays

Radioactivity Assays. Samples of tissue homogenate and incubation media were precipitated with trichloroacetic acid (TCA) at 10% final concentration, or 0.5 N perchloric acid (PCA), or 0.5 N PCA -- 0.5% phosphotungstic acid (PTA) and kept at least 2 hr at 0-4° C. The precipitates were then pelleted by centrifugation and washed at least twice by resuspension and repelleting in the corresponding cold acid. Drained pellets were dissolved in 0.3 ml 88% formic acid (where TCA or PCA served as acid precipitant) or 0.5 ml N-chlorosuccinimide

(where PCA-PTA served as precipitant). Acid soluble fractions and the dissolved precipitates were counted in either a p-dioxane base liquid scintillation fluid (Bray, 1960) or in a toluene-Triton X-100 (5:1 v/v) base scintillation fluid (Patterson and Greene, 1965) using a Nuclear Chicago Mark I liquid scintillation spectrometer. Counting rates were corrected (where designated dpm) for quenching using an external standard and for background.

Gel slices dissolved in 20% H_2O_2 and aliquots of protein hydrolysates (used for amino acid analysis of whole content, whole membranes, and gel bands of content and membranes) were counted in the toluene-Triton X-100 mixture (containing 0.4% 2,5-diphenyloxazole (PPO) and 0.05% 1,4-bis-2-(4-Methyl-5-phenyloxazolyl)-benzene (Dimethyl POPOP)). The ^{14}C-label was counted at 75-80% efficiency in either a Nuclear Chicago Mark I or a Beckman LS-250 liquid scintillation spectrometer. Comparison within each type of samples indicated uniformity of quenching; thus these data were corrected only for background and remain expressed as counts per minute (cpm).

Chemical Assays. DNA content of lobules was determined by Burton's method (Burton, 1956) on hot (80° C) 0.5 N PCA extracts of washed PCA precipitates of samples of tissue homogenate. Calf thymus DNA was used as standard.

RNA content of homogenates and subcellular fractions was assayed according to the procedure of Blobel and Potter (1968) in which RNA is determined by spectrophotometry on alkaline extracts (0.3 N KOH) of washed PCA precipitates. RNA concentration (mg/ml) was calculated from OD_{260} readings using the extinction coefficient $E^{1\%}_{1cm} = 312$ determined by Munro and Fleck (1966). Bovine plasma albumin (1 and 2 mg/ml) was selected as assay blank in an effort to control for alkaline extraction of material other than ribonucleic acid having substantial absorbance at 260 nm.

Estimations of total protein were performed by three different procedures. Acid precipitates (TCA, PCA, or PCA-PTA) of homogenates

or subcellular fractions were dissolved in 1 N NaOH and assayed according to: 1) the procedure of Lowry et al. (1951), or 2) the microbiuret assay developed from procedures presented by Chase and Williams (1968) and by Gornall et al. (1949) using bovine plasma albumin as a standard, or 3) a procedure based on quantitating amino acid analyses of protein hydrolysates using the fluorescamine method (Stein et al., 1973). This procedure, which assays amino acid residues individually, was applied to hydrolysates of gel bands and to aliquots of whole membrane proteins and whole content proteins.

Enzymatic Assays. Amylase was assayed according to the method of Bernfeld (Bernfeld, 1955) on samples of homogenates and subcellular fractions diluted with 0.2% Triton X-100 containing 0.02 M sodium phosphate (pH 6.9) and 0.02 M NaCl. The assay depends on the liberation of maltose (having reducing groups) from the 1% soluble starch substrate. One unit of amylase activity is defined as: the amount of enzyme which liberates 1 μmole of maltose per min at 30° C.

Deoxyribonuclease (DNase) was assayed using the Kunitz hyperchromicity assay (Kunitz, 1950) as described by Price et al. (1969) at pH 5.0. Highly polymerized calf thymus DNA served as substrate, and Worthington bovine DNase I (2000 units/mg) was used as standard. One unit of DNase is defined as the activity causing an increase in OD_{260} of 1.0 per min per ml (Price et al., 1969) at 25° C.

Ribonuclease (RNase) was assayed according to the pH 7 procedure of Kalnitsky et al. (1959). Yeast RNA (Sigma Fraction VI) was used as substrate and incubation was carried out for 4 min at 37° C. One unit of enzyme activity corresponds to the release of 1.0 OD_{260} unit of acid (25% PCA - 1% uranyl acetate) - soluble oligonucleotide.

Neutral lipase was assayed precisely as prescribed by Bradshaw and Rutter (1972) using as substrate β-naphthyl nonanoate in the presence of sodium taurocholate. The assay quantitates the release of β-naphthol through subsequent reaction with fast blue RR, the extent of color development being determined spectrophotometrically at 540 nm.

β-naphthol concentration was calculated using a millimolar extinction coefficient of 0.16. One unit of lipase activity releases 1 μmole β-naphthol per min at 37° C. Purified porcine pancreatic lipase (kindly supplied by Dr. M. F. Maylie) was used as standard.

Peroxidase was assayed for in granule lysates by the procedure of Klebanoff (1965) using O-dianisidine and 0.3% H_2O_2 at both pH 5 and pH 7. Horseradish peroxidase 5-10 ng/ml served as control for the assay.

The presence or absence of protease activity at pH near neutrality in concentrated granule hypotonic lysates was assessed using the Azocoll assay (Mandl et al., 1953) which follows spectrophotometrically at 520 nm the soluble dye released proteolytically from Azocoll.

Cytochrome oxidase was assayed in subcellular fractions using the procedure of Cooperstein and Lazarow (1951) as modified by Peters et al. (1972), except that reactions were run at 25° C. 0.1 ml of appropriately diluted fractions was added to 1 ml of 41 μM cytochrome C substrate (90% in the reduced form), and the decrease in OD_{550} with time was monitored in a Gilford spectrophotometer equipped with a linear-scale recorder set at a zero absorbance corresponding to substrate fully oxidized with potassium ferricyanide and a full-scale absorbance corresponding to fully reduced substrate (OD_{550}~0.70). Zero order kinetics were approximated by serially diluting (in two-fold increments) enzyme-containing fractions until the plots of the initial decrease in optical density showed little curvature and their slopes for consecutive dilutions changed by a factor of two, indicating half the amount of enzyme. The activity of cytochrome oxidase was converted to units of μmoles cytochrome c oxidized per min using a micromolar extinction coefficient for reduced cytochrome C of 0.019.

N-acetyl-β-glucosaminidase (NAβ-gase) was determined for all subcellular fractions by the method of Findlay et al. (1958). The substrate used was p-Nitrophenyl-N-acetyl-β-glucosaminide, and the assay quantitates the release of p-nitrophenol which in a second step is

determined (in alkaline glycine buffer) by absorbance at 400nm (micromolar extinction coefficient for p-nitrophenol, 0.018). Activity units are μmoles p-nitrophenol liberated per min at 37° C.

Cathepsin D was assayed using the protocol of Gianetto and de Duve (1955). The substrate was a solution of denatured hemoglobin at pH 3.6, and the assay follows the release into acid (TCA)-soluble form of tyrosine-containing peptides; the degradation products are quantitated in the TCA-containing solutions after alkalinization by the assay of Lowry et al. (1951). The activity is expressed as chromogenic equivalents of 1 mg/ml albumin solution simultaneously subjected to Lowry assay.

MATERIALS

L-[4,5-^{3}H]leucine (specific activity 50-58 Ci/mmole, reconstituted [^{14}C]algal protein hydrolysate, and isoproterenol hydrochloride were purchased from Schwartz/Mann Inc., Orangeburg, N. Y. Nutrient Mixture F12 was obtained in liquid form from Grand Island Biological Co., Grand Island, N. Y., and in powder form from Schwartz/Mann Inc. Ilford L4 liquid photographic emulsion was obtained from Ilford Ltd., Ilford, Essex, England. Acrylamide, N,N'-methylene-bisacrylamide and N,N,N',N'-tetramethylethylene diamine (TEMED) for gel electrophoresis were obtained from Eastman Organic Chemicals, Rochester, N. Y. Ammonium persulfate and sodium dodecyl sulfate, also for gel electrophoresis, were obtained from Sigma Inc., St. Louis, Mo. Fluorescamine was synthesized by the chemical research division of Hoffman-LaRoche Inc., Nutley, N.J., and was used solely at the Roche Institute of Molecular Biology. Constant boiling hydrochloric acid for protein hydrolysis was obtained from Pierce Chemical Company, Rockford, Ill.

III. RESULTS

RESULTS

As shown in Fig. 2, the rabbit parotid is composed of closely packed acini. Ducts are few in number, and blood vessels and cellular elements of the connective tissue occur sparsely in the narrow interacinar and interlobular spaces. A single cell type, the acinar cell, is the main constituent of the tissue and accounts for 91% of the cell volume of the gland. The remainder consists of ducts (3%), blood vessels (4%), and connective tissue cells (2%).* Evidently, the parotid is considerably less heterogeneous than the liver; it is slightly more homogeneous than the pancreas having the further advantage over the latter of containing a single secretory cell type.

Morphology of Acinar Cells

The acinar cells have the shape of irregular truncated pyramids whose apices are directed toward the acinar lumina. At the subcellular level they have the characteristic organization of cells producing and accumulating protein for secretion. As illustrated by Fig. 3, they have a well-developed rough endoplasmic reticulum (RER) arranged in arrays of parallel cisternae and a large population of polysomes and ribosomes which is predominantly membrane bound. Their Golgi complex is extensive and consists of the usual elements (stacks of cisternae, small peripheral visicles, and large condensing vacuoles), but its location and orientation are more variable than in other exocrine cells (Palade *et al.*, 1962; Jamieson and Palade, 1967a; Wuhr *et al.*, 1969; Neutra and Leblond, 1966). The secretory granules which occupy half or more of the cell volume even in animals fed *ad libitum*, are large and variable in dimensions (1.1-1.5 μ diameter) and irregular in

*Volume approximations are weight percentages determined from 12 light micrographs (total tissue area 2×10^4 μ^2) by cutting out and weighing the profiles of tissue elements.

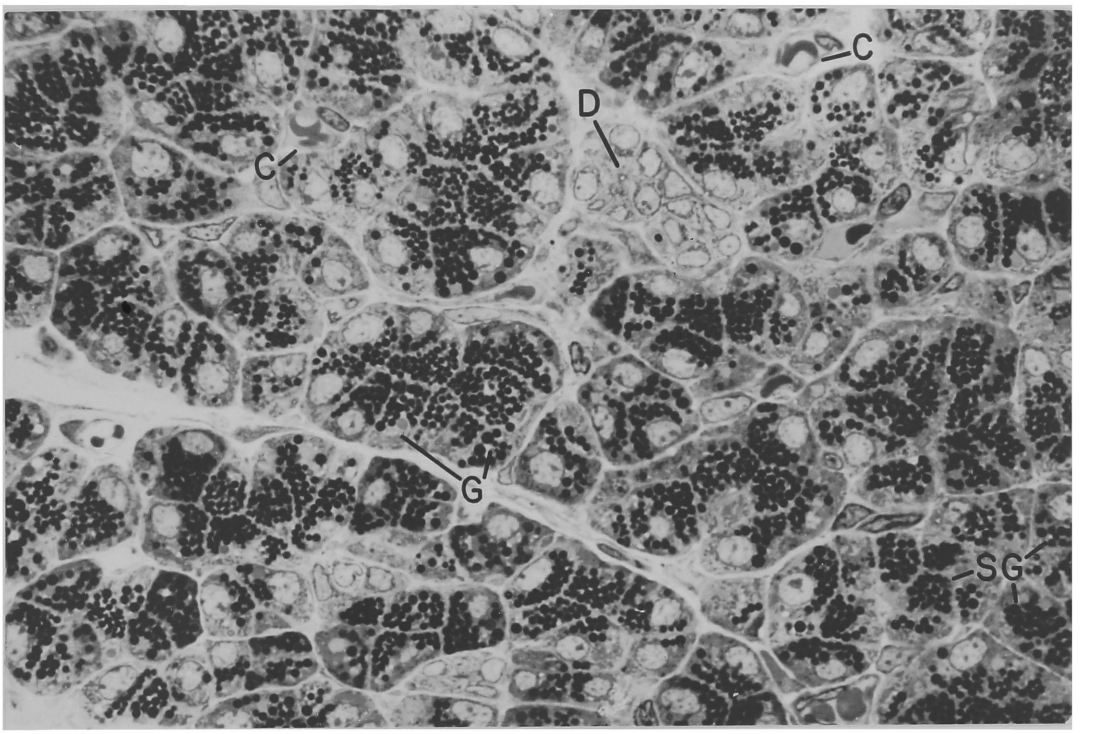
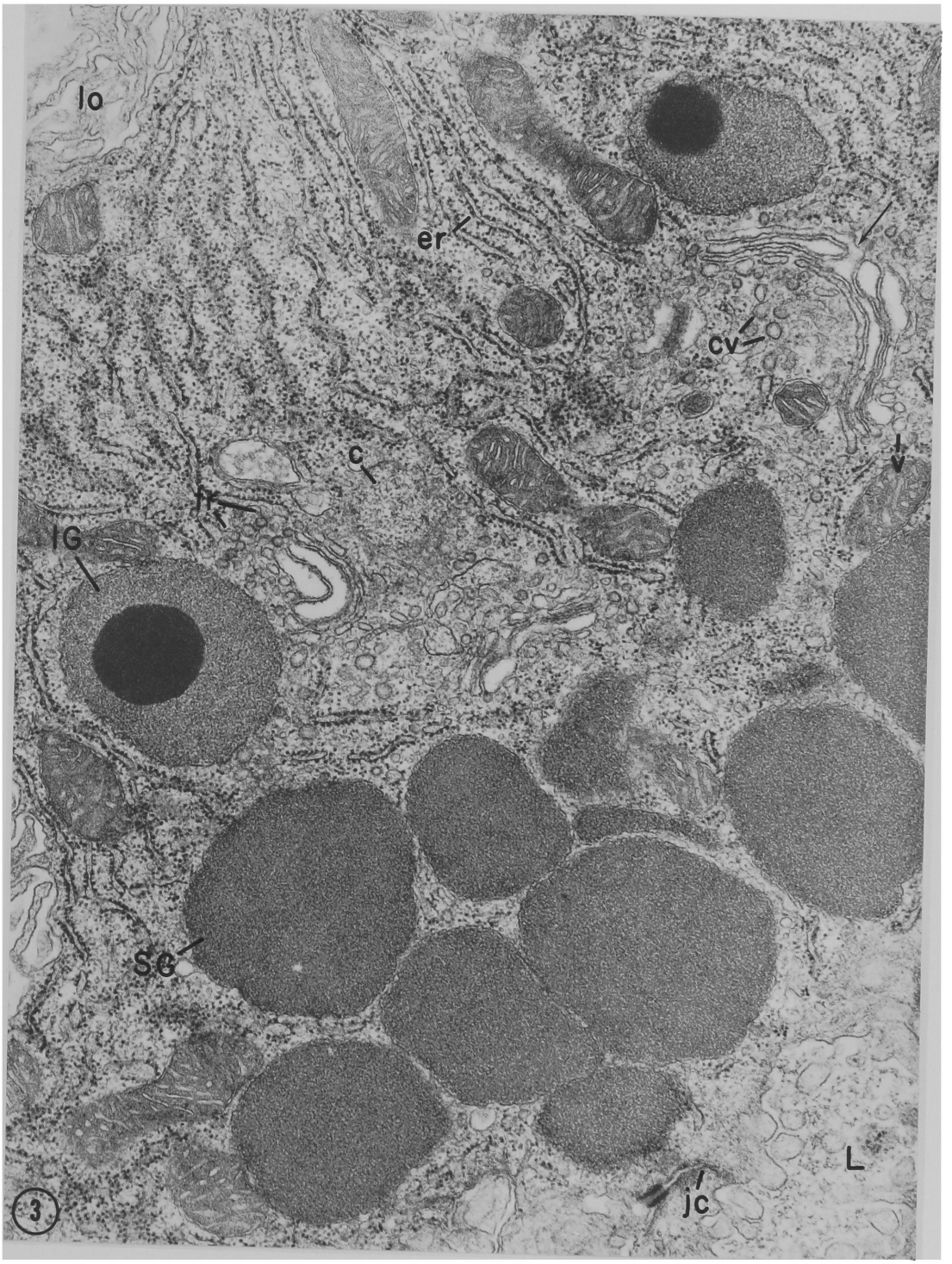


Fig. 2. Light micrograph of parotid tissue showing the compact organization of the lobules. Acini are almost exclusively composed of acinar cells containing basally located nuclei and ergastoplasm and extensive apical accumulations of secretory granules (SG). Faintly stained areas representing Golgi complexes (G) are observed both subapically and immersed in the granule population. Profiles of acinar lumina are not evident at the cellular apices due to the distended state of the cells. Occasional ducts (D) and capillaries (C) penetrate the interacinar and interlobular spaces. Epon section (0.5 μ) of fresh tissue fixed initially with 4% glutaraldehyde in 0.1 M K phosphate (pH 7.2) and postfixed in 1% OsO_4 in the same buffer; stained with 1% methylene blue in 1% Na borate. X 730.

Fig. 3. Electron micrograph of the apical portion of a rabbit parotid acinar cell. Note the parallel arrays of RER cisternae (er); the RER-Golgi transitional elements (tr); the components of the Golgi complex -- peripheral vesicles (v), parallel flattened cisternae showing interconnections (arrow), and condensing vacuole (c); the coated vesicles (cv) located mostly near the exit side of the Golgi complex; the immature granules (IG) containing heterogeneously packed content; the mature secretory granules (SG), irregularly shaped and closely packed; the acinar lumen (L) and junctional complexes (jc); and the lateral plasma membrane outfoldings (lo). Initial fixation in 4% glutaraldehyde-0.15 M Na cacodylate (pH 7.2). X 25,000.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shape. They are closely packed and frequently deformed by contact with one another. According to their content they can be classified in two groups. The first has a homogeneous content, occupies the apical region proper, and represents mature granules. The second has a heterogeneous content (see legends of Figs. 15 & 16), is located subapically usually in the vicinity of the Golgi complex and, as will be shown, represents immature granules. The acinar lumina appear to be either branched or highly contorted since in sections a single cell often appears associated with more than one luminal profile (Fig. 12). The first alternative is supported by the light microscopy literature in which branched intercellular secretory capillaries have been described in the mammalian parotid (Zimmerman, 1927). The cell surface is provided with microvilli on the apical aspect, with typical junctional complexes located laterally in the immediate vicinity of the lumen and with an elaborate system of narrow interdigitating outfoldings extending over the entire lateral surface basal to the complexes. Only the basal aspect is partially free of such protrusions. The nucleus is located in the basal half of the cell, usually surrounded by arrays of RER cisternae.

Morphology of In Vitro Incubated Parotid Lobules

Light and electron microscope observations of lobules incubated for up to 10 hr showed that the general organization of lobules, acini, and acinar cells was quite well preserved provided no initial damage was incurred by the lobule during dissection. If medium was adequately gassed with humidified oxygen-carbon dioxide at 37° fine structure was indistinguishable from that of unincubated tissue. There was little evidence of cell damage which when present caused: a) swelling of individual RER cisternae and disorganization of cisternal arrays; b) swelling and vesiculation of Golgi cisternae; c) nuclear pycnosis; and d) formation of large cytoplasmic vacuoles.

Incorporation of [^{3}H]leucine into Total Lobule Proteins during In Vitro Incubation

When parotid lobules were incubated at 37° C in gassed F12 supplemented with 5 μCi/ml L-[^{3}H]leucine, label was incorporated into protein at a constant rate, very similar to that observed with pancreatic slices (Jamieson and Palade, 1971) for at least 10 hr (Fig. 4). Thus the protein-synthesizing apparatus remained generally and consistently active in lobules subjected to prolonged incubation.

Pulse-Chase Characteristics of Parotid Lobules

The lobule system satisfied an important requirement for the study of the intracellular transport and packaging of secretory proteins. It facilitated the introduction of a short, well-defined pulse (shorter than the shortest time intervals involved in transport from one intracellular compartment to another) of labeled precursor followed by the rapid and efficient washout of unincorporated label by transfer of the tissue to a chase medium. In this manner it was possible not only to identify the appearance of newly synthesized protein at each step of the transport and packaging pathway, but also to assess the degree of concentration attained in packaging protein for export and to estimate the thoroughness of drainage of each compartment.

Dissected lobules were subjected in succession to prepulse incubation at 0° C for 10 min in leucine-free medium, equilibration at 0° C for 10 min in tracer-containing medium, pulsing by warming to 37° C for 4 min, extensive washing with ^{1}H-leucine-supplemented chase medium, and finally chase incubation for times up to 80 min. The results (Fig. 5) showed that chase incubation was successful in promoting both rapid washout of PCA-PTA soluble radioactivity and cessation of incorporation of radioactive leucine into proteins. The chase was fully satisfactory. In the first 5 min, 78% of the initial amount of unincorporated label was washed out from parotid lobules. Labeled precursor remaining in lobules after cessation of chase incubation was two orders of magnitude below the initial post-pulse value;

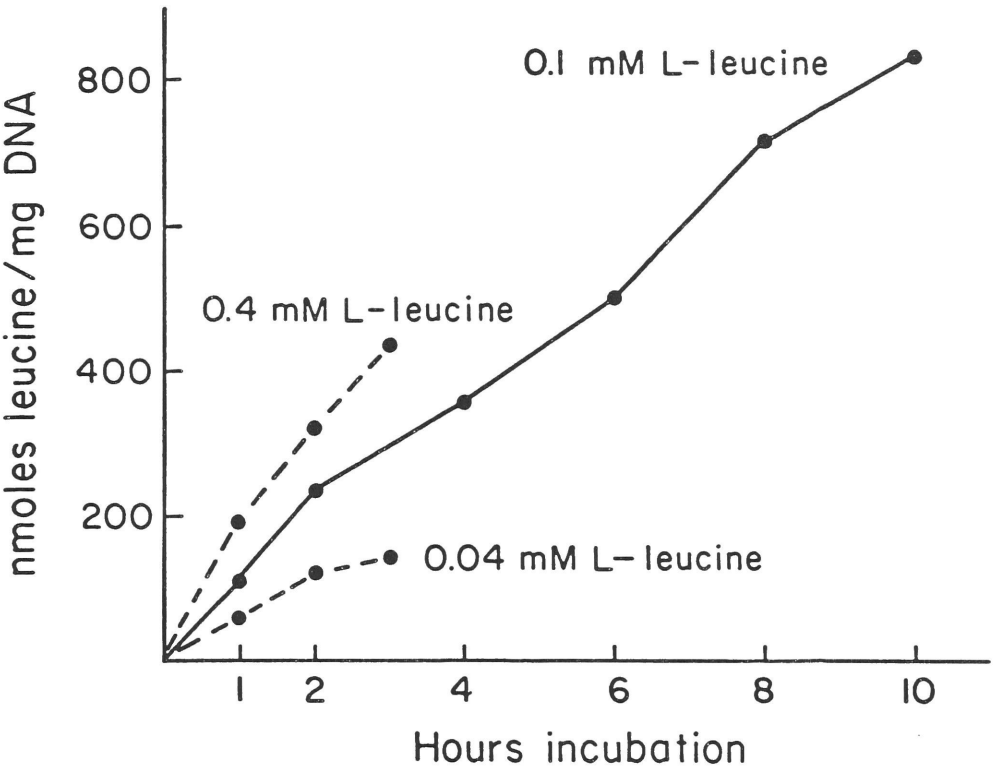


Fig. 4. Incorporation of L-[^{3}H]leucine into total lobule proteins. Lobules were incubated in F12 (containing 0.1 mM L-^{1}H-leucine supplemented with 5 μCi/ml [^{3}H]leucine (58 Ci/mmole). At the indicated times the incubation was terminated and lobules and incubation medium were separated for assay. Aliquots of the medium were precipitated with TCA (10% final concentration) after adding 1 mg/ml carrier bovine plasma albumin. The lobules were homogenized in water, and homogenate samples were precipitated with 10% TCA for assay of radioactivity and 0.5 N PCA for DNA determinations. Total leucine incorporated into protein was calculated from the disintegrations per minute (dpm) found in proteins in the lobules plus the medium and from the specific radioactivity of [^{3}H]leucine in the medium. The solid line depicts results obtained for parotid lobules when the concentration of leucine in the incubation medium was 0.1 mM. The broken lines represent data obtained with guinea pig pancreatic slices incubated in amino acid supplemented KRB containing 5 μCi/ml [^{3}H]leucine (58 C/mmole) and either 0.04 or 0.4 mM ^{1}H-leucine (see Fig. 8 of Jamieson and Palade (1971)).

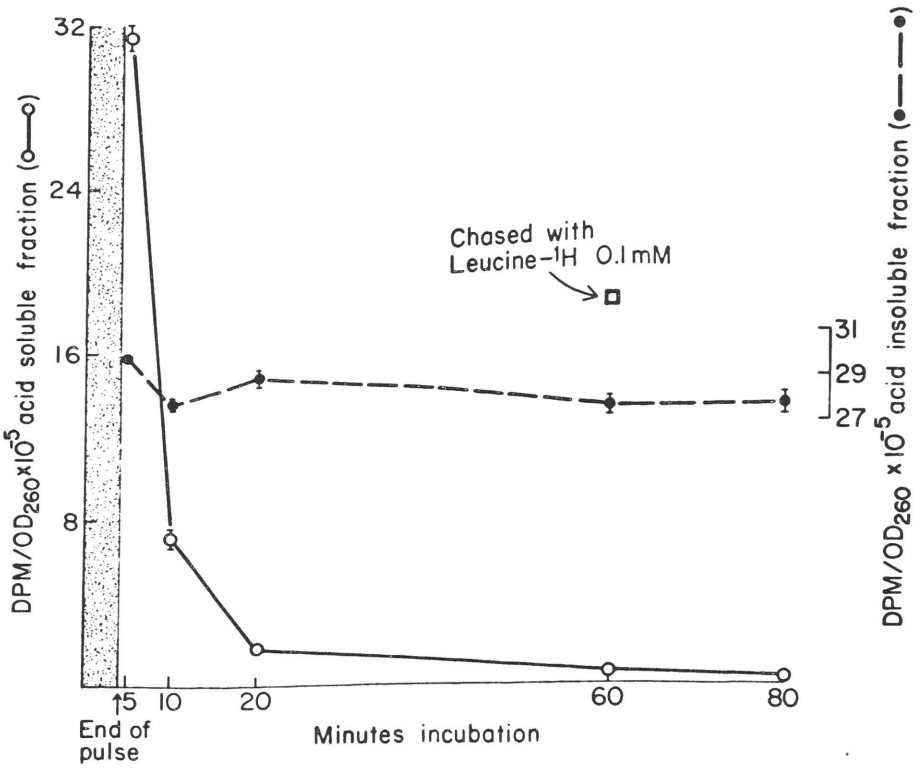


Fig. 5. Kinetics of pulse labeling of parotid lobule proteins. The pre-pulse incubation medium was KRB (25 ml) supplemented with all amino acids except leucine. The pulse medium contained 50 μCi/ml L-[4,5-^{3}H]leucine (1 μM) and ^{1}H-leucine (4 μM). The wash and chase medium was F12 with added ^{1}H-leucine to a final concentration of 4 mM. The pulse: chase ratio of leucine concentration is comparable to that used in the autoradiographic experiments. The chase of unincorporated [^{3}H]leucine (cold PCA-PTA soluble radioactivity) is indicated by the open circles and solid line. Incorporated [^{3}H]leucine (cold PCA-PTA insoluble radioactivity) is shown by the filled circles and broken line. The results (averaged from duplicate samples) are expressed relative to OD_{260} of hot PCA extracts of samples of tissue homogenates initially precipitated and washed with cold PCA. A single sample (shown by the open square) was chased up to 60 min with F12 containing 0.1 mM ^{1}H-leucine and demonstrates the reduced chaseability of acid insoluble radioactivity at lower leucine concentrations.

apparently the balance had equilibrated with the chase medium by isotope and volume dilutions, and further synthesis of labeled proteins was too small to cause a significant increase in acid insoluble radioactivity (Fig. 5).

For assaying the results of a pulse-chase experiment, it is necessary to achieve complete precipitation of the proteins and glycoproteins of the samples. Experiments carried out to assess the efficiency of precipitation showed that TCA and PCA alone gave only partial precipitation, and that the best results were obtained by using PCA-PTA as an acid precipitant. Figure 6 gives the results of an experiment in which PCA was compared to PCA-PTA. Gel filtration of the acid soluble extracts prepared from homogenates of lobules removed from incubation at early (1 min post-pulse) and late (61 min post-pulse) chase time points shows: 1) at 1 min post-pulse OD_{280}-absorbing material was excluded from the Sephadex G10 column especially where PCA alone was used as acid precipitant; however, it was not labeled; the bulk of the radioactivity appeared in smaller molecules which were included in the column; 2) at 61 min post-pulse a large amount of radioactively labeled, OD_{280}-absorbing material in the PCA soluble fraction was excluded from the column. Excluded as well as included radioactivity and absorbance at 280 nm were greatly reduced in the supernate of PCA-PTA precipitates. The PCA-PTA insoluble pellet showed a specific radioactivity 83% higher than that of the PCA insoluble pellet at this time point.

The incomplete precipitation attained with PCA gives misleading results which mimic incomplete chase (since unincorporated as well as incorporated label remains in the supernate) and loss of newly synthesized protein from the tissue (observed as a decrease in PCA-insoluble radioactivity with extended incubation). PTA is therefore a necessary additive to precipitate large labeled molecules which progressively accumulate at later time points since these proteins may become more soluble in time as a result of glycosylation. Sulfosalicylic acid, another precipitant described to be effective for glycoproteins failed to improve results obtained with PCA or TCA alone.

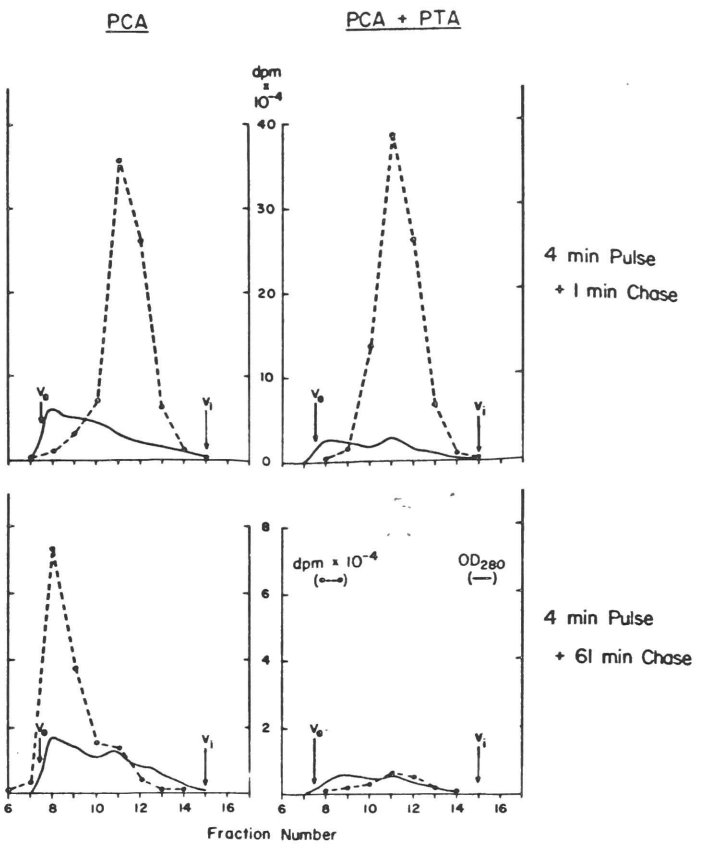


Fig. 6. Comparison of PCA and PCA-PTA as acid precipitants for pulse-labeled proteins from parotid lobules. Lobules were labeled for 4 min in F12 supplemented with 50 μCi/ml [^{3}H]leucine (~1 μM) and incubated post-pulse for 1 min and 61 min. At these times lobules were removed from incubation, homogenized in distilled water, and aliquots of each homogenate were precipitated with PCA and PCA-PTA. The acid soluble supernates were harvested and each subjected to gel filtration on Sephadex G10. Radioactivity and OD_{280} profiles for these filtrations are presented in the four panels of this figure. The data can be compared from panel to panel since the amount of tissue constituting each homogenate was found to be nearly identical by measuring OD_{260} and DNA (Burton, 1956) in hot PCA extracts of acid insoluble precipitates. Evidently, at 1 min post-pulse, OD_{280}-positive material excluded from G10 appears in higher amounts in PCA supernates than in PCA-PTA supernates but radioactivity in both types of acid soluble fraction is comparable in quantity and is included within the column. At 61 min post-pulse, however, radioactivity and OD_{280}-positive material are excluded from the G10 column in substantial amounts for filtration of the PCA supernate; however, the addition of PTA brings about a much more effective precipitation of the radioactivity and OD_{280}-positive material potentially excluded from Sephadex G10.

In Vitro Stimulation of Secretion In Parotid Lobules

Incubated parotid lobules showed excellent sensitivity to stimulation by isoproterenol, an isopropyl analogue of adrenalin known to act as a potent secretagogue for the parotid. To determine the optimal isoproterenol concentration for the system used, parotid lobules were pulse labeled and then incubated post-pulse in the absence (control) or presence of concentrations of isoproterenol ranging from $1 \times 10^{-8} - 1 \times 10^{-4}$ M and the amounts of protein radioactivity discharged into the medium followed over a period of 5 hr. The family of curves in Fig. 7 identifies 1×10^{-6} M as the minimum concentration which gives maximum discharge at maximal rates. Note that increasing the concentration of isoproterenol one order of magnitude above the optimal 1 μM did not modify the kinetics of discharge. In experiments requiring long term incubation without replenishment of medium, and thus secretagogue or experiments in which an amount of tissue greater than ten dissected lobules per flask was subjected to stimulation, the concentration of isoproterenol was increased to up to 5×10^{-6} M with confidence that the higher secretagogue concentration would not have an inhibitory effect on discharge.

Morphologic examination of lobules during continuous in vitro stimulation with 1 μM isoproterenol vividly portrayed the sensitivity of parotid tissue to sympathomimetic agents and its potential for massive discharge of packaged secretory protein. Cells of the lobule shown in Fig. 8 have received twenty minutes stimulation. Lumina are distended and packed with released secretion while cellular integrity appears unaffected although individual cells have a considerably reduced complement of secretion granules. What is particularly striking is the large number of luminal profiles and the extensive cellular surface area delimiting them and thus apparently available for exocytosis; this reinforces the earlier discussion of highly branched secretory capillaries. The insert in Fig. 8 demonstrates exocytosis in progress. The membrane of a secretion granule is seen to have become continuous with the cellular apical membrane following their interaction and fusion;

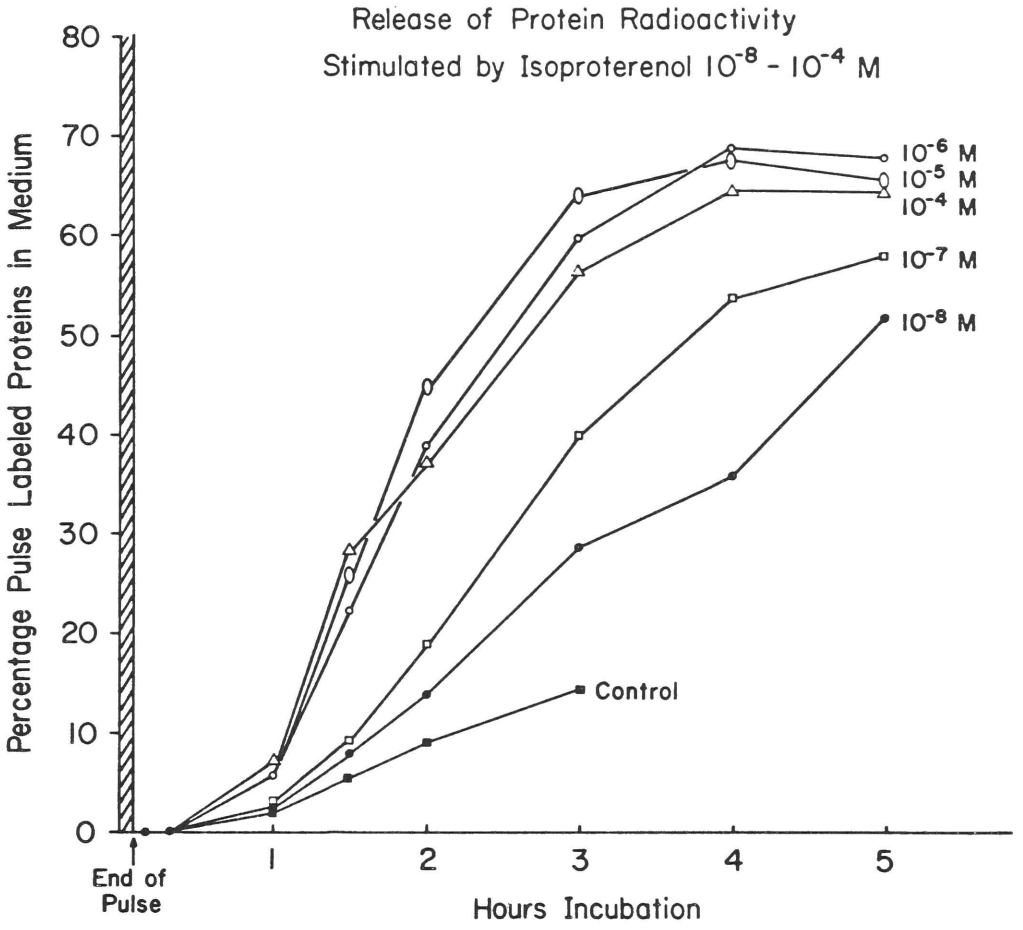
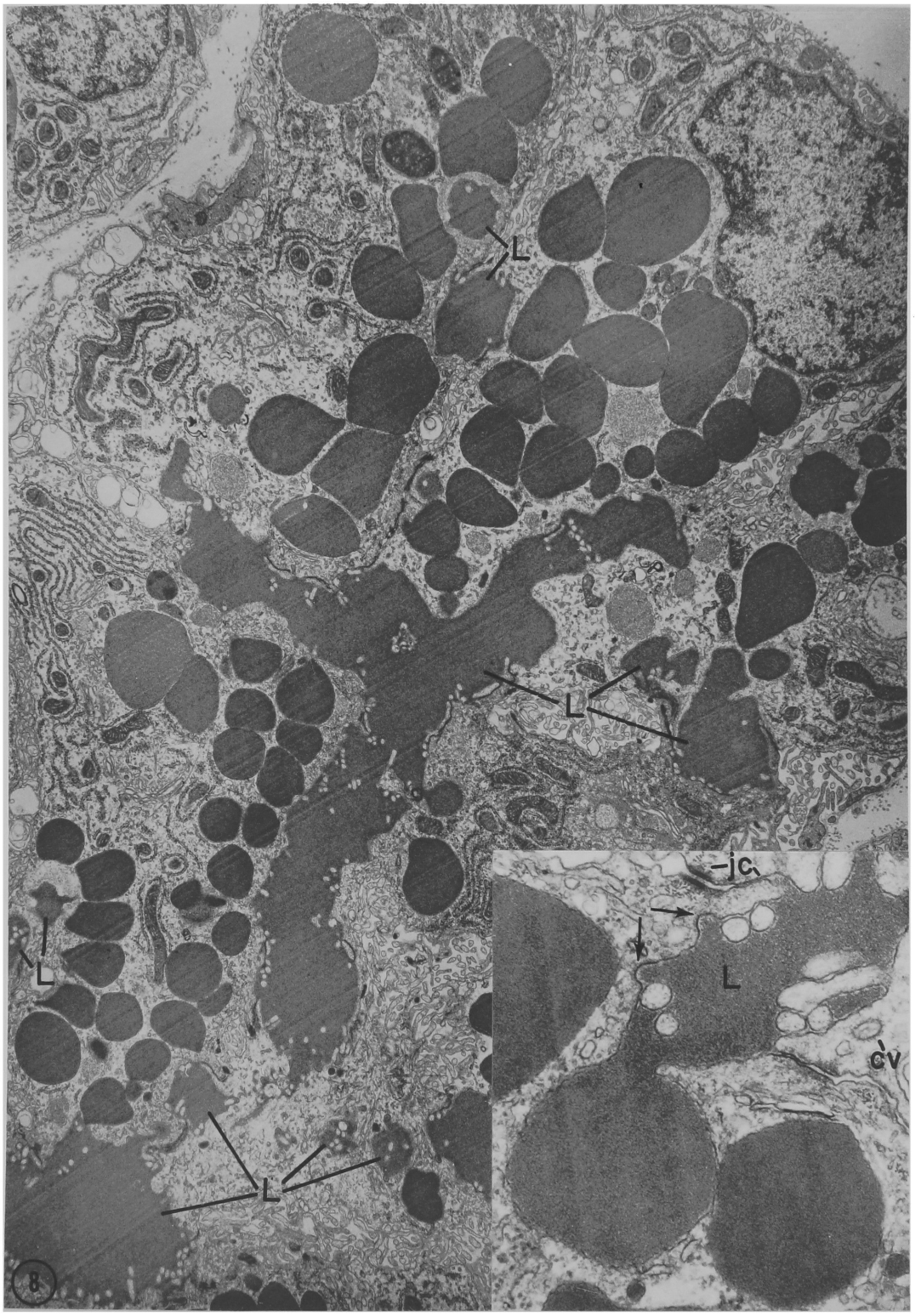


Fig. 7. Release of [^{3}H]leucine-labeled secretory protein in response to different doses of isoproterenol. Lobules were pulse labeled for 4 min in [^{3}H]leucine-supplemented (300 μCi/ml) pulse medium, washed and incubated post-pulse in F12 supplemented with ^{1}H-leucine (4 mM) in the absence or presence of concentrations of isoproterenol differing by an order of magnitude from 1 x 10^{-8} M to 1 x 10^{-4} M. At specified times during incubation 2 ml aliquots of media were removed and replaced with fresh medium containing stimulant (to compensate for secretagogue depletion by medium removal and by oxidation). The medium samples collected during incubation and the tissue at the end of incubation were assayed for acid insoluble radioactivity. The percentage release of labeled proteins was calculated from the data obtained and is plotted versus time of incubation.

Fig. 8. Electron micrograph of a portion of a parotid acinus 20 min after the onset of stimulation with 1 μM isoproterenol. The expanded acinar lumen (L) delineated by apical cell membrane and fused granule membrane evidently is highly branched or contorted, the multiple luminal profiles cut both longitudinally and in cross-section indicating that a large proportion of the apical surface area of each cell is apparently available for exocytosis under conditions of massive discharge. Based on its electron opacity, the released content within the lumen appears to be as concentrated as that still retained within granules. The inset shows a secretion granule in the act of releasing its content to the lumen by the process of exocytosis. Coated vesicles (cv) are often seen in the vicinity of acinar lumina especially under conditions of stimulated discharge, and the expanded apical cell membrane is marked by discrete coated regions (indicated by arrows). Junctional complex (jc). X 9,000. (Inset X 34,000).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consequently, the packaged granule content has gained access to the extracellular luminal space.

By ninety minutes isoproterenol stimulation, the acinar cell population was practically devoid of recognizable secretion granules, as shown in Fig. 9, and once distended apical lumina have returned to nearly original size, the microvilli on apposing luminal faces once again becoming interdigitated. A few immature granule-like structures were evident in the vicinity of Golgi regions.

Synthesis of Secretory Versus Non-Secretory Proteins

To study the intracellular transport of secretory proteins requires not only that newly synthesized protein be pulse labeled for a well-defined short time interval, but also that rates of synthesis for exportable proteins be considerably different (higher) from those of non-exportable proteins (Jamieson and Palade, 1967). To assess whether there was a large difference in the relative amounts of [^{3}H]leucine incorporated into secretory and non-secretory proteins in parotid acinar cells, lobules were subjected in succession to pulse labeling, chase incubation of sufficient duration to label secretory granules and further incubation in the presence or absence of 1 μM isoproterenol to determine the percentage of incorporated radioactivity and amylase discharged (Jamieson and Palade, 1971). Figure 10 shows that through the first 2 hours of incubation in the presence of isoproterenol 61% of the protein radioactivity and 90% of the total amylase are released into the incubation medium. During subsequent incubation from 2 to 4 hours an additional 5% of both incorporated radioactivity and amylase appears in the medium at a rate comparable to that of secretion from control (unstimulated) lobules. Thus during the pulse approximately two-thirds of the labeled protein precursor is incorporated into secretory protein eventually exported from acinar cells. The remaining one-third of the radioactivity represents both non-secretory protein retained for intracellular use and additional secretory protein not yet cleared from the intracellular transport and lobular duct systems.

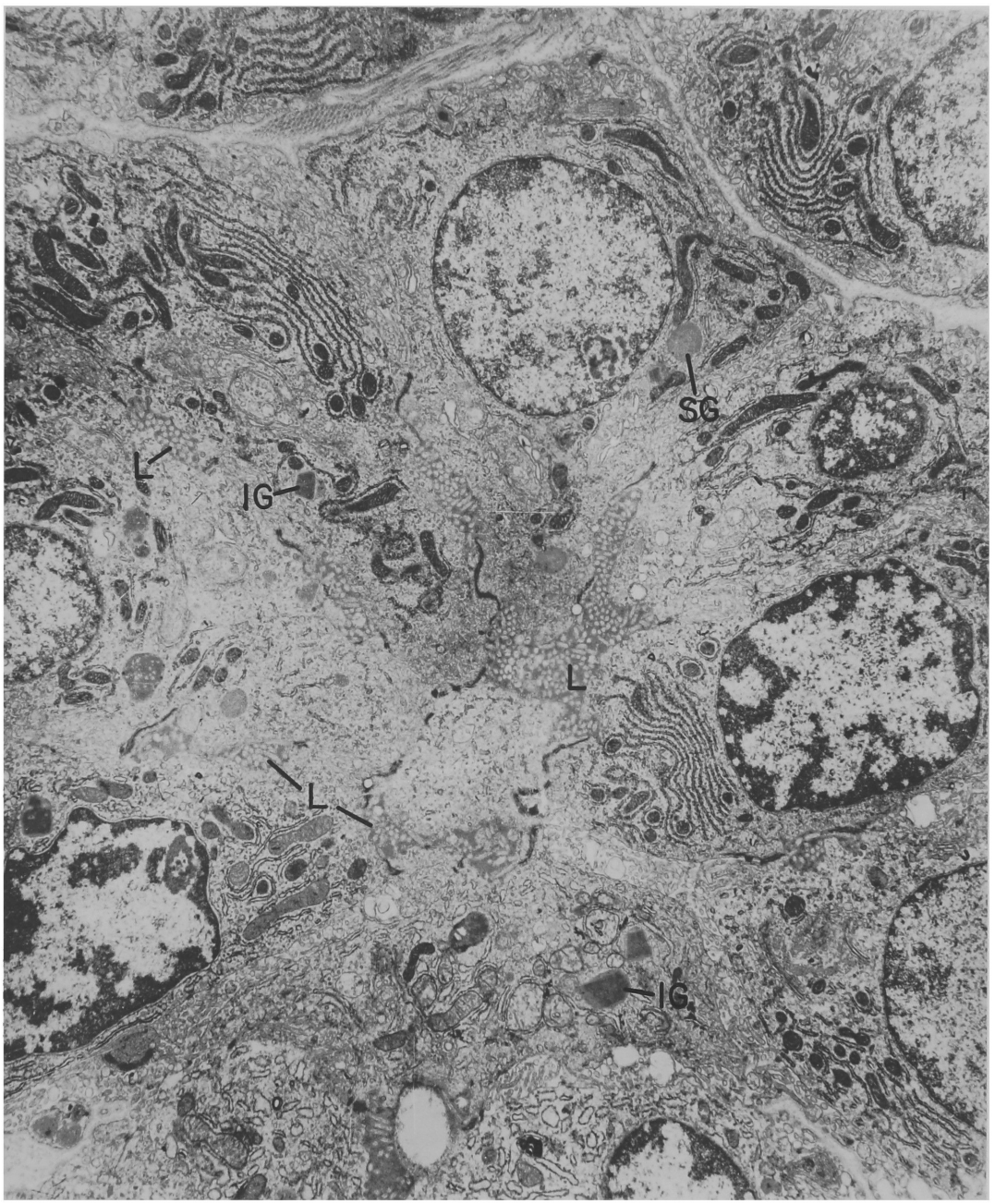


Fig. 9. Electron micrograph of an acinus of a parotid lobule after 90 min incubation in the presence of 1 μM isoproterenol. Evidently, the tissue is practically devoid of secretion granules (SG), and the apical lumen (L), although it apparently still contains released secretion, has undergone a large decrease in volume with microvilli on apposing luminal faces reassuming the interdigitating arrangement characteristic of unstimulated tissue. Immature granule (IG). X 8,500.

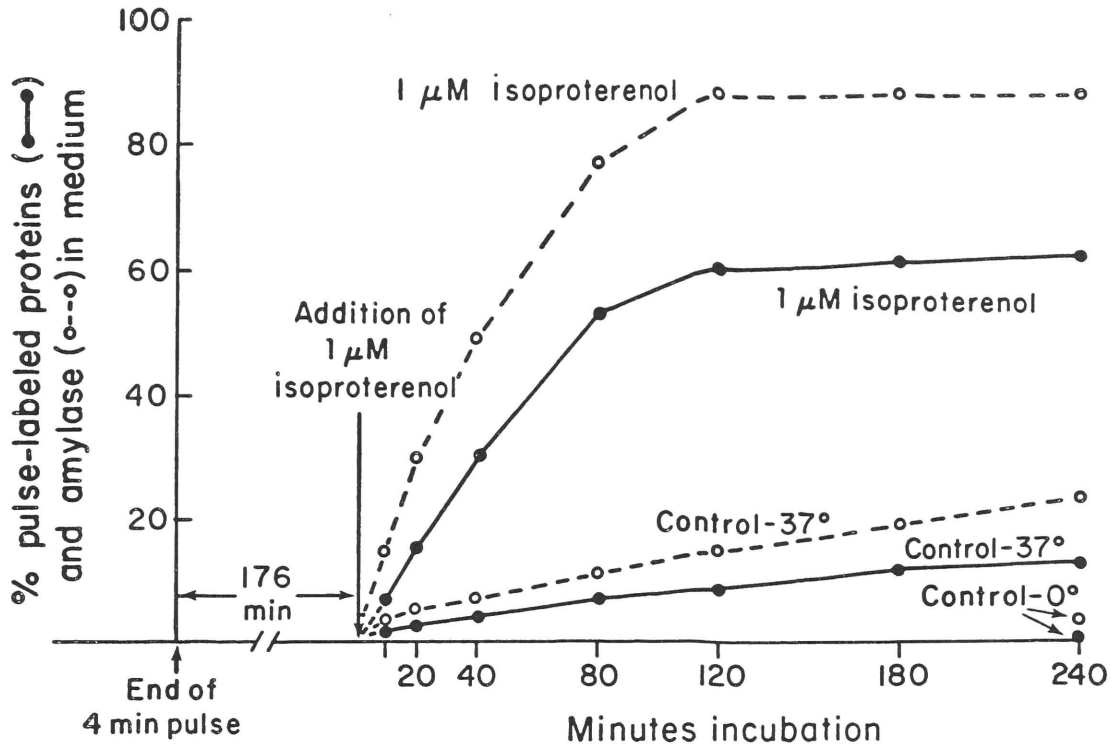


Fig. 10. Discharge of [^{3}H]leucine-labeled secretory protein and amylase from parotid lobules. Lobules were labeled for 4 min in pulse medium containing 300 μCi/ml L-[4,5-^{3}H]leucine (5.4 μM) washed and chase incubated in F12 supplemented with ^{1}H-leucine (4 mM final concentration) for 176 min to allow the labeled secretory proteins to reach the secretion granule stage, and then divided into three groups and incubated further in leucine supplemented F12 in the presence (at 37°) or absence (at 0° and 37°) of 1 μM isoproterenol to stimulate discharge of granule content. At the specified times, 2 ml aliquots of incubation medium were removed and immediately replaced by the same volume of fresh medium. Isoproterenol was included in the replacement medium to compensate for secretagogue depleted by medium removal and by oxidation. The medium aliquots collected during incubation as well as the tissue at the end of incubation were assayed for protein radioactivity and amylase. Percentage release of labeled protein and amylase was calculated from the data obtained. Note the very low level of release of amylase from lobules during prolonged 0° exposure, which demonstrates that the enzyme leakage observed by Schramm et al. (1965) is not a problem in the present system. For the sake of graphical clarity, only the final points for the 0° control have been plotted. Release at 0° of labeled protein and amylase increases monotonically to the final values of 0.5% and 4%, respectively.

Binding of Soluble (Unincorporated) L-[4,5-^{3}H]leucine during Fixation for Autoradiography

Aldehydes were found to be obligatory as primary fixatives for a satisfactory structural preservation of parotid tissue. Osmium tetroxide alone, even in hypertonic solutions did not prevent the swelling, explosion, and extraction of secretory granules. Since significant binding of unincorporated [^{3}H]leucine and [^{3}H]glucosamine during fixation in the presence of glutaraldehyde has been reported in autoradiographic studies of Ashley and Peters (1969) on rat liver and Wuhr et al. (1969) on rat thyroid, it was necessary to assess the extent of binding of soluble label during tissue fixation. Such binding represents a potential source of error in autoradiography for specimens fixed either at the end of the pulse or after a short chase incubation.

Parotid lobules were pulsed for 4 min under three different conditions: 1) normal pulse at 37° C, 2) pulse at 37° C in the presence of 5×10^{-4} M cycloheximide (CHI) known to inhibit protein synthesis by 95-98% in guinea pig pancreatic slices (Jamieson and Palade, 1968), and 3) pulse at 0°C. After the pulse, lobules were fixed in several changes of either 2% glutaraldehyde, 2% formaldehyde or 10% formaldehyde (Ashley and Peters, 1969). The fixed tissue was weighed and dissolved in hot 1 N NaOH; the digest was assayed for radioactivity. Label bound in the absence of protein synthesis (pulse conditions 2 and 3) is assumed to approximate the level of chemical (fixative) binding expected in autoradiographic experiments. The results are presented in Table III as percentage of the specific radioactivity of specimens pulsed under normal conditions and fixed post-pulse in 10% formaldehyde. The table shows that the presence of glutaraldehyde in the fixative increased the specific activity of bound label by 57% for tissue pulsed under normal conditions and increased binding eight-fold in tissue pulsed at zero degrees in the absence of protein synthesis. Chemical binding of the [4,5-^{3}H]leucine by the fixative used throughout the autoradiographic experiments (10% formaldehyde, 0.175 M potassium phosphate, pH 7.2) accounted for, at maximum, 10% of the tissue-associated radioactivity.

Table III

Binding of Unincorporated [^{3}H]leucine to Tissue by Aldehyde Fixatives

Experiment	Sample	Aldehyde Fixative	% Specific Activity
I	1. Normal Pulse	10% formaldehyde	100*
	2. Pulse in CHI	10% formaldehyde	10
	3. Pulse at 0°	10% formaldehyde	2
II	1. Normal Pulse	10% formaldehyde	100*
	2. Pulse at 0°	10% formaldehyde	6
	3. Normal Pulse	2% formaldehyde + 2% glutaraldehyde	157
	4. Pulse at 0°	2% formaldehyde + 2% glutaraldehyde	46

*100% is arbitrarily chosen as the specific activity of label found in lobules subjected to normal pulse incubation followed by fixation in 10% formaldehyde. It corresponds to specific activities of 142,000 disintegrations per min/mg wet weight in Experiment I and 75,300 disintegrations per min/mg wet weight in Experiment II.

# # #

For all conditions the pulse medium consisted of KRB supplemented with amino acids, L-[^{3}H]leucine being present at a concentration of 300 μCi/ml (5.4 μM); for condition 2, Experiment I only, cycloheximide (CHI) was added to a final concentration of 5×10^{-4}M. The pre-pulse incubation medium was leucine-free. Aldehyde fixation was performed for 22 hr in either 10% formaldehyde buffered by 0.2 M (Expt. I) or 0.175 M (Expt. II) phosphate (pH 7.2) or in 2% formaldehyde, 2% glutaraldehyde 0.175 M potassium phosphate (pH 7.2).

This figure can be expected to decrease with increasing times of chase incubation. Thus in autoradiographic experiments silver grains primarily localized label incorporated into protein, and errors caused by chemical binding were negligible.

Autoradiographic Studies

Since it was found that the lobule system could satisfactorily be pulse labeled, that a large difference existed between the rates of synthesis of exportable and non-exportable proteins, and that conditions of fixation minimizing binding of unincorporated [^{3}H]leucine could be obtained, autoradiographic studies were performed to determine the rates of intracellular transport and the efficiency of compartment drainage.

Parotid lobules were pulse labeled for 4 min with L-[4,5-^{3}H] leucine (300 μCi/ml; 5.4 μM) and incubated post-pulse for times ranging from 1 to 356 min in chase medium containing 4 mM ^{1}H-leucine. During tissue fixation and subsequent preparation for electron microscopy, label extraction was monitored in parallel samples by following the radioactivity which appeared in fixing and dehydrating fluids and comparing it with that remaining in completely processed, Epon-infiltrated tissue subsequently dissolved in hot 1 N NaOH. The dissolved tissue accounted for 90% of the total radioactivity recovered, the aldehyde fixatives for 6-7%, and the subsequent osmium tetroxide fixative and dehydrating and embedding fluids for 3%. Thus label retention during tissue processing was nearly complete.

Light Microscope Autoradiography. Thick section, 0.5 μm, were cut from tissue at all time points and prepared for autoradiography. These specimens indicated in a general way the movement of incorporated [^{3}H]leucine from basal cytoplasm (chase: 1-6 min) to the centrosphere region (chase: 11-56 min) and finally to secretory granules located primarily in the basal part of the apical granular mass (chase: 56 min and longer). Adequate grain density at the light microscope level was obtained with 6 days of exposure for samples chased for 1-116 min and with 9 days of exposure for samples chased for 176-

356 min. Thus approximately 6 and 9 weeks of exposure were required for autoradiography at the electron microscope level.

Light microscope autoradiography showed that lobules were not uniformly labeled during the [^{3}H]leucine pulse: a gradient of label from the periphery to the center of the lobules was observed with centermost acini showing lower grain density. Nevertheless, the rate of intracellular processing of labeled protein was uniform throughout the thickness of the lobule despite the uneven penetration of the [^{3}H]leucine precursor. Thus intracellular transport of incorporated label was carried out in synchrony in the entire cell population.

Electron Microscope Autoradiography. Electron microscope autoradiography was performed on thin sections cut from the same material used for light microscope autoradiography.

After 1 min chase (Fig. 11) the label was localized over the cytoplasmic regions occupied by the RER with relatively few grains appearing over the Golgi complex and secretory granules.

By 11 min chase (Fig. 12) a large fraction of the label had converged to the smooth membranes of the Golgi complex, although the majority of the grains was still associated with the RER.

After 26 min chase incubation (Fig. 13) labeled protein was primarily associated with the Golgi complex. Autoradiographic grains marked all the elements of the complex including its stacked cisternae but were mainly concentrated over distended cisternae and condensing vacuoles located on the exit side of the stacks. At this time the RER was noticeably less labeled, and labeled granules were very few in number. Thus the concentrative function that the Golgi complex plays in the packaging of secretory product had become evident.

By 36 min chase (Fig. 14) the concentrated secretory material associated with cisternae and condensing vacuoles on the Golgi exit face was heavily labeled. Multiple concentrative sites were evident in single acinar cells. The RER had few residual grains and secretory granules were still devoid of label.

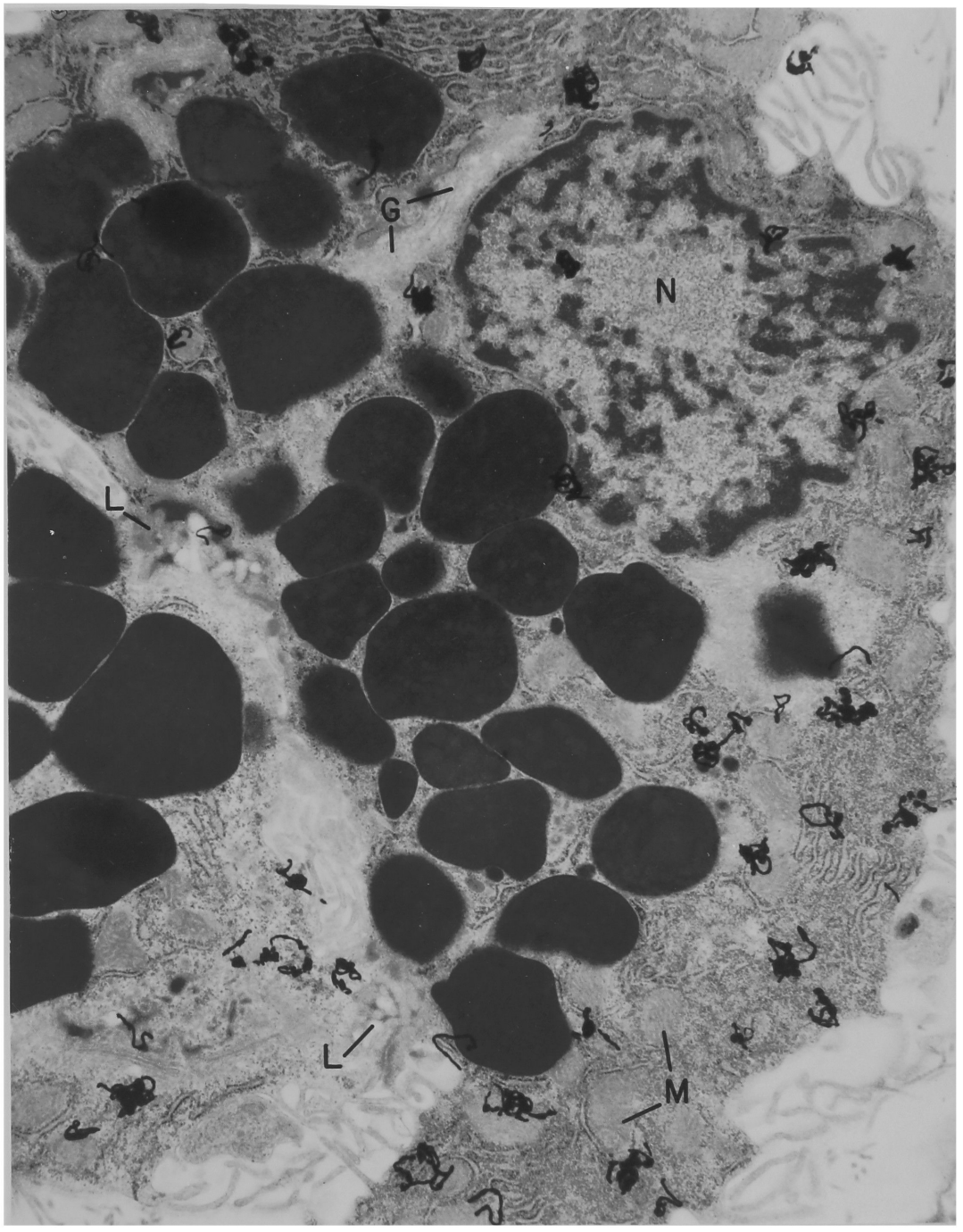


Fig. 11. Autoradiograph of an acinar cell after 1 min chase incubation following a 4 min pulse with [^{3}H]leucine. The autoradiographic grains are located almost exclusively over the RER. Note the two profiles of acinar lumina (L) associated with this cell. The nucleus (N), RER cisternae, and particularly the intercellular spaces show the effect of partial water removal by the hypertonic 10% formaldehyde fixation. Mitochondrion (M); Golgi complex (G). X 20,000.

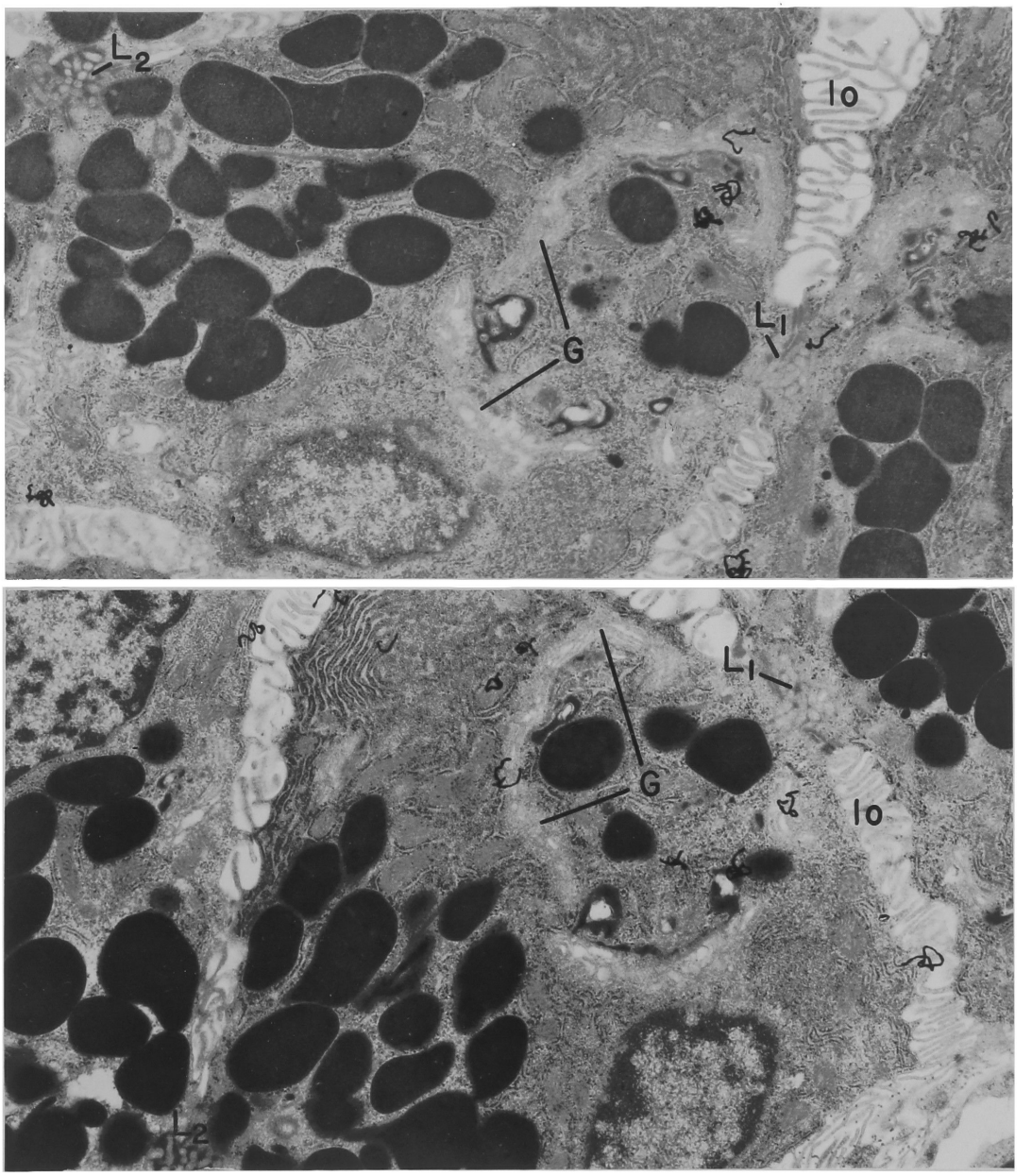


Fig. 12. Autoradiograph of two nearly successive sections of the same cells after 11 min chase incubation. Note that the long stacks of Golgi cisternal elements (G) are labeled and that labeling patterns are different in the two sections. The extensive Golgi complex seen in the two sections is probably associated with the acinar lumen L_1. Its position and orientation relative to the main mass of secretion granules of the cell appear to be the reverse of those typically observed in the guinea pig pancreas. The main mass of granules is polarized toward the acinar lumen L_2. It may extend over the Golgi complex in a different plane and/or may be associated with another Golgi complex located at a different level within the cell. Lateral outfoldings (lo). X 12,000.

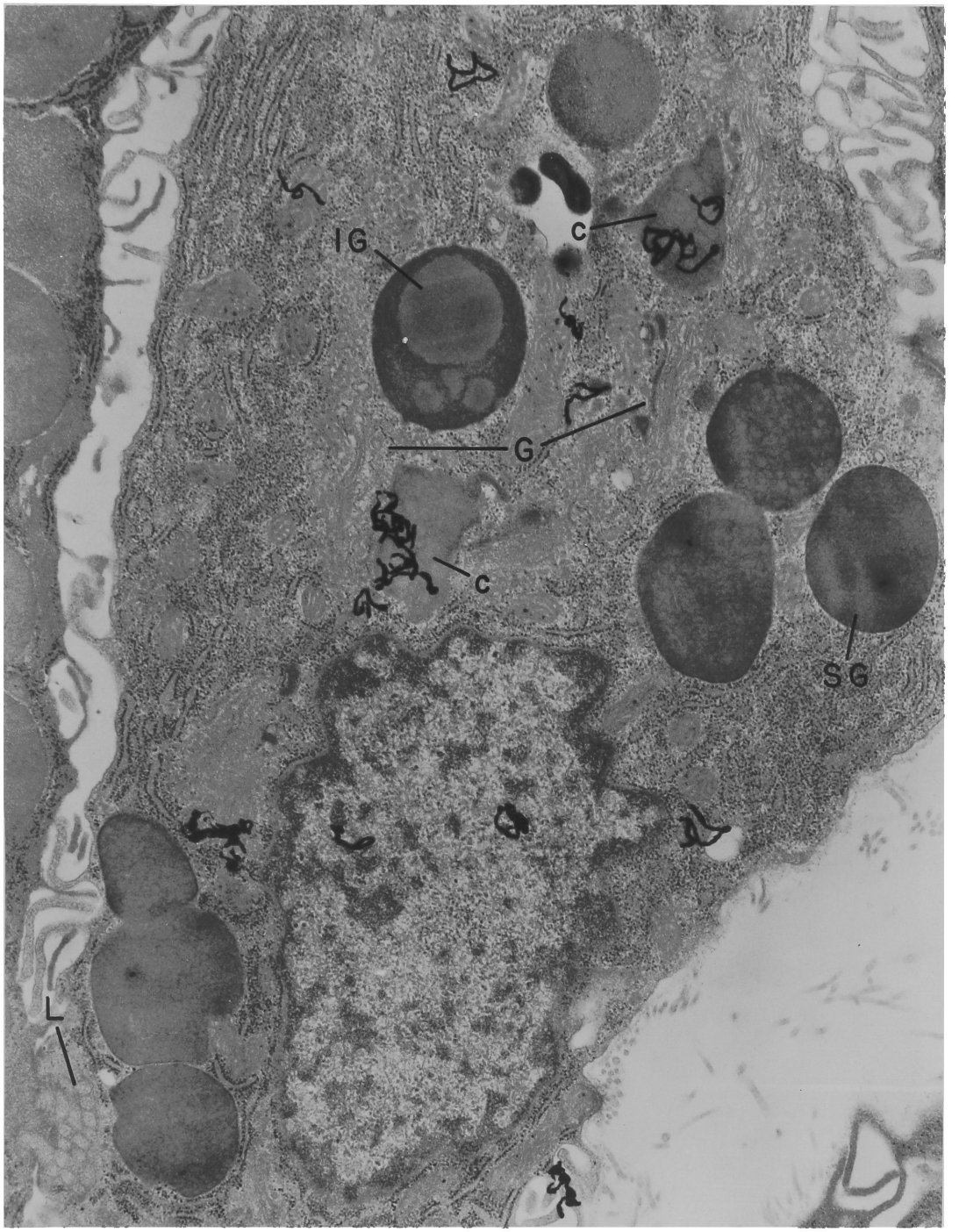


Fig. 13. Autoradiograph of a parotid acinar cell after 26 min incubation in chase medium. The concentrative function of the Golgi complex has become evident since numerous autoradiographic grains are seen over condensing vacuoles (c). The label has migrated in large part from the RER, but secretory granules (SG) are still unlabeled. Golgi cisternae (G); immature granules (IG); acinar lumen (L). X 20,000.

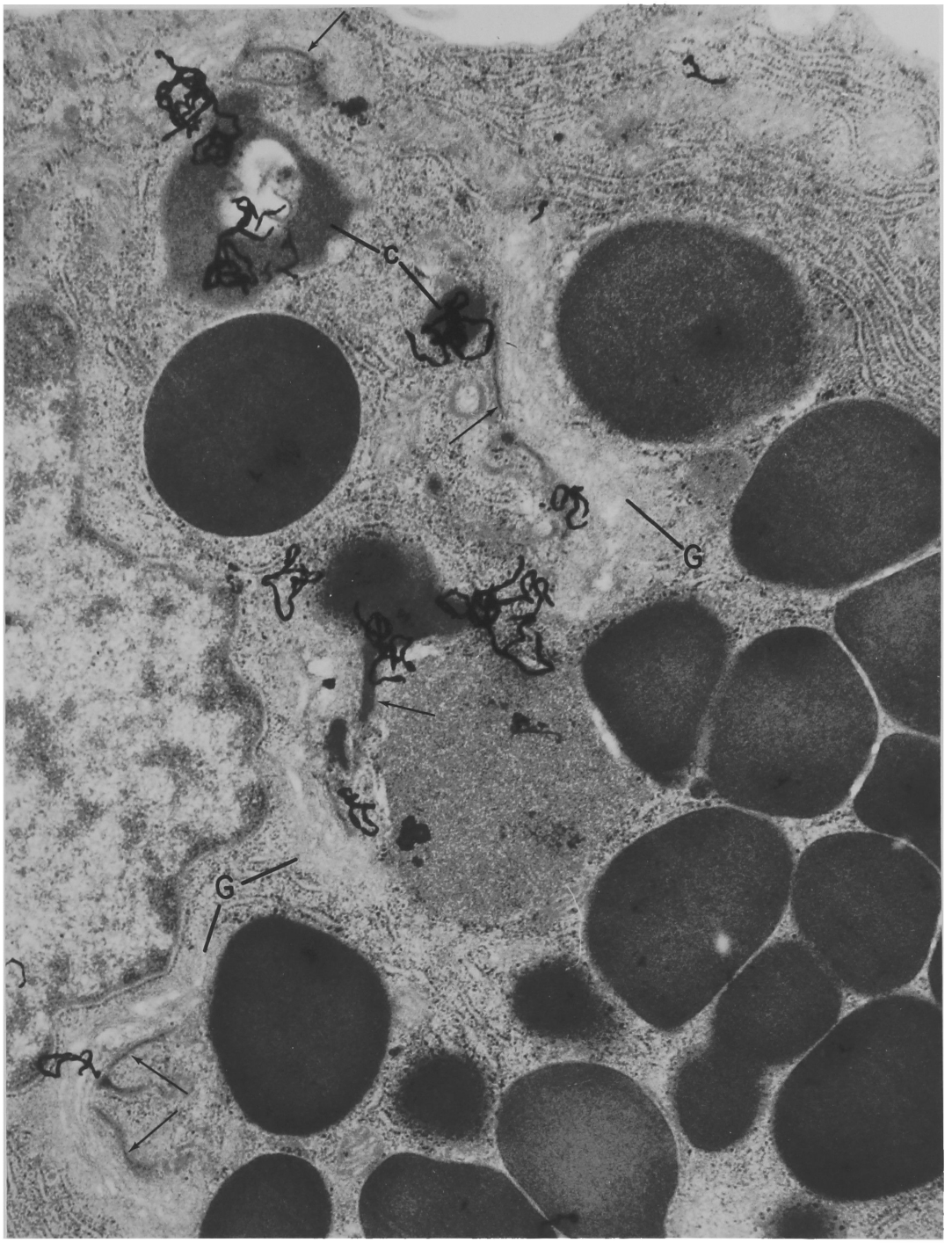


Fig. 14. Autoradiograph of a portion of an acinar cell 36 min post pulse. Multiple concentrative sites on the exit side of the Golgi complex are indicated by the presence of condensing vacuoles (c) and cisternae (arrows) containing content of high density. The label marks these condensing vacuoles and exit sides of the stacks. Label remaining over the RER is minimal. Golgi cisternae (G). X 31,000.

After 56 min and especially after 86 min (Fig. 15) and 116 min (Fig. 16) chase, the label was concentrated over granules which: a) were close to, but not directly associated with, the Golgi region, b) had not yet joined the cell's granule storage population, and c) possessed an obvious substructure, possibly reflecting uneven concentration of secretory product. On the basis of these three characteristics and especially considering the time of their labeling, these structures can be identified as immature secretory granules. After 116 min chase some label still trailed over the Golgi complex, but silver grains were not yet associated in significant numbers with mature, homogeneously dense granules. During late stages of intracellular transport the rabbit parotid acinar cell was slower than the guinea pig pancreatic exocrine cell, in which after chase incubation of the same duration mature pancreatic zymogen granules were both heavily labeled and randomized in each cell's granule population.

At 176 min (Fig. 17) and later, the label was found in progressively greater proportions over granules which had attained homogeneous density and had become part of the cell's accumulated granule population, but most of the labeled granules remained basally located (removed from the cell apex) within this population. It appeared as if granule movement relative to one another was severely restricted, probably because of tight packing. Autoradiographic grains overlying immature granules markedly decreased from 176 min to 356 min chase.

To quantitate the autoradiographic grain distribution over the various subcellular components, grain counts were performed on low magnification electron micrographs, and the results are given in Table IV and Fig. 18. The wavelike passage of pulse-labeled secretory protein through intracellular compartments: RER → Golgi → immature granules → mature secretion granules is readily observed. After 1 min chase the bulk of the radioactive label was found associated with cytoplasmic regions occupied by the RER (~78%). At subsequent times during chase incubation there was a progressive decrease of label associated with the RER to a level of about 20% of the total grains. The residual label

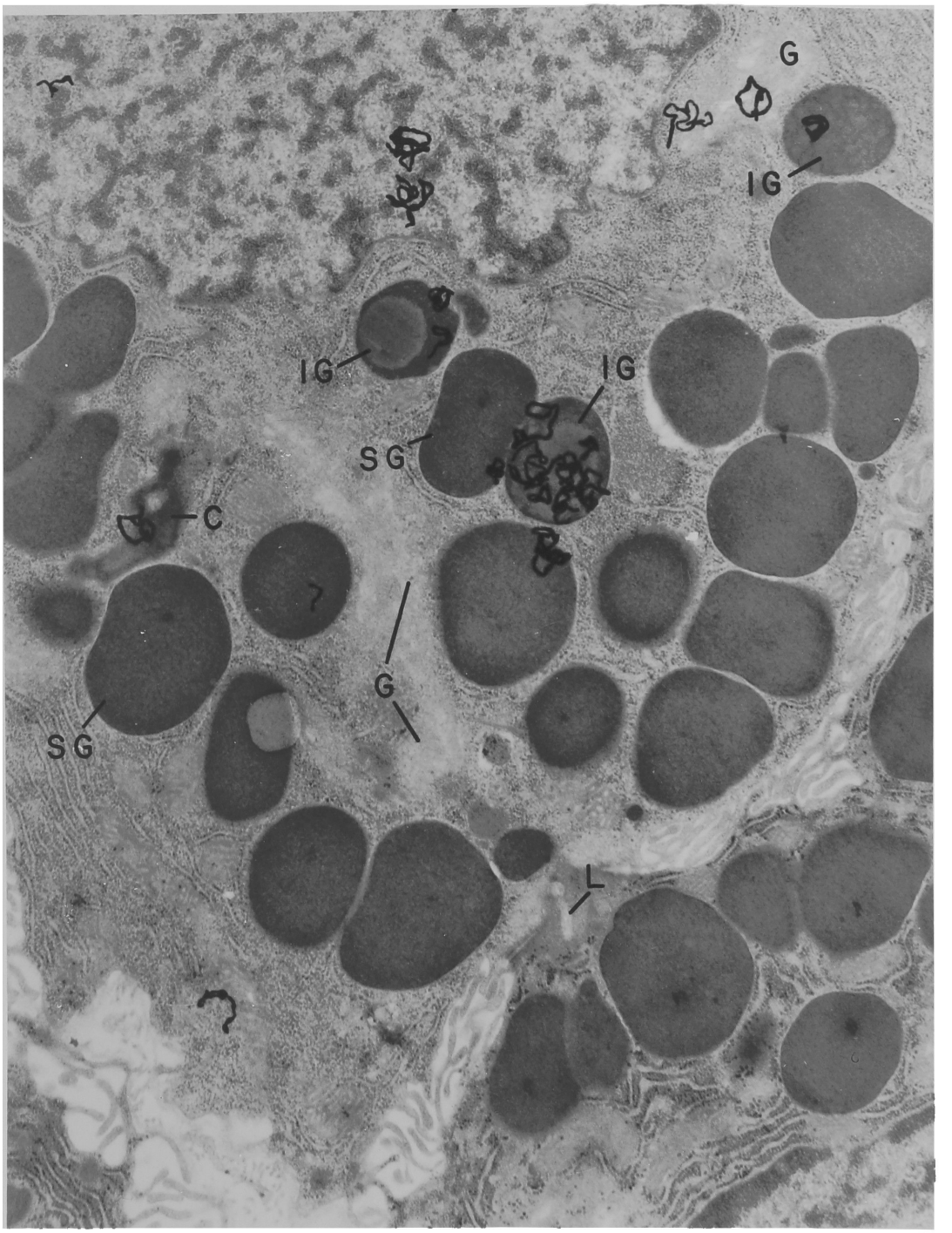


Fig. 15. Autoradiograph of part of an acinar cell after 86 min chase incubation. Immature granules (IG) recognizable by their heterogeneously packed content are highly labeled while the Golgi complex (cisternae (G) and condensing vacuoles (c)) is partially cleared of labeled proteins. Mature secretory granules remain unlabeled.
Lumen (L). X 17,000.

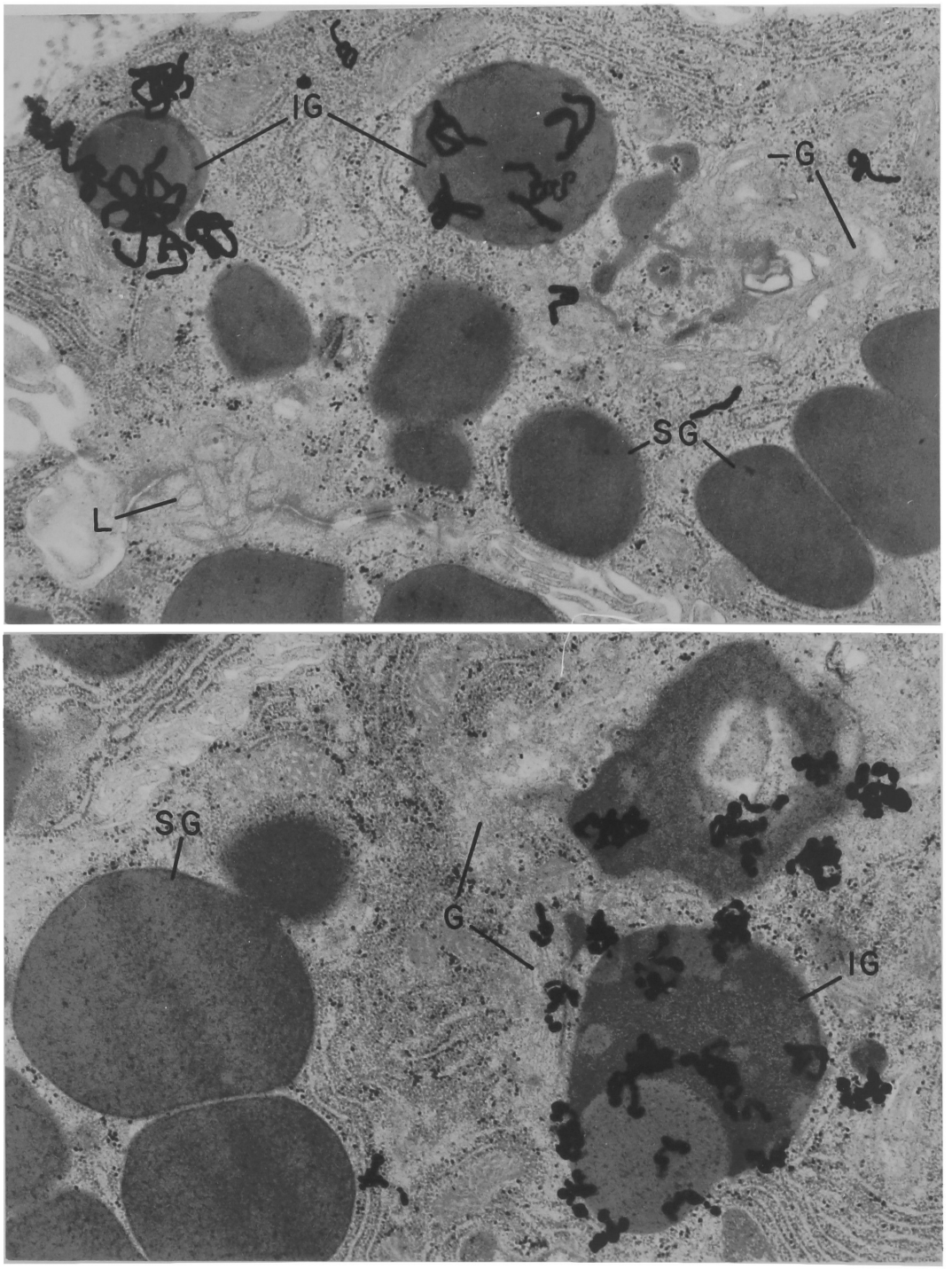


Fig. 16. Two autoradiographs of portions of acinar cells 116 min post pulse. They demonstrate the retardation of intracellular transport of secretory proteins in the rabbit parotid relative to the guinea pig pancreas. Mature secretion granules (SG), highly labeled at this time in pancreatic exocrine cells, still are devoid of label, while immature granules (IG) are marked by highly concentrated autoradiographic grains. Golgi cisternae (G); lumen (L). Both pictures: X 20,000.

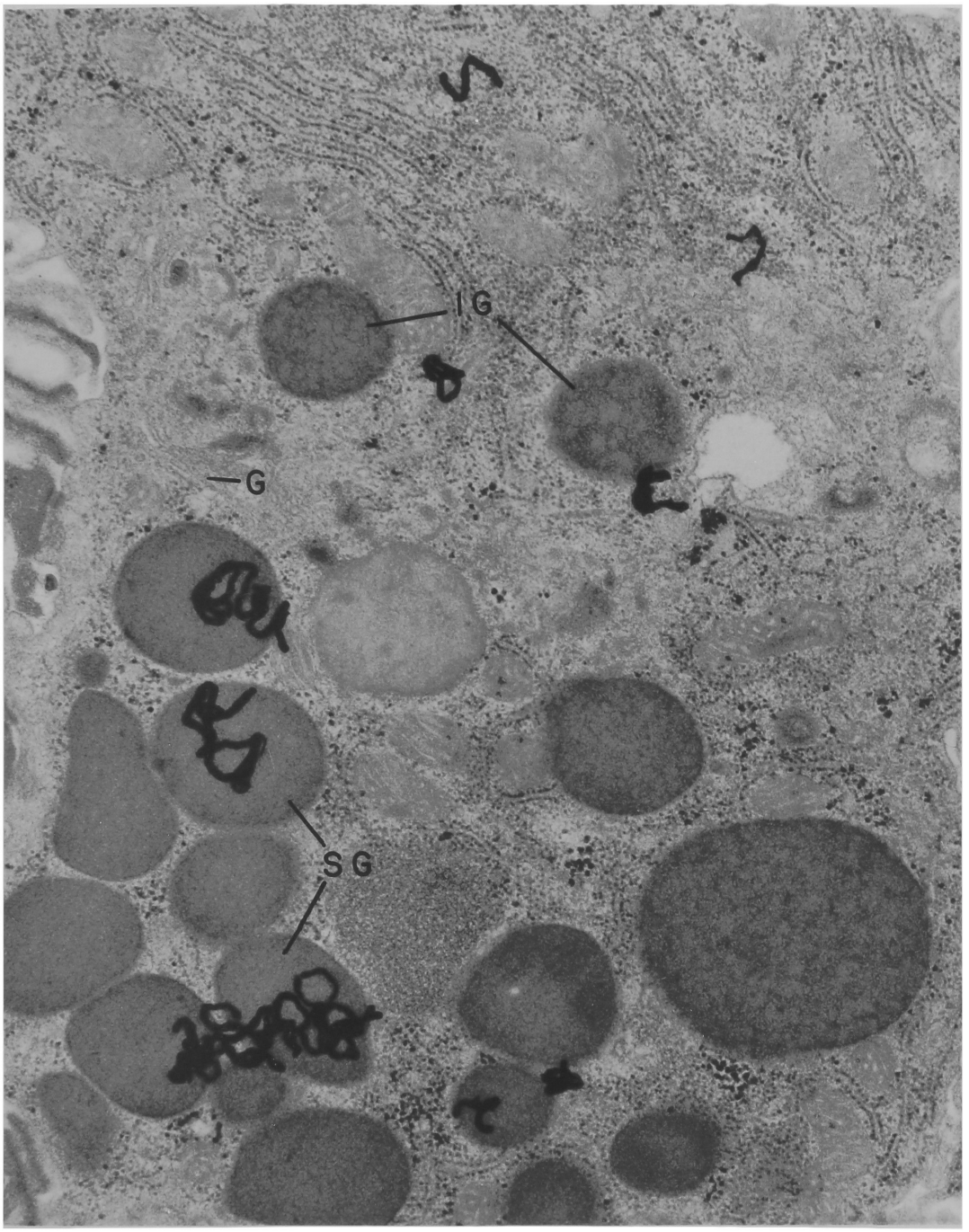


Fig. 17. Autoradiograph of part of a parotid acinar cell chase-incubated for 176 min. Autoradiographic grains are concentrated over secretory granules (SG) that have attained homogeneous packing of their content, have joined the mass of storage granules of the cell, and hence, are considered mature secretion granules. The immature granules (IG) are no longer highly labeled, indicating that the crest of the wave of incorporated label has passed this stage of intracellular transport. Golgi cisternae (G). X 25,000.

Table IV

Distribution of Autoradiographic Grains over Cell Components

% of Autoradiographic Grains[†]
Chase Incubation Following a 4-Min Pulse

	1 min	6 min	11 min	16 min	26 min	36 min	56 min	86 min	116 min	176 min	236 min	356 min
Rough Endoplasmic Reticulum	77.7	66.7	58.6	49.8	31.8	24.4	19.4	19.2	17.7	22.5	25.7	19.1
Golgi Complex												
Periphery including entrance cisternae	6.7	11.8	20.5	24.1	27.4	22.0	16.0	8.3	7.5	5.2	1.3	2.2
Exit cisternae and condensing vacuoles	0.9	3.5	1.3	3.3	22.0	40.3	35.9	19.2	16.1	4.2	2.8	0.9
Immature Secretion Granules	--	1.4	0.9	1.6	3.5	4.2	18.4	40.4	49.4	16.9	9.3	2.9
Mature Secretion Granules	6.7	6.1	6.2	7.4	6.8	2.8	4.5	7.1	7.4	35.2	49.5	58.5
Nuclei	3.3	4.9	6.4	7.9	6.4	5.2	3.3	3.9	4.7	10.3	8.3	12.4
Mitochondria	4.6	4.7	6.6	5.9	2.1	1.0	2.4	1.8	--	4.7	2.6	3.6
Number of Grains Counted	817	491	454	1740	971	1480	749	661	612	213	540	582

Underlined Numbers indicate maximum accumulation of grains over the corresponding cell component.

[†]These data were obtained in two overlapping experiments.

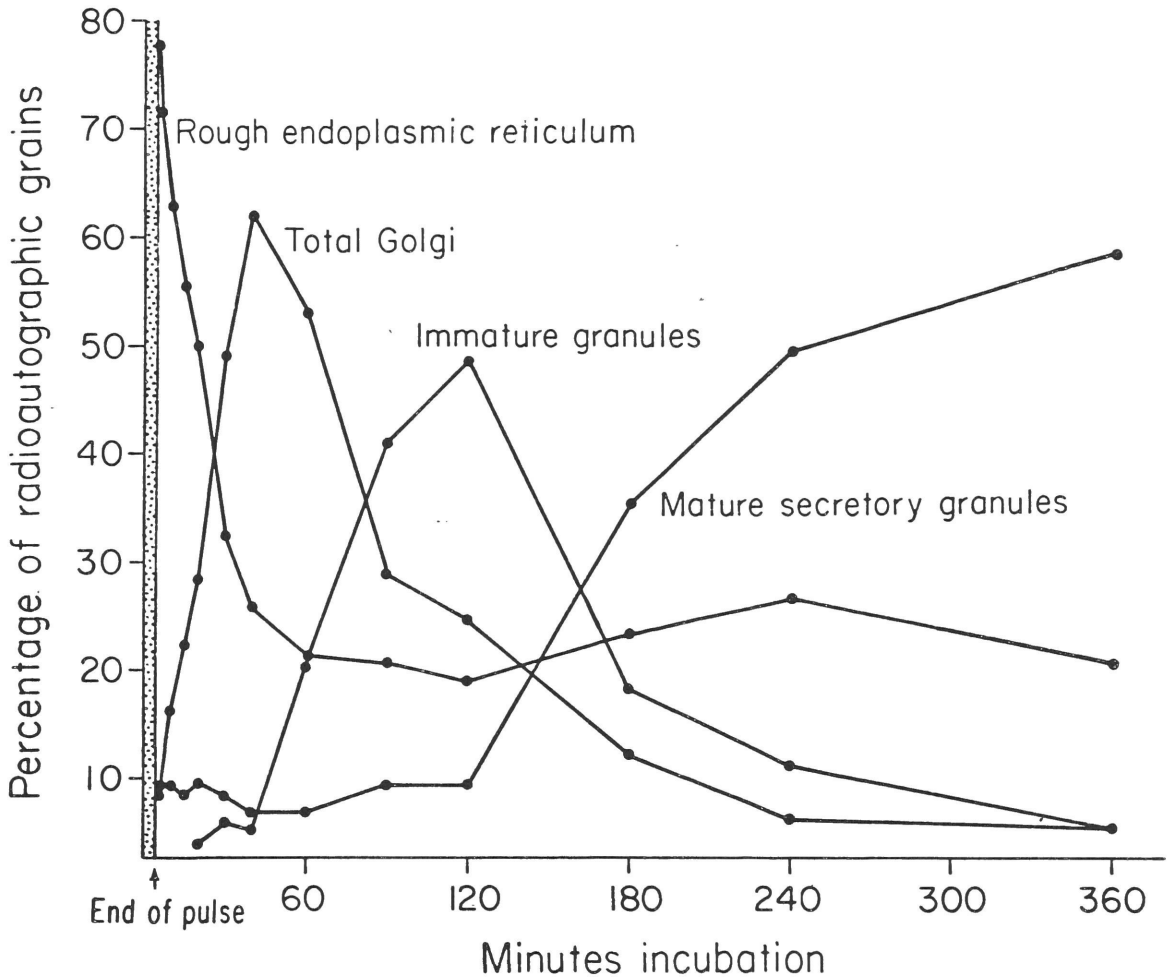


Fig. 18. Wavelike movement of pulse labeled secretory protein through intracellular compartments. A plot of the quantitative data of Table IV for cellular components involved in transport. Summation of percentages at each time point shows that 75-80% of the incorporated label moves with the wave against the RER background of ~20%.

presumably represents nonexportable protein (Siekevitz and Palade, 1960).

With the decrease of label associated with the RER there was a concomitant increase in Golgi-associated label which reached a maximal level (62%) by 36 min chase. At this time, labeled secretory protein was maximally concentrated in vacuoles in direct contact with the exit side of the Golgi complex. After prolonged chase very few autoradiographic grains remained over the Golgi complex, indicating efficient drainage.

The Golgi-associated condensing vacuoles developed into heavily labeled immature granules which accounted for 50% of the grains counted at 116 min chase incubation. Granule maturation and migration into the accumulated granule population proceeded for the duration of the chase past 116 min. The thoroughness of conversion of immature granules (Table IV and Fig. 18) suggested that the intracellular transport system was completely functional throughout the 6 hr chase.

The labeling of nuclei and mitochondria (structures not implicated in intracellular transport) was generally low and variable (nuclei: 3.3-12.4% and mitochondria: <1-6.6%). No significant label was ever seen in acinar lumina, corroborating both the observation of extremely few labeled granules at the cell apex and the low level of release of protein radioactivity from unstimulated lobules over prolonged periods of incubation, 10-15% (Fig. 10)

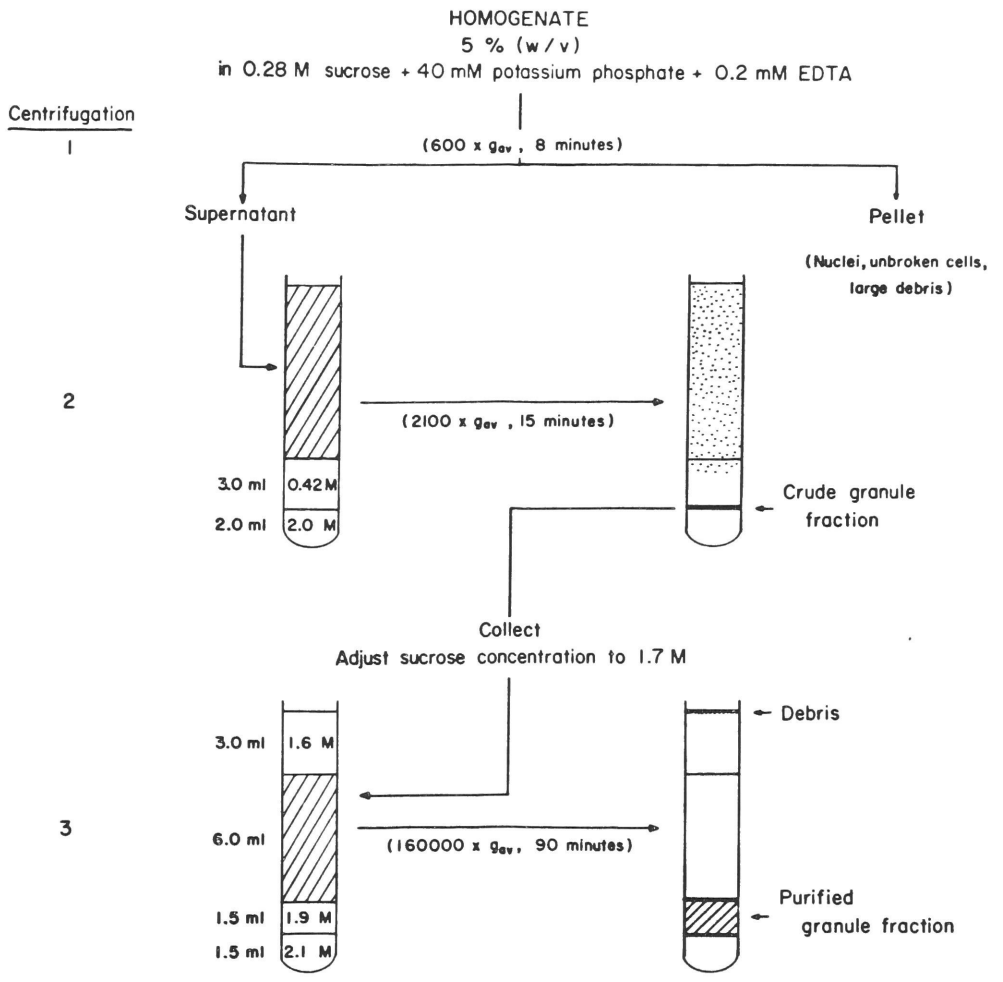
In summary, intracellular transport of newly synthesized secretory proteins in the rabbit parotid acinar cell occurs along the same basic pathway described in the pancreatic acinar cell, namely: RER -- Golgi -- secretory granules. The efficiency of compartment drainage and the lack of reappearance with time of label in compartments already drained suggested that the transport process is unidirectional (vectorial). Prior to the Golgi condensing vacuole stage the proteins are processed at the same rate in both glands, but secretory granule maturation in the rabbit parotid is greatly prolonged relative to matura-

tion in the guinea pig pancreas. It is thus possible to distinguish kinetically and morphologically an immature granule stage in the transport pathway which is interposed between the condensing vacuole and mature secretory granule stages.

Isolation and Characterization of a Secretion Granule Fraction from Rabbit Parotid Tissue

Procedures conventionally used for the isolation of secretion granules of approximately 1 μm in diameter all rely on rather unsatisfactory means for reducing mitochondrial contamination which involve surface rinsing and repeated washing by resuspension of a granule pellet obtained by differential centrifugation. These operations reduce granule yield and are only partially successful in reducing contamination by mitochondria and other elements. Contamination by mitochondria in the granule fraction usually amounted to $\geq$ 2% of the initial total homogenate activity (Amsterdam *et al.*, 1971; Greene *et al.*, 1963; Kirshner *et al.*, 1973; Meldolesi *et al.*, 1971). Therefore a new granule isolation protocol was developed with the intent of obtaining through a simple procedure an effective reduction of contamination by other cellular organelles at minimal expense to the yield of secretion granules. At all stages, a light microscope outfitted with Nomarski differential interference contrast optics proved to be very useful for visually monitoring fractionation and qualitatively assessing contamination. The steps involved in this protocol have been enumerated in the Methods section of this thesis (Page 14) and appear in Fig. 19 in diagrammatic form. The total homogenate prepared as described on Page 14 was fractionated by first pelleting large debris, unbroken cells, and nuclei at 600 x g_{av} for 8 min. Under these conditions the resulting pellets were overlaid with a fine white layer of secretion granules lending assurance that most, if not all, larger structures -- red blood cells and nuclei -- had already been sedimented. The resulting supernate was layered over a discontinuous gradient which upon centrifugation (15 min at 2100 x g_{av}) yielded a crude granule fraction, appearing at the 0.42-2.0 M

Isolation Protocol for Secretion Granules of the Rabbit Parotid Gland



All sucrose solutions (except homogenization medium) contain 40 mM potassium phosphate and 1 mM EDTA

Fig. 19. All centrifugal forces (g_{av}) refer to the values at the center of the tubes. The tubes for centrifugations 2 and 3 were filled to capacity.

sucrose interface. The bulk of the mitochondria and smaller subcellular organelles fail to penetrate the 0.42 M sucrose layer under these conditions. The crude granule fraction was collected from the 2.0 M sucrose cushion interface with a Pasteur pipet and was then diluted, using 2.1 M sucrose to an approximate sucrose concentration of 1.7 M. After thorough mixing the diluted crude granule fraction was loaded centrally in a high molarity discontinuous sucrose gradient. The final centrifugation at 160000 x g_{av} for 60 min produced a purified granule fraction distributed throughout the 1.9 M sucrose layer with debris, primarily mitochondria and large pieces of membrane floating through the 1.6 M layer.

Several features of this protocol should be emphasized.

1. Potassium phosphate buffer at pH 7.2 was present in all sucrose solutions at a final concentration of 40 mM. Granules are totally labile in unbuffered sucrose, and examination of granule stability as a function of phosphate concentration indicated that a minimum of 20 mM potassium or sodium phosphate tested over the range of pH 6.5-7.6 was required to maintain intact granules. Granule lysis could be observed in the light microscope at lower phosphate concentrations. Other salts such as potassium chloride, sodium bicarbonate, and HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) were completely unsatisfactory as substitutes for potassium phosphate.

2. Secretion granules readily stick to both glass and cellulose nitrate. Therefore the glass centrifuge tubes used in steps one and two of the centrifugation schedule were siliconized, and polyallomer centrifuge tubes were used in place of cellulose nitrate tubes for the final high speed centrifugation.

3. The use of high molarity sucrose cushions as seen in centrifugations two and three as an alternative to pelleting the secretion granules at any stage was extremely helpful in reducing contamination induced by physical contact and aggregation between granules and other subcellular organelles, especially mitochondria. When pelleting of

granules by velocity sedimentation was attempted in early phases of these studies mitochondria were observed to adhere strongly to granule surfaces, the intimate association being maintained throughout vigorous resuspension of granule pellets. The tenacious association could be largely overcome by procedures which avoid pelleting of granules and other cell particulates.

4. Finally, the inclusion of EDTA at a final concentration of 1 mM complemented the use of sucrose cushions in effectively reducing aggregation between granules and between granules and other subcellular organelles. The efficiency of EDTA can be readily visualized in the light microscope. It should be pointed out, however, that EDTA was used in the initial phases of fractionation at 0.2 mM final concentration. Nuclei are stabilized by divalent cations, so until nuclei are removed by the initial centrifugation, EDTA is kept at a lower concentration. This precaution was necessary since the only visual subcellular contaminant of the granule fraction was an occasional partially extracted nucleus.

Morphology of the Isolated Granule Fraction

Fixed pellets of secretion granules were studied systematically throughout their thickness and were found to be homogeneous as portrayed by Fig. 20, a representative low magnification electron micrograph.

Immediately evident in this figure are the large variety of irregular granule shapes similar to the variety observed in intact cells (Fig. 3) and the absence of recognizable mitochondria and microsomes present at higher incidence in secretion granule fractions obtained by repeated differential sedimentation. As demonstrated by Fig. 21, this rabbit parotid granule fraction contains recognizable immature secretion granules, and the higher magnification further indicates that the limiting membranes of granules are well preserved, showing a reduced incidence of myelin figure formation (at the expense of membrane phospholipids) when compared to pancreatic zymogen granules (Meldolesi *et*

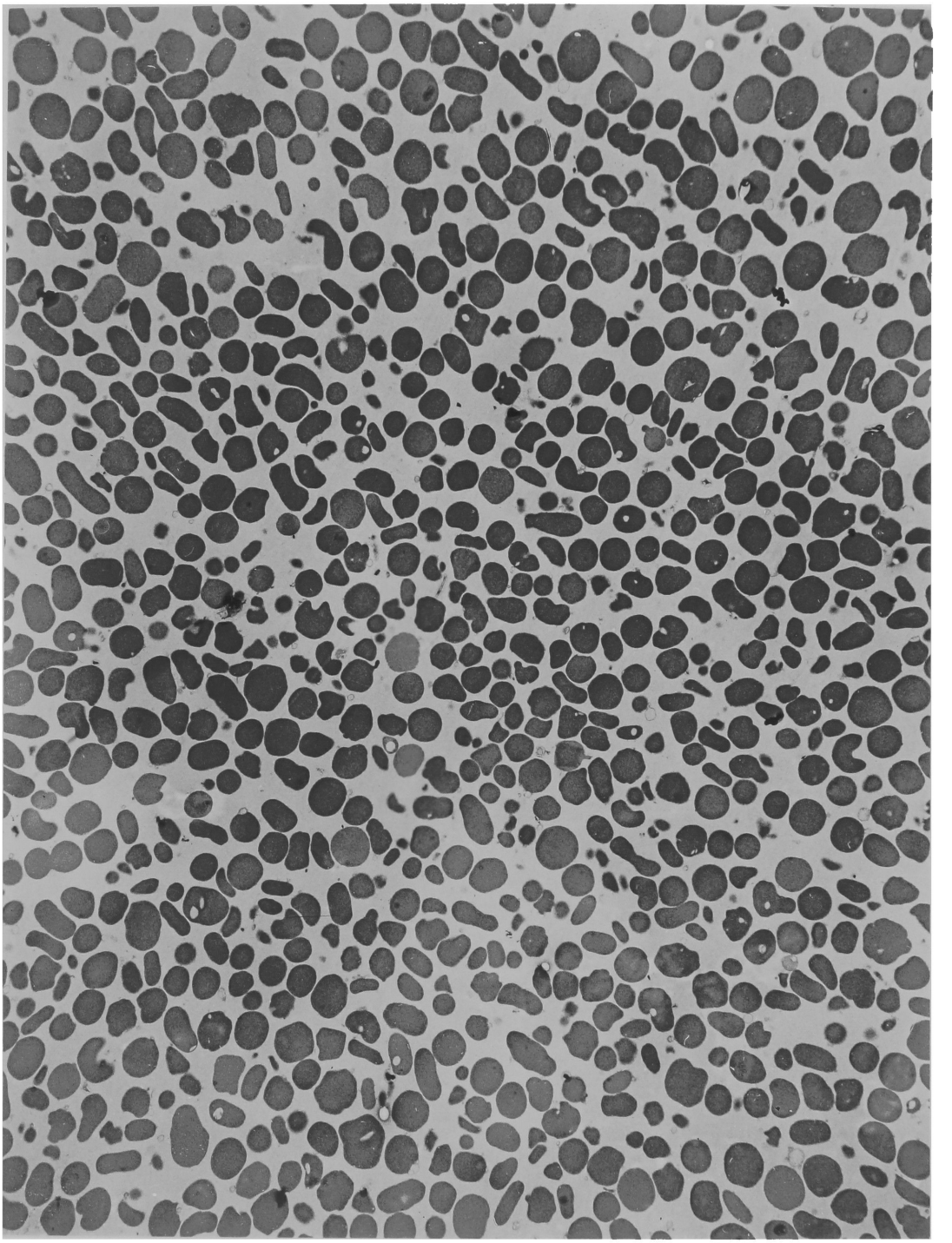


Fig. 20. Representative electron micrograph of the purified secretion granule fraction. Aliquots of the granule fraction were fixed with phosphate-buffered aldehydes in suspension, then post-fixed in osmium tetroxide and further processed for microscopy as pellets as described in the text. Note the variety of unusual granule shapes seen in all parts of the pellet. X 5,000.

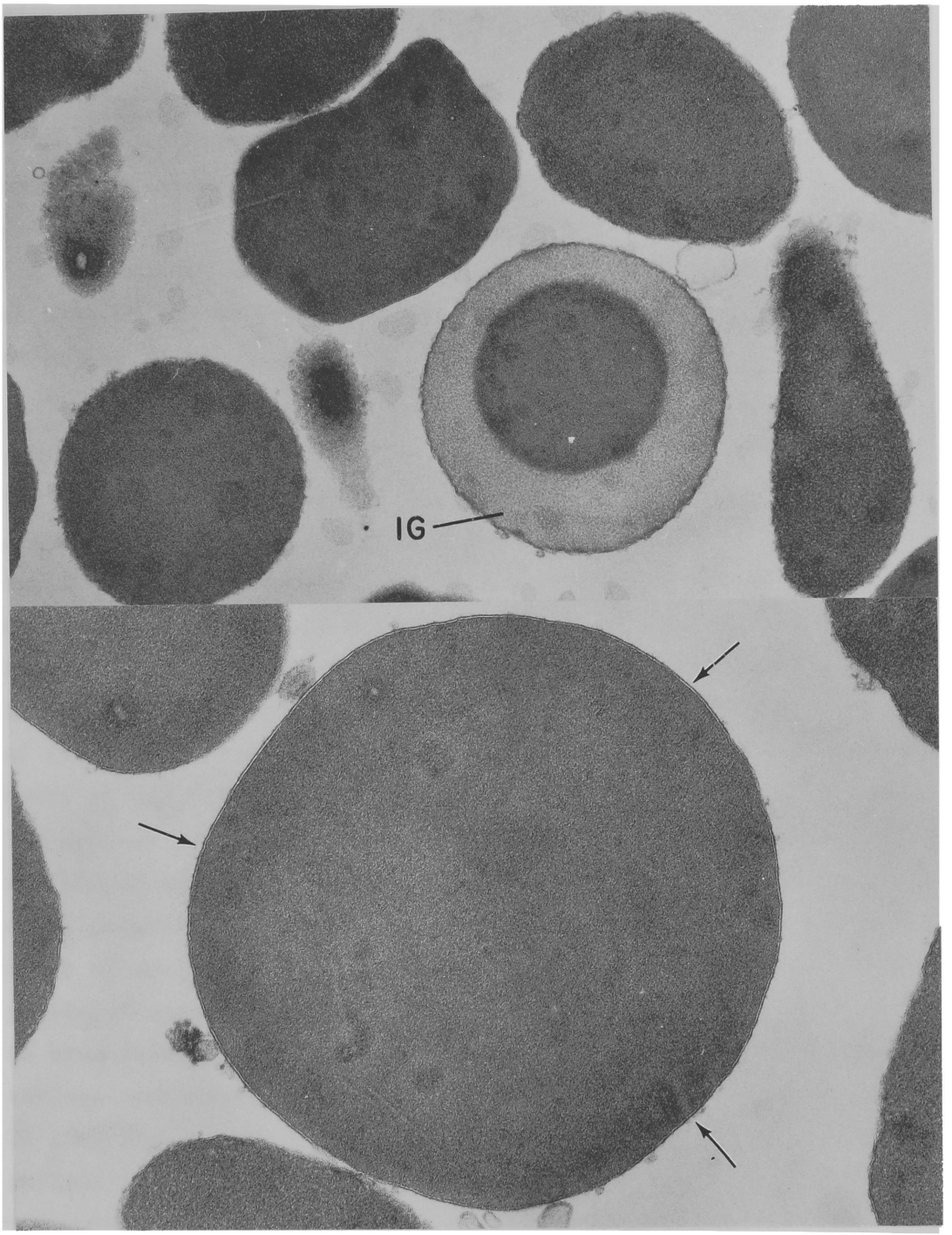


Fig. 21. The secretion granule fraction at higher magnification. (a) The fraction contains immature secretion granules (IG) recognized by their heterogeneous content. (b) The membranes of the granules are well preserved, the trilaminar structure being readily observed (arrows), especially in samples stained in-block with magnesium uranyl acetate prior to alcoholic dehydration. (a) X 40,000; (b) X 61,000.

al., 1971).

The Yield of Secretion Granules

An estimate of the proportion of the total secretion granule population recovered in the purified granule fraction was obtained by determination of the distribution of one of the secretory enzymes, i.e., α-amylase taken as a granule marker. The underlying assumption is that in the intact cells the bulk of this enzyme is located within secretion granules.

Presented in Table V is a representative amylase distribution and specific activity profile for the entire fractionation scheme. The percentage distribution data are expressed in two forms: a) percent relative to the fraction of origin at each step in the protocol facilitating an assessment of recovery for each step, and b) percent of the total homogenate activity. 12.1% of the total amylase activity of the homogenate was recovered in the purified granule fraction in the experiment given in Table V, and the amylase specific activity of the granule fraction normalized to protein assayed according to Lowry et al. (1951) was four times the homogenate specific activity. The average yield of purified granules for eight different experiments was 15% of the total homogenate amylase which compares favorably with other granule yields reported in the literature (Amsterdam et al., 1971; Greene et al., 1963; Meldolesi et al., 1971).* These percentage yields of granules based on amylase activity distribution are likely to be underestimates for two reasons: 1) they are not corrected for amylase underrecovery at each step of the fractionation protocol, and 2) they are calculated on the assumption already stated, that is, amylase is localized in the tissue exclusively within secretion

*Secretion granule yield based on recovery of amylase activity in the purified granule fraction relative to amylase activity present in intact granules of the tissue homogenate becomes 25% (on the average) when the non-sedimentable (postmicrosomal supernatant) amylase activity is assumed to represent enzyme derived entirely from breakage of secretion granules during homogenization and is thus subtracted from the total homogenate value.

Table V

Amylase Distribution in the Granule Isolation Scheme

Fraction	Amylase Percentage Distribution			Percentage of Homogenate Amylase	Specific Activity Units[a] mg Protein (Lowry)
Homogenate	100.0 [b]			100.0 [b]	75
Pellet Spin 1	27.2			27.2	46
Supernate Spin 1	62.6	(100.0)		62.6	94
Post Granule Supernate Spin 2		66.4		41.5	84
Crude Granule Fraction		21.9	(100.0)	13.7	200
Purified Granule Fraction			89.0	12.1	292
Debris & Residual Sucrose Spin 3			7.1	1.0	n.d.*
[c] Mitochondria & Microsomes				2.6	30
[c] Post Microsomal Supernate				37.6	96

[a] Units: μmoles maltose released per 3 min at 30° C.

[b] 100% Homogenate corresponds to 14,625 amylase units.

[c] Post granule supernate spin 2 hr at 160,000 x g_{av} to yield mitochondria and microsomes (Pellet) and post microsomal supernate.

* not determined.

granules; this assumes that contributions of other compartments and of the glandular lumina to the total activity of the tissue is negligible. In any case, the yields were sufficiently high that the purified granule fraction could be considered representative of the total granule population.

Further examination of the data in Table V, particularly with reference to amylase appearing in the postmicrosomal supernate and in the pellet of the first centrifugation provides a rough estimate for: 1) the extent of granule breakage incurred during homogenization — ~40% of the amylase activity is found in soluble form in the final supernate (most of it represents leakage from broken granules, but part of it is accounted for by amylase already discharged into the acinar lumina at the time of tissue homogenization (see footnote on page 66)), and 2) the incompleteness of cellular breakage during homogenization: > 25% of the total homogenate amylase activity is found in the pellet of the first centrifugation. If it is assumed that a) the bulk of amylase activity is associated with secretion granules and b) all cells of the tissue had been broken during homogenization, then a calculation (performed in the manner of de Duve et al. (1959) of the percentage of the secretion granule population which sediments under the conditions of the initial centrifugation of the homogenate indicates that ~15% of the granules (hence, the amylase activity) are expected in the pellet. Thus a minimum of 10% of the parotid acinar cells remain unbroken after homogenization.

Contamination of the Purified Granule Fraction by Other Cellular Organelles

Biochemical assessment of contamination of the secretion granule fraction by other subcellular organelles was carried out following choice of cytochrome oxidase as a marker enzyme for mitochondria, RNA as a chemical marker for rough microsomes and nuclear envelope, and N-acetyl-β-glucosaminidase and acid proteolytic cathepsin D-like activity as diagnostic for lysosomes. The identification of potential

proteolytic activity has additional significance where chemically unaltered membranes are the ultimate object of investigation. The distributions of marker activities for each of the steps comprising the granule isolation protocol are tabulated in Table VI.

Cytochrome oxidase contamination in the granule fraction was reduced to a level of about 0.2% of the initial homogenate value, an order of magnitude better than comparable data available in the literature (Amsterdam et al., 1971; Greene et al., 1963; Kirshner et al., 1973; Meldolesi et al., 1971). The final cytochrome oxidase specific activity is less than one-tenth that of the homogenate and less than one-two hundredth the specific activity expected for a purified mitochondrial fraction.

RNA, used to determine the distribution of rough microsomes, nuclei and nuclear envelope in the fractions yielded by the protocol in Fig. 19 was reduced in the purified granule fraction to 0.1% of the initial homogenate value. The specific activity of the granule fraction -- 3.6 mg RNA per g protein -- is two times lower than that of the purest pancreatic zymogen granule fractions available (Greene et al., 1963; Hokin et al., 1955; Meldolesi et al., 1971) and tenfold lower than that of the parotid secretion granule fraction of Amsterdam et al. (1971). A typical pancreatic rough microsomal fraction (Jamieson and Palade, 1967a) has an RNA specific activity more than two-fold that of the homogenate, corresponding to approximately 40 times the specific activity of the purified fraction of rabbit parotid secretion granules.

N-acetyl-β-glucosaminidase and cathepsin D-like acid proteolytic activity are effectively segregated, although not quite in parallel, from the secretion granules by both discontinuous gradient centrifugations. N-acetyl-β-glucosaminidase was reduced to 0.3% of the initial homogenate value in the purified granule fraction and had a final specific activity one-eighth the initial homogenate value and less than one-hundreth the specific activity expected for a purified rat liver lysosomal fraction (Leighton et al., 1968). Determination (in a

Table VI

Assessment of Contamination of the Secretion Granule Fraction by Cellular Organelles

Fraction	Cytochrome Oxidase [a] (Mitochondria)			RNA [b] (Microsomes Plus Nuclear Envelope)			N-acetyl-β-Glucosaminidase [c] (Lysosomes)			Acid Protease [d] (Cathepsin D) (Lysosomes)		
	Act.	%	S.A.	mg RNA	%	RNA/ protein	Act.	%	S.A.	Act.	%	S.A.
Homogenate	92.2	100.0	0.32	3.84	100.0	59.6	4.00	100.0	19.6	1.76	100.0	8.6
Pellet Spin 1	9.6	10.4	0.10	1.94	50.5	43.6	1.33	33.0	18.1	0.62	35.1	8.4
Supernate Spin 1	87.4	94.7	0.04	2.04	53.0	51.6	2.43	61.0	16.7	1.09	62.0	7.5
Post Granule Supernate Spin 2	63.2	68.5	0.38	1.64	42.7	48.0	1.78	44.5	16.3	1.00	57.8	9.2
Crude Granule	6.5	7.0	0.42	0.14	3.7	60.0	0.05	1.2	5.7	0.07	4.0	8.2
Purified Granule	0.25	0.27	0.025	< 0.004	0.1	3.6	0.01	0.3	2.4	0.04	2.3	8.2
Debris Spin 3	6.3	6.8	4.00	0.004	0.1	--	0.08	2.0	67.0	0.15	8.5	(125.0?)

Act. = Activity; % = percentage Distribution of Activity; S.A. = Specific Activity.

[a] Cytochrome oxidase: activity expressed as μmoles cytochrome C oxidized · min $^{-1}$. Specific activity: activity mg · protein $^{-1}$. The values above represent one of four determinations of cytochrome oxidase distribution. For the four different experiments the cytochrome oxidase activity of the purified granule fraction represented 0.17-0.30% of the total homogenate activity.

[b] RNA: content expressed as mg RNA. Normalization: mg RNA · g protein $^{-1}$.

[c] N-acetyl-β-glucosaminidase: activity expressed as μmoles β-naphthol released · min $^{-1}$. Specific activity: activity · g protein $^{-1}$. The above data represent one of two lysosomal distribution experiments. In both experiments 0.03% of the homogenate N-acetyl-β-glucosaminidase activity was recovered in the purified granule fraction.

[d] Cathepsin D: activity expressed as release into soluble form of chromogenic equivalents of 1 mg/ml albumin · min $^{-1}$. Specific activity: activity · g protein (Lowry)$^{-1}$.

single experiment) of the distribution of cathepsin D-like acid protease activity showed that 2.3% of the initial homogenate total activity was recovered in the purified secretion granule fraction. Note, however, that unlike N-acetyl-β-glucosaminidase, acid protease does not show decreases in specific activity for the crude granule and purified granule fractions relative to the values for the fractions from which the granules were derived. This feature is probably an artifact of the low levels of total activity for these granule fractions rather than an indicator of the presence of a latent, granule-associated acid protease. However, further investigation using a more sensitive assay is necessary to test for any true proteolytic activity which is potentially detrimental to the polypeptides of granule membranes.

In each of these assays at least one-third of the entire purified granule preparation was processed as a single sample and with the exception of cytochrome oxidase, levels of activity detected were at or near the limit of sensitivity of the assay. Therefore, recoveries of contaminant activities in Table VI which are satisfactory for initial stages of fractionation were quite variable and in most cases did not approach 90-100% of the total activity in the final granule purification step.

In summary, the fraction of rabbit parotid secretion granules obtained by this new protocol not only exhibits satisfactory morphologic purity, but at the more general biochemical level is quite thoroughly freed of subcellular contaminants at acceptable expense to the yield of granules.

Other Enzymatic Activities Associated with Granules

Additional characterization of the secretion granule fraction was limited to assaying for a number of potential secretory enzymes. All assays were carried out on hypotonic lysates of the fraction.

Protease activity at neutral pH was not detectable using an assay based on dye released from Azocoll (Mandl *et al.*, 1953) which should detect less than 1 μg/ml trypsin equivalents. Neutral lipase assayed

using as substrate β-naphthyl nonanoate in the presence of sodium taurocholate (Bradshaw and Rutter, 1972) gave a positive reaction barely above the colorimetric level of the blank only when a large fraction (1/4 - 1/3 the quantity of a single experiment) of the total granule protein was assayed as a single sample. These assays confirmed the initial assumption that the parotid might represent a promising exocrine system for the study of undegraded intracellular membranes.

Peroxidase, a secretory enzyme extensively studied in rat parotid (Herzog and Miller, 1970) was not detected at either pH 5.5 or 7 in rabbit parotid confirming observations by Thomson and Morrell (1967) as well as making it unnecessary to include in fractionation media the antiperoxidatic agent DPPD (N,N'-diphenyl-p-phenylenediamine) (Hochstein and Ernster, 1963) which has been used by Amsterdam et al. (1971) as preventive for possible peroxidation of membrane lipid.

The nucleases, DNase and RNase, however, were both found to be present in lysates in easily detectable amounts increasing to three the number of known secretory enzymatic activities. DNase had a specific activity in the lysate of 19 units per mg Lowry protein which can be compared to a value of ~2000 units/mg for Worthington purified bovine pancreatic DNase I. RNase has a lysate specific activity of about 70 units/mg Lowry protein, whereas purified porcine pancreatic ribonuclease A has a specific activity of 3000 units/mg. If it is assumed that the specific activities of these purified nuclease standards are identical to the specific activities of the respective purified parotid nucleases, then it can be calculated that DNase would account for about 1% and RNase about 2% of the total exportable protein packaged in parotid secretion granules. However, these estimates should be regarded with caution on two accounts: a) protein in pancreatic standards and lysates was determined by different methods (gravimetric and Lowry assays respectively), b) recent work by Lundblad et al. (1973) demonstrated that antibodies raised against pancreatic RNase and parotid DNase (both of bovine origin) failed to inhibit the enzymatic activities of the respective parotid and pancreatic counterparts where con-

trols -- pancreatic RNase reacted with antipancreatic RNase and parotid DNase reacted with antiparotid DNase -- showed strong inhibition of enzymatic activity.

The search for activities associated with the secretion granule content was not carried past this point since primary interest was focused on the resolution of lysed granules into their limiting membranes and packaged content requisite to studies of the biosynthesis of membrane and content proteins.

Granule Lysis and Separation into Membranes and Content

To lyse secretion granules an alternative to the lysis at mildly alkaline pH used for pancreatic zymogen granules (Greene et al., 1963; Hokin, 1955; Meldolesi et al., 1971) was sought since rabbit parotid granules were found to be reasonably stable in the range pH 7.5-8.0. Instead, the instability of rabbit parotid granules to hypotonic media was exploited in developing a lysis procedure. To reduce the high tonicity of the solution in which the purified granule fraction is suspended, a procedure based on stepwise dilution and pressure dialysis in an Amicon ultrafiltration cell was devised. In this manner it was possible: a) to avoid the long time periods required for standard dialysis against media low in salt concentration as were used by Schramm and collaborators (Amsterdam et al., 1971) for lysis of rat parotid secretion granules, and b) to prevent fragmentation of membranes resulting from too abrupt a change in tonicity which occurs when granules are pelleted from high molarity sucrose and resuspended directly in lysis medium. The detailed stepwise lysis schedule has been described in the Methods section (Page 16). In summary, granules collected initially in 1.9 M sucrose, 40 mM potassium phosphate, 1 mM EDTA pH 7.2 were added to the Amicon chamber along with three volumes of lysis medium (20 mM sodium bicarbonate, 0.5 mM EDTA pH 7.2). Sucrose molarity at this stage was approximately 0.5. Then by successive concentration and dilution the sucrose molarity was reduced to 0.25 and finally to 0.08 at which point complete clearing of the lysate

occurred. The lysate was finally concentrated to a small volume before resolution into membranes and secretory content.

In the case of pancreatic zymogen granules of the guinea pig, the procedure for resolving granule lysates into membranes and content took into account the unusually low density of granule membranes. These membranes, which had accumulated free fatty acid secondary to the action of endogenous pancreatic lipase on tissue triglycerides and membrane phospholipids (Meldolesi et al., 1971), banded at a 0.7-1.0 M sucrose interface while residual mitochondria pelleted through the 1.0 M sucrose during centrifugation. Since rabbit parotid secretion granules contain no significant lipase activity, membranes of lysed granules would not be expected to undergo any significant shift to lower density. Resolution of the final concentrated hypotonic lysate into membranes and packaged content was achieved using the two-step centrifugation scheme shown in Fig. 22. The first discontinuous sucrose gradient containing layers of 0.2 M, 0.6 M and 1.5 M sucrose was designed so that the slightly hypotonic 0.2 M layer would provide a wash of the ghost fragments prior to any resealing of ghosts anticipated to take place in the 0.6 M layer. The initial centrifugation brings membranes to the 0.6-1.5 M sucrose interface, the soluble granule content remaining in the original load. The second centrifugation where diluted membranes are pelleted through 0.6 M sucrose is similarly intended as a membrane wash.

Assessment of the Degree of Separation of Content and Membrane

Enzymatic assessment of the degree of separation of granule membranes and secretory content achieved by the above protocol was carried out by determining the amount of residual amylase activity present in the purified granule membranes relative to that present in either the granule content or unlysed granules. A representative amylase distribution is presented in Table VII. Consistently, membranes were freed of amylase to a level of 0.03-0.07% of the initial total granule com-

The Separation of Membranes and Content from Secretion Granule Lysates

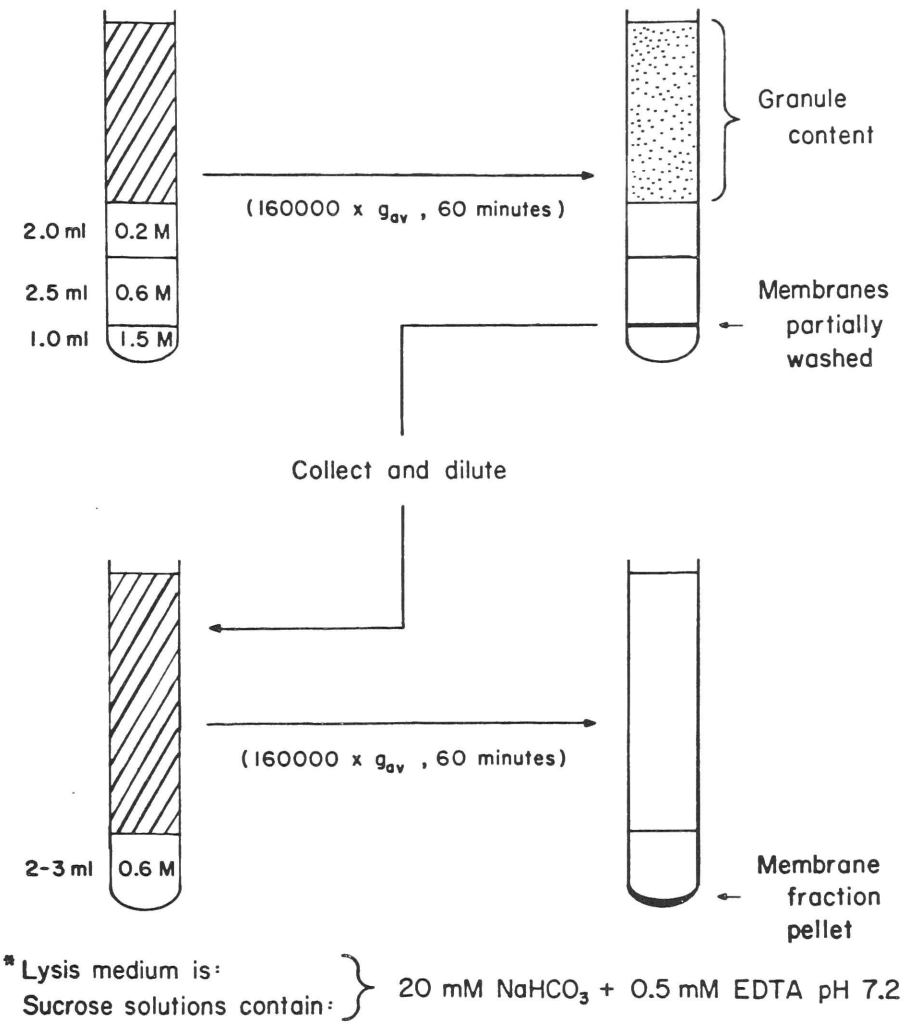


Fig. 22. Centrifugal forces (g_{av}) refer to the values at the center of the tubes, all tubes being filled to capacity. Sucrose solutions contain 20 mM $NaHCO_3$, 0.5 mM EDTA, pH 7.2.

Table VII

Removal of Amylase from Secretion Granule Membranes

Fraction	Units Amylase Activity	Percentage Distribution
Secretion Granules	1670.	100.0
Granule Content	1608.	96.3 *
Granule Membranes	1.1	0.06

*The recovery of granule amylase activity after concentration of the lysate in an Amicon chamber varied from 66-100% in 8 experiments. To consistently recover over 90% of the amylase activity at this step, it is necessary to siliconize the walls of the chamber and to use a sieving membrane that has been exposed to granule protein previously: (more than 10% of the total granule amylase activity has been found to stick to a sieving membrane that has not been conditioned with protein prior to use).

plement suggesting on the basis of a single major secretory protein, a very satisfactory separation.

Morphology of the Membrane Fraction

Figure 23, a low magnification micrograph of the membrane fraction, shows a homogeneous preparation of unusual oblong structures that appear to be collapsed or folded membrane vesicles. Throughout the thickness of the pellet, cut to expose the entire cross-section of sedimented material, the appearance did not differ at all (only the spacing of vesicular elements differed) from that seen in Fig. 23, a field taken from the middle of the pellet. Higher magnification (as seen in the insert of Fig. 23) revealed that the membranes were indeed quite clean, and generally organized in closed vesicles as indicated by the absence of free ends. At many points the membranes of these vesicles showed typical bilayer structure, and at certain points along the inner aspect a fine fibrillar material (marked f) is evident.

Contamination of the Membrane Fraction by Other Subcellular Organelles

Attempts to assess the degree of contamination of the membrane fraction using marker assays for other subcellular organelles were, for the most part, unsuccessful since the use of half the entire membrane fraction per assay failed to give values above the assay background. Cytochrome oxidase, the mitochondrial marker, was, however, readily detectable somewhat above assay background in amounts representing 16-20% of the total activity associated with the granule fraction.

In summary, the membrane subfraction did not appear as a collection of spherical completely extracted "ghosts", but as a population of collapsed vesicles with a fuzzy (fine fibrillar) inner lining. Nevertheless, the morphological survey showed that the subfraction was homogeneous, and the biochemical assays suggested -- on the basis of

Fig. 23. Electron micrograph of the granule membrane subfraction, taken from the center of the pellet, but representing in composition all levels of the pellet. Evidently, the membranes do not have the appearance of spherical granule ghosts. Instead, they are, for the most part, collapsed and infolded vesicles. The inset shows a portion of the pellet at higher magnification. The trilaminar structure of normally-cut membranes is indicated by arrows. At many points along the inner aspect a fine fibrillar material (f) can be seen which appears to form a quasi continuous layer in regions of folding. X 15000; (Inset X 62000).

results obtained for a single major secretory protein -- that the removal of the content was satisfactory.

Quantitation of Total Proteins by Different Assays

Comparison of amounts of protein and protein-based specific activities between different subcellular fractions is highly dependent on the nature of the protein assay, i.e., its reliability in expressing true protein quantitation. The assay of total protein devised by Lowry *et al*. (1951) is primarily indicative of protein tyrosine content, and absolute quantitation is subject to the variability in frequency of tyrosine residues between different proteins. The microbiuret assay of total protein provides quantitation at a more generalized level since it assesses the amount of polypeptide backbone based on structural similarity to biuret; however, its widespread use has been hindered by its lower sensitivity in comparison to the Lowry assay, a necessary consideration where only small amounts of samples can be spared for determination of total protein. The caution which must be exercised in attaching significance to absolute values of protein estimations is dramatically demonstrated by Table VIII which shows assessment of protein in homogenate, secretion granules, and granule membranes as determined by the Lowry and microbiuret assays. While assays for total granule protein happen to give the same absolute value, there is an order of magnitude difference between absolute values obtained for the same membrane fraction by the two different assays. In the same manner the homogenate from which the granules were derived reflects nearly a three-fold difference in absolute amount of protein. As indicated in the legend below the table, absolute values of contaminant cytochrome oxidase specific activity in the total granule and membrane fractions are affected in an analogous manner. Thus for any comparison of absolute values of specific activities between different subcellular fractions composed of different spectra of proteins, it is mandatory that the denominator of the specific activity truly reflects quantitation of the protein present. For examination of specific radioactivities of granule membranes and secre-

Table VIII

Comparison of Protein Quantitation by Different Assays

Fraction	Lowry Assay		Microbuiret Assay	
	mg Protein	Percentage Distribution	mg Protein	Percentage Distribution
Homogenate	117.0	100.0	338.0	100.0
Secretion Granules	14.1	12.0 (100.0)	14.1	4.2 (100.0)
Granule Membranes	0.05	(0.3)	0.52	(3.7)

Membrane cytochrome oxidase:

$\mu moles \cdot min^{-1} \cdot mg\ Lowry\ protein^{-1}$: 0.066

$\mu moles \cdot min^{-1} \cdot mg\ Biuret\ protein^{-1}$: 0.0061

Granule cytochrome oxidase:

$\mu moles \cdot min^{-1} \cdot mg\ Lowry\ or\ Biuret\ protein^{-1}$: 0.0013

tory content in biosynthetic studies, to be presented in the last sections of this thesis, values for total protein were obtained by quantitating all amino acids in protein hydrolysates. The approach was chosen to avoid lack of sensitivity or misleading results of the type illustrated in Table VIII.

Polyacrylamide Gel Electrophoresis of Granule Membranes, Granule Content, and Secretion Collected by Cannulation of Parotid Ducts

Polyacrylamide gel electrophoresis in discontinuous [pH] gels in the presence of sodium dodecyl sulfate was performed according to Shapiro et al. (1967). Representative Coomassie Blue-stained 11% gels of granule membranes, granule content, and material collected by cannulation of parotid ducts after in situ stimulation are presented in Fig. 24 accompanied by a logarithmic molecular weight scale based on the mobilities of β-galactosidase, albumin, ovalbumin, chymotrypsinogen, and cytochrome C. The staining patterns for granule content and duct collection are quite similar and show a surprising number of bands representing exportable proteins considering the paucity of identified enzymatic activities. Although there was considerable variability in the amounts of various secretory components from experiment to experiment, three bands in the content are distinguished as major components having molecular weights of approximately 58000, 33000, and 12000.* The pattern observed for granule membrane, however, is considerably more complex with 20-30 Coomassie Blue-staining bands being readily visible, the majority having molecular weights greater than 30000. Of particular interest is a single band of estimated molecular weight 40000 which stands out as a major component. Another interesting property of two low mobility bands

*Molecular weights should clearly be considered approximations since electrophoretic mobility in SDS polyacrylamide gels in addition to being an approximate log-linear function of molecular weight is dependent on the polysaccharide content (Glossmann and Neville, 1971; Marchesi et al., 1971) and on the volume of sample loaded on each gel which in these experiments was by no means uniform.

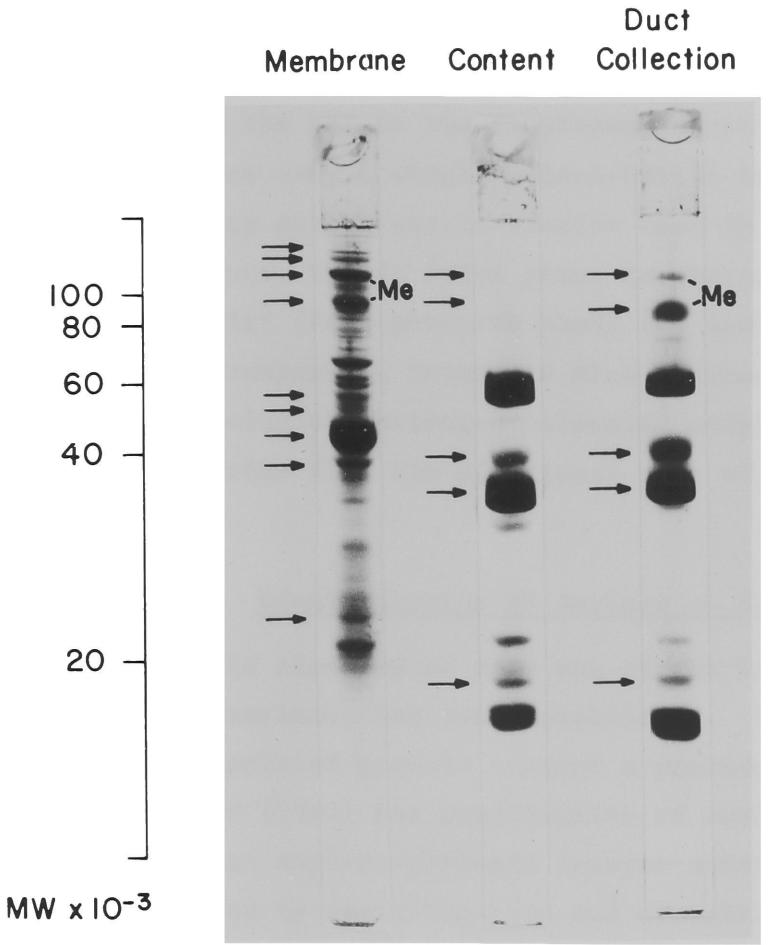


Fig. 24. 11% polyacrylamide gel profiles of granule membrane, content, and secretory protein collected by cannulation of parotid ducts. Samples for electrophoresis, dissolved in SDS, were reduced with 50 mM dithiothreitol (or 1% 2-mercaptoethanol) (2 min, 100° C), and sample loads were ~125 µg, ~100 µg, and ~140 µg protein (Lowry) for membrane, content, and duct collection respectively. Loads for gels reacted with the PAS-technique were 150-200 µg protein (Lowry). (Me) denotes bands that stain metachromatically with Coomassie Blue.

evident in gels of the secretion obtained from cannulated parotid ducts (designated Me in Fig. 24) and a couple of high molecular weight species present in gels of granule membranes (also designated Me) is that they stain pink with Coomassie Blue. This metachromatic staining is unstable, fading with time, but is reestablished by restaining indicating qualitatively that loss of visibility of these components is not a result of protein loss from the gel to the isopropanol-acetic acid fixative. The granule content has only a single metachromatic band corresponding to the higher mobility metachromatic species seen in the secretion collected from the duct, but it faded prior to photographing this gel. Periodic acid-Schiff (PAS)-positive bands are indicated by an arrow alongside the corresponding Coomassie Blue stained band. Note that the major content species of estimated molecular weight 58000 and 12000 are PAS negative and thus fill the additional role of internal controls in this assay.

Identification of Amylase on Gels

The enzymatic identity of only one of the bands present in the granule content, amylase, has been established. This was achieved by applying to the isolated granule content a procedure developed by Schramm and Loyter (1966) for purification of amylase by alcoholic precipitation of an amylase-glycogen (enzyme-substrate) complex. The precipitate, washed by centrifugation and dissolved in sodium dodecyl sulfate, was compared by gel electrophoresis to its supernate (i.e., the fraction of the content left soluble after ethanol precipitation of the amylase-glycogen complex) and to untreated granule content as seen in Fig. 25. Evidently, the 58000 molecular weight band has been selectively extracted, the balance of the content showing very little retention, hence barely visible staining of this species.*

*Figure 25 suggests that in addition to amylase, the content has lost the pink staining band of molecular weight ~95000 during ethanol precipitation. In fact, a band was initially present at the corresponding location, but it faded away by the time the gel was photographed. Since the band was considerably weaker than in untreated content (in relation to the relative staining intensities of other content

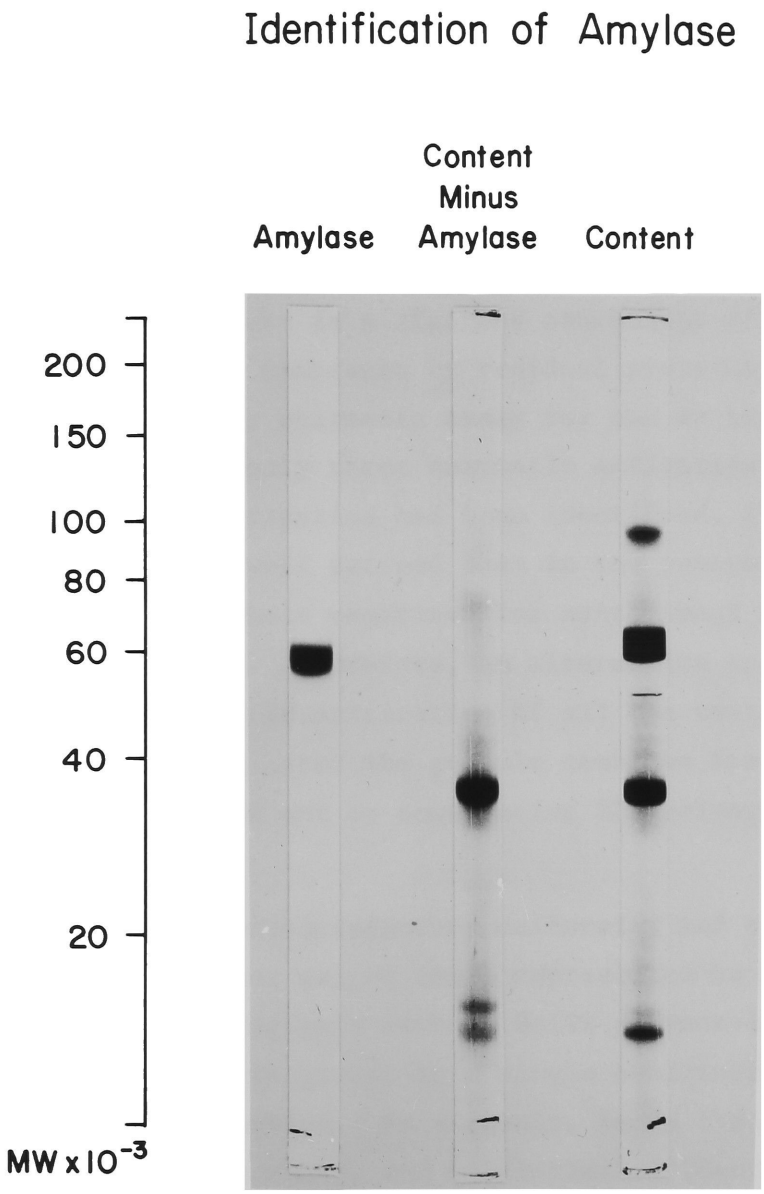


Fig. 25. Polyacrylamide gradient (8-12%) gel profiles of amylase isolated by alcoholic precipitation of content in the presence of glycogen (as described in the Methods), content not susceptible to precipitation (the supernate of the precipitation step), and the same content untreated for amylase separation (control). Amylase is identified as the major Coomassie Blue staining species having an electrophoretic mobility corresponding to a molecular weight of 55,000 - 60,000 daltons.

Comprehensive Identification of Residual Content Polypeptides Contaminating Secretion Granule Membranes

The experimental approaches already described (i.e., pulse-chase labeling of newly synthesized proteins, isolation of secretion granules and their subfractionation into granule content and granule membrane) can be combined to investigate the rates of protein synthesis for the content and membranes of secretory granules. The necessary prerequisite to a study of this nature is a rigorous assessment of the degree of contamination of granule membranes by residual proteins in a more comprehensive manner than by enzymatic assay for one or two constituent secretory proteins. Since only three enzymatic activities associated with the secretion granule fraction had been identified, the territory remained considerably less well defined than in the pancreas, and exhaustive examination of granule membranes for contaminant enzymatic activities was not possible. Therefore, an alternative approach was followed which aimed at the identification of all the content (secretory) proteins which contaminated the granule membrane fraction, the identification being carried out by comparative SDS polyacrylamide gel electrophoresis.

To obtain disc gels of satisfactory uniformity and optimal porosity for the entire molecular weight range represented in both membranes and content, resolving gels with an 8-12% polyacrylamide continuous gradient were made in groups in a single container as described in detail in the Methods section. As a result, bands throughout the molecular weight range were sharp, and mobilities of identical proteins were quite comparable from gel to gel.

Complete assessment of content contaminants was achieved in two complementary types of experiments.

I. To carry out the first type radioactively labeled granule

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species), it is probable that part of it was lost by absorption to glass surfaces during the additional step introduced by the amylase precipitation procedure.

content was prepared by pulse labeling lobules with [^{14}C]amino acids, chase incubating the tissue until the incorporated label had reached mature secretion granules, isolating and finally lysing the granules to obtain the labeled soluble extract.* This radioactive extract was then used for two mixing experiments: 1) an isolated granule fraction was lysed in the presence of roughly 0.1 μCi of this radioactive extract, and the membranes were subsequently isolated and processed for gel electrophoresis, 2) in the second type of experiment, isolated granule membranes solubilized in SDS were mixed just prior to gel electrophoresis with radioactive extract (an amount corresponding to one-fifth the radioactivity loaded on gels of extract alone). These two types of experiments should establish which bands in the gel electrophoresis pattern of a usual membrane fraction represent content contaminants. Such bands should be easily identifiable by: a) their co-migration with radioactivity, b) their increased absorbancy at 550 nm and c) their identical mobility to bands present in the gel-electrophoretic pattern of the content. The results of the two mixing experiments are presented in Fig. 26 accompanied by the radioactivity and optical density (Coomassie Blue) profiles given by a gel electropherogram of the radioactive extract used in these experiments. Also included, as control, is the staining pattern of the gel of an unlabeled, untreated granule membrane fraction. A comparison of the four composites in this figure reveals that content contaminants not removed by the extraction procedure can be easily identified in control membrane preparations since, as expected, they have the same mobility as radioactivity peaks and amplified optical density peaks in the mixing experiments.

Amylase, for example, recognized at molecular weight ~58000 in the electropherogram of the extract was present as a substantial band in the gel profile of untreated membranes even though enzymatic assay showed that 99.97% of the total granule complement had been removed post-lysis. The gel pattern of isolated membranes mixed with radio-

*The term extract rather than content will be used in part in the rest of the paper for reasons that soon will become evident: the extraction procedure employed does not fully remove the content.

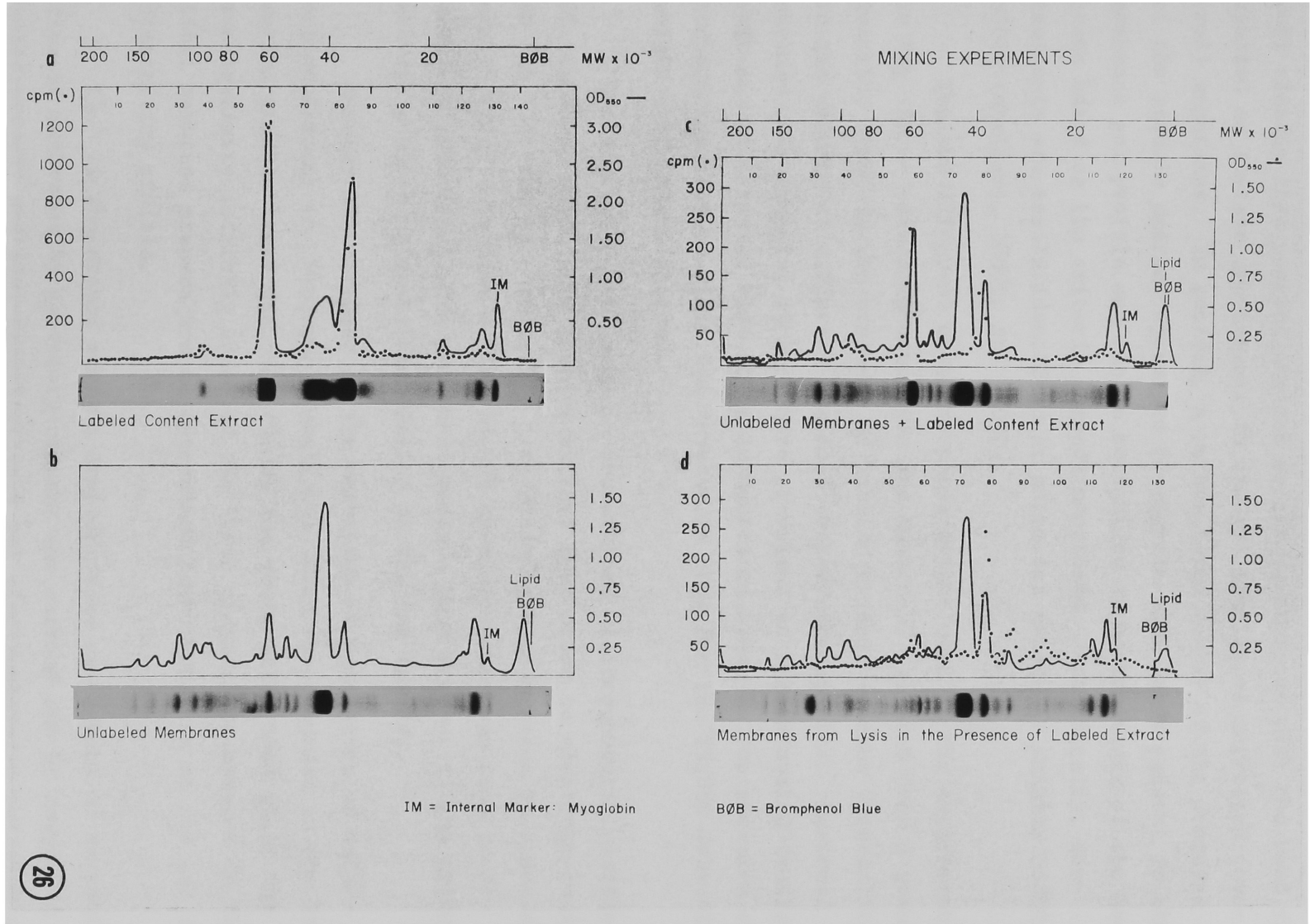
Fig. 26. Mixing experiments used to identify bands in electropherograms of granule membrane subfractions that represent residual content contaminants. Panels (a), (c), (d) of the figure are composites of gel photographs, OD_{550} scans of the staining pattern (—), and radioactivity profiles ( ) obtained by slicing and counting the gels. Panel (b) is a composite which has no radioactivity profile. Gels are polyacrylamide continuous (8-12%) gradients.

(a) [^{14}C]granule extract which was used for both mixing experiments that are presented in panels (c) and (d).

(b) Unlabeled (control) gel profile of the membrane subfraction. By comparison of this profile with that of the two mixing experiments (panels (c) and (d)) it is possible to identify which bands comprising a usual membrane subfraction represent or co-migrate with residual content contaminants.

(c) Mixing experiment in which labeled extract ($^1/_5$ the amount of panel (a)) was added to SDS-solubilized granule membranes (the identical membrane preparation viewed in panel (b)) just prior to electrophoresis. Contaminants can be recognized as co-migrating with radioactivity and having an amplified staining intensity in comparison to bands present in the profile of panel (b).

(d) Mixing experiment in which ~0.1 μCi [^{14}C]granule extract was added to secretion granules in the Amicon chamber prior to lysis. The electrophoretic pattern of the membrane subfraction separated from the ensuing lysate is presented in this panel. Bands which co-migrate with radioactivity can be recognized as containing residual content contaminant.


MIXING EXPERIMENTS
a
200 150 100 80 60 40 20 BØB MW x 10⁻³
cpm(•)
OD550
IM
BØB
Labeled Content Extract
b
Lipid
BØB
IM
Unlabeled Membranes
c
Unlabeled Membranes + Labeled Content Extract
d
Membranes from Lysis in the Presence of Labeled Extract
IM = Internal Marker: Myoglobin
BØB = Bromphenol Blue

active granule extract just prior to electrophoresis demonstrates very clearly that content amylase co-electrophoreses with the band recognized as amylase contaminant in the gel profile of untreated (control) membranes. In general, a rather large number of the proteins of the granule extract appear to co-migrate with bands present in the membrane gel profile and can be recognized as contaminants of the membrane based on the criteria already mentioned. Specifically, contaminants are easily identified at estimated molecular weights 95000, 58000 (amylase), 33000, and 12000.

The two different membrane preparations used in the experiments in Fig. 26 -- unlabeled membranes and membranes isolated from a granule fraction lysed in the presence of labeled extract -- show variability in the amounts of different residual contaminant species. Residual amylase, for example, is much more prevalent in the unlabeled (control) membrane preparation than it is in membranes lysed in the presence of radioactive extract while the reverse is true for the 33,000 molecular weight contaminant species.

Further, as can be seen in the stained electrophoretic profile of membranes mixed with labeled extract just prior to electrophoresis in Fig. 26, the metachromatic, pink-staining band present in granule membranes at molecular weight 95,000 co-electrophoreses with radioactivity associated with the metachromatic band present in the granule extract suggesting possible identity of the two species.

Finally, an estimate of the magnitude of adsorption of radioactive extract to the granule membranes which were exposed to the label during granule lysis was provided by the observation that 1% of the radioactivity initially added to the lysis chamber was present in the final membrane preparation as determined from summation of the gel radioactivity profile.

II. In the second study a comprehensive assessment of contamination of membranes by secretory content was carried out by comparing qualitatively and semi-quantitatively gel electrophoretic patterns of granule membranes, granule extract, whole granules unlysed, and se-

cretory protein collected by cannulation of parotid ducts. Material collected from the ducts of glands stimulated *in situ* by systemic administration of isoproterenol provided a reference for the identification of all granule protein destined for secretion under physiologic conditions. Moreover, the relative amounts of various secretory proteins present in the collected secretion and in unlysed granules allowed direct and independent assessment of the thoroughness of the non-physiologic means (20 mM $NaHCO_3$, 0.5 mM EDTA pH 7.2) of extracting granule content proteins and thereby separating them from granule membranes. The comparison of gel electropherograms of granule membranes and extracted content with the two references -- protein released into ducts and protein of unlysed granules -- is presented in Fig. 27. Since protein in the membrane fraction represents at most 3-4% of the total granule protein, nearly the entire population of granule membranes of a single isolation was subjected to electrophoresis on a single gel. On the other hand, since content represents the bulk of granule-associated protein, very small fractions (less than 1%, ~100 μg (Lowry)) of: 1) the total duct collection, 2) the total granule population of a single isolation, and 3) the total soluble extract of a single lysis experiment were subjected to electrophoresis on the appropriately designated gels. From this study, it is evident that all bands which stain metachromatically with Coomassie Blue and not just the band of highest mobility (identified in the mixing experiments) belong to the secretory content. In stained profiles of gels of secretion collected from ducts and especially in those of unlysed granules these bands represent a considerable fraction (roughly estimated at 20-30%) of the staining intensity of the entire profile indicating that in recovering secretory content post-lysis, a considerable amount of these high molecular weight metachromatically staining species were lost by tenacious adsorption to the granule membranes and probably (because of the quantities involved) by adsorption to the walls of the Amicon lysis chamber.

Combination of sonication with extraction by NaCl solutions at

Identification of Granule Proteins Destined for Export

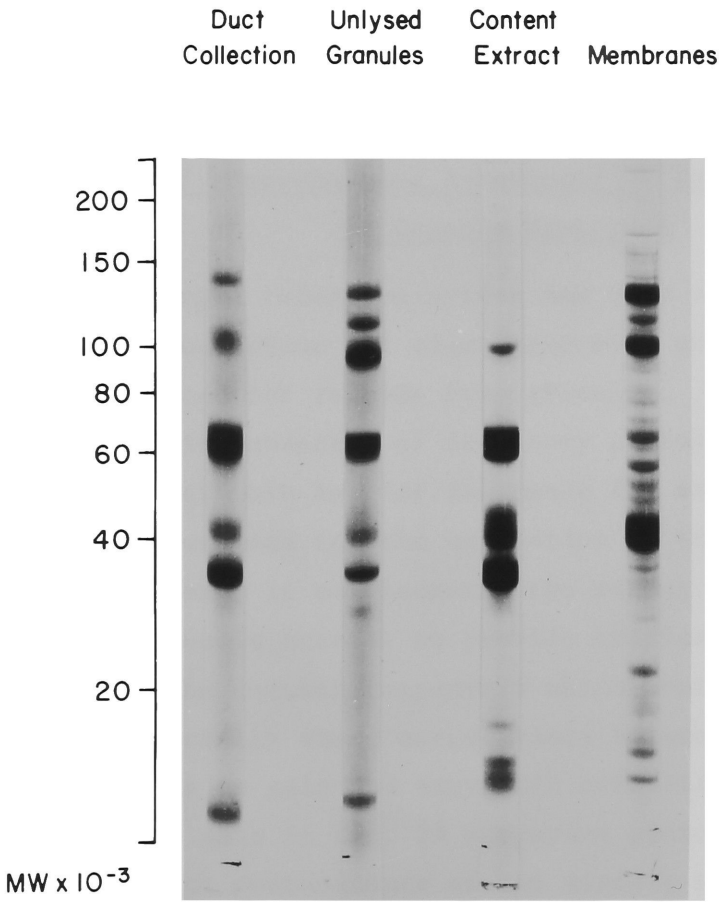


Fig. 27. The physiological reference system for comprehensive identification of all secretory proteins of the acinar cells of the rabbit parotid gland. The gel staining profile of secretory protein collected from cannulated ducts and the profile of unlysed granules indicate the entire spectrum of proteins destined for export. Evidently, the metachromatically staining species (designated Me) present in the membrane between estimated molecular weights 95,000 - 130,000 daltons are all proteins destined for export; however, they remain tenaciously adsorbed to membranes following the non-physiologic process of granule extraction. Note in the staining pattern of duct collection that one metachromatic species is missing. A faint band was present but faded before photographing the gel; however, the bulk of this species may have been lost in processing the collected parotid saliva for gel electrophoresis.

low (50 mM) as well as high (500 mM) concentrations (used to free membranes of other systems of loosely adsorbed proteins (Rosenberg and Guidotti, 1968; Adelman et al., 1973)) or sonication in the presence of the mild chaotropic agent sodium bromide (0.25-0.50 M) (Dandliker et al., 1967; MacDonald and Ronzio, 1972) did not succeed in further reducing adsorption of these proteins to granule membranes.

Summary of Comprehensive Assessment of Content Contaminants in Granule Membranes

A physiologic reference system has been used which uniquely defines on high resolution gel electrophoretic patterns which proteins are truly destined for release from granules. Not only did it establish the entire spectrum of secretory proteins, but additionally it provided a reliable base of reference for assessing the efficiency of the procedures used for the extraction of the content of the granules. Furthermore, it complemented the mixing experiments performed with labeled granule extract to provide critical means for the identification of the content components which contaminate the granule membranes, especially where correlations between polypeptides visible as stained bands on gels and enzymatic activities have not been established. The gels in Fig. 28 summarize pictorially the bands identified as content contaminants of the granule membrane fraction. The arrows indicate bands representing or containing secretory contaminants in the membrane fraction; the remaining bands are considered to be truly membrane associated polypeptides. The companion gel of unlysed granules represents less than one percent of the total granule fraction of a single isolation.

Examination of Protein Biosynthetic Rates for Granule Membranes and Content

The less than complete resolution of granules into membranes and content and, in some cases, the difficulty of unambiguously identifying a protein as belonging to the membrane must be recognized as significant experimental limitations. At this point, it was necessary to

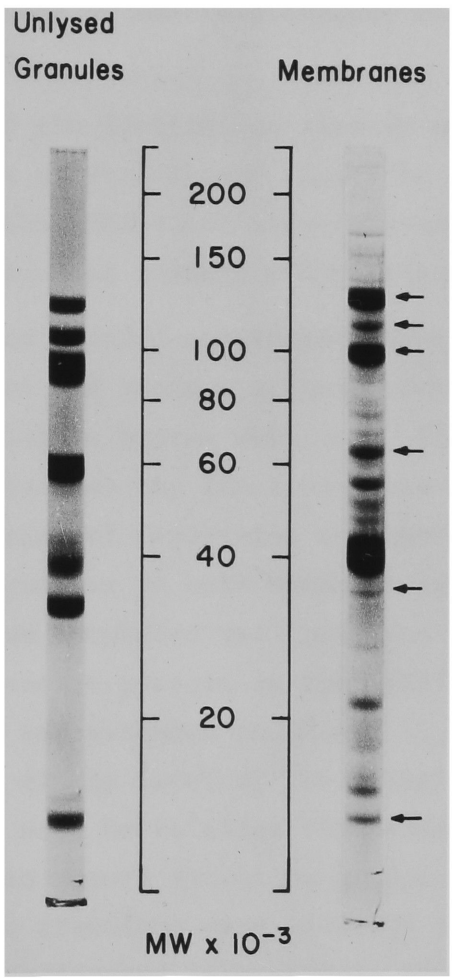


Fig. 28. The Coomassie Blue-stained species present in gel electropherograms of the membrane subfraction which are readily identified as contaminants representing residual granule content by comparison to the polypeptide spectrum of unlysed granules. Gels are polyacrylamide continuous (8-12%) gradients.

choose between performing a more thorough investigation of procedures for content extraction with the aim of achieving complete resolution of membrane from content or accepting the present level of residual contamination and devising methods for determining rates of synthesis for membrane proteins which would allow correction for (or exclusion of) contributions made by adsorbed content proteins. The latter alternative was chosen.

On account of the limitations already mentioned, the data in the concluding part of this thesis should be considered as providing tentative, rather than definite, answers concerning the rates of synthesis of membrane and content polypeptides.

In experiments carried out to determine the relative rates of protein synthesis of the various granule components, the techniques developed for the lobule system were used with the following modifications or adjustments: a) the label used was a mixture of [^{14}C]amino acids, chosen in hopes of maximizing and randomizing the incorporation of radioactive precursors in both membrane and content proteins; b) the amount of tissue incubated was increased to the equivalent of 4 glands to provide enough protein in the final subfractions; c) the period of labeling was extended to 30 min to introduce sufficient label for analysis at the level of individual gel bands and was followed by a "chase" of 4 hr to allow the radioactive secretory proteins to be processed into mature secretion granules; and d) at the end of the "chase" granule fractions were isolated (as described in the Methods) and subfractionated into granule membranes and granule extract (content). No special precautions were taken to insure efficient chase of soluble unincorporated label from the lobules, since it was assumed that any extra incorporation into proteins destined to become granule content or membrane should be affected in parallel if the same precursor pool supports synthesis of both types of proteins. Hence, the chase medium was not supplemented with high concentrations of the whole spectrum of the corresponding ^{12}C-amino acids. Separate determination indicated that pulse-chase incubation of similarly large

batches of lobules in proportionately smaller volumes of medium than used in the characterization of the lobule system was not detrimental to tissue integrity. After the 4 hr incubation a total of 30% of the tissue associated amylase had been released into the pulse and chase media (15% to the pulse medium) which is only slightly higher than the 4 hr release from control lobules in Fig. 10.

Two types of experiments were performed on the membrane and content subfractions of labeled granules obtained as indicated above.

1. Specific Radioactivity at an Average Level. The objective of the first experiments was to determine specific radioactivity at an average level, that is, for the whole mixture of proteins of the content subfraction (granule extract) and the whole mixture of proteins of the membrane subfraction, including a correction of the latter value for adsorbed content. The necessary correction is straightforward:

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\frac{\text{Membrane Subfraction CPM}}{\text{Protein}} - \frac{\text{Adsorbed Content CPM}}{\text{Protein}}}{\frac{\text{Membrane Subfraction}}{\text{Protein}} - \frac{\text{Adsorbed Content}}{\text{Protein}}}$$

As developed in Appendix Ia, this equation can be converted into the form:

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\text{Membrane Subfraction Specific Radioactivity} - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein}}}{1 - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein}} \times \frac{}{\text{Specific Radioactivity Granule Extract}}}.$$

The data for the equation in this latter form are provided by assaying total radioactivity and protein in the membrane and granule extract subfractions and by determining the fraction of the total membrane radioactivity representing content contaminant in a membrane gel profile, thus circumventing the laborious determination and subtraction of the radioactivities and protein of individual contaminant bands. One of the two gel profiles used for this type of estimate is shown in Fig. 29 and shows in crude quantitative fashion how radioactive are the

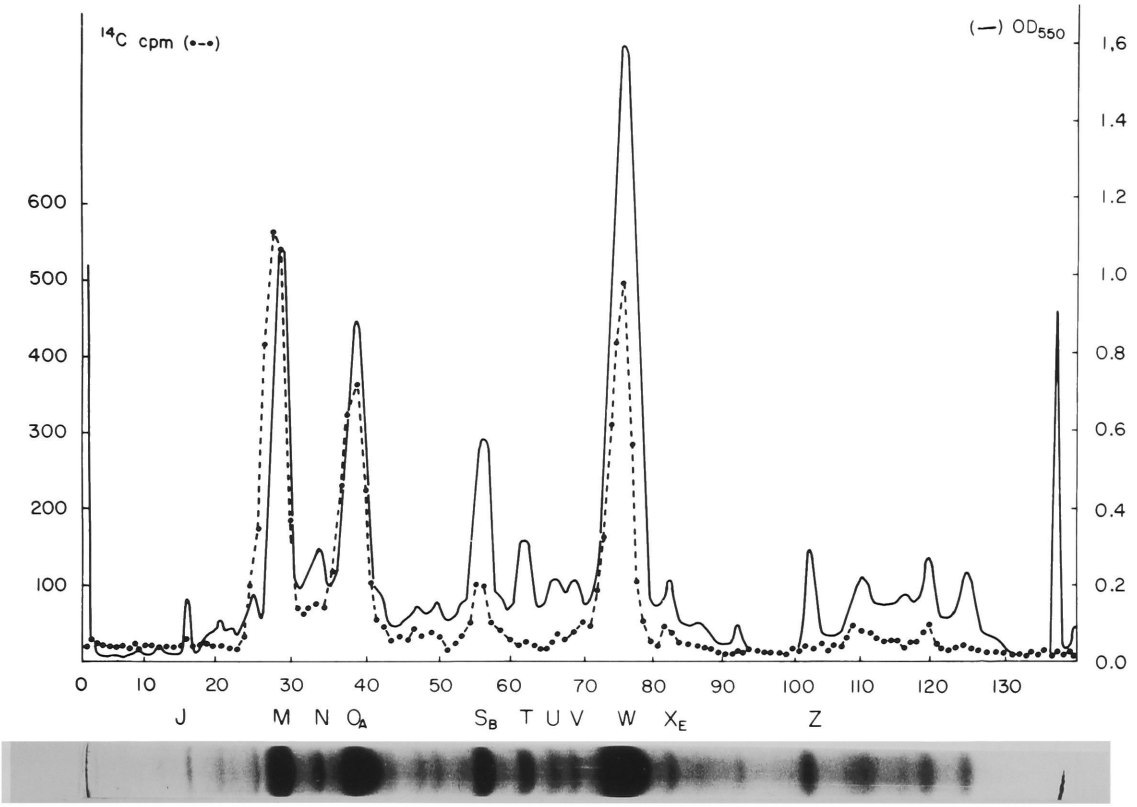


Fig. 29. Composite of a gel photograph, a staining intensity scan at OD_{550} (—), and a radioactivity profile (--) for the granule membrane subfraction used to correct one of the experimental determinations of the average membrane specific radioactivity (mentioned in the text and calculated in Appendix Ib). Membranes were prepared from secretion granules obtained from lobules (equivalent to 4 parotid glands) that had been labeled for 30 min in F12 containing 25 μCi/ml reconstituted algal [^{14}C]protein hydrolysate and incubated post-"pulse" for 4 hr in F12. Certain bands have been labeled according to the nomenclature presented in Fig. 30. Note that bands with the exception of W which are presumed to be membrane-associated (T, U, V, Z) co-electrophorese with no recognizable radioactivity peaks. This is the level to which Winkler et al. (1972) extended their analysis of radioactivity associated with chromaffin granule membranes. Note further, that band W and bands recognized as containing content contaminant (M, N, O_A, S_B, and X_E) co-migrate with peaks of radioactivity. The gel is a polyacrylamide continuous (8-12%) gradient.

residual content contaminants in relation to bands ascribed as true membrane components. (Consider, for example, bands designated M, O_A, S_B versus bands designated T, U, V, Z).

In view of the magnitude of content adsorption to granule membranes already demonstrated, the correction presented in equation form above is expected to be quite sensitive to small uncertainties in the subtracted quantities, especially if content contaminants obscure bona fide membrane bands of significantly different specific radioactivity. Nevertheless, the analysis was carried through on the experiment illustrated in Fig. 29 and on a duplicate experiment in hopes of obtaining consistent values for corrected specific radioactivity of membrane proteins for comparison to the specific radioactivity of the content (extract) proteins. Calculations (presented in detail in Appendix Ib) for the two experiments gave ratios of corrected membrane specific radioactivity to content (granule extract) specific radioactivity of ~1.0 in one case and <0.5 in the other. Therefore, it was concluded that this approach to the analysis of biosynthetic rates was unreliable, and further efforts were oriented toward a second type of experiment which involved the direct examination of specific radioactivities of individual membrane and content polypeptides resolved initially by gel electrophoresis. Evidently, specific radioactivity contributions of content contaminants are, by the nature of this experiment, handled individually and separately from specific radioactivities of polypeptide bands identified as true membrane components.

2. Specific Radioactivity of Polypeptides Comprising Individual Bands in Gel Electropherograms. Specific radioactivities of polypeptides found in individual gel bands representing: a) content (granule extract), b) content contaminating the membrane profile, and c) presumed true membrane components were determined. The bands analyzed are indicated in Fig. 30 by the offset letters A through E as content; M, O_A and S_B as residual content contaminants in membranes; and J, T, U plus U, and W for membrane. In this manner, biosynthetic rates could be compared directly for membrane and content

Gradient Gels (8-12%)

Granule Content and Membranes

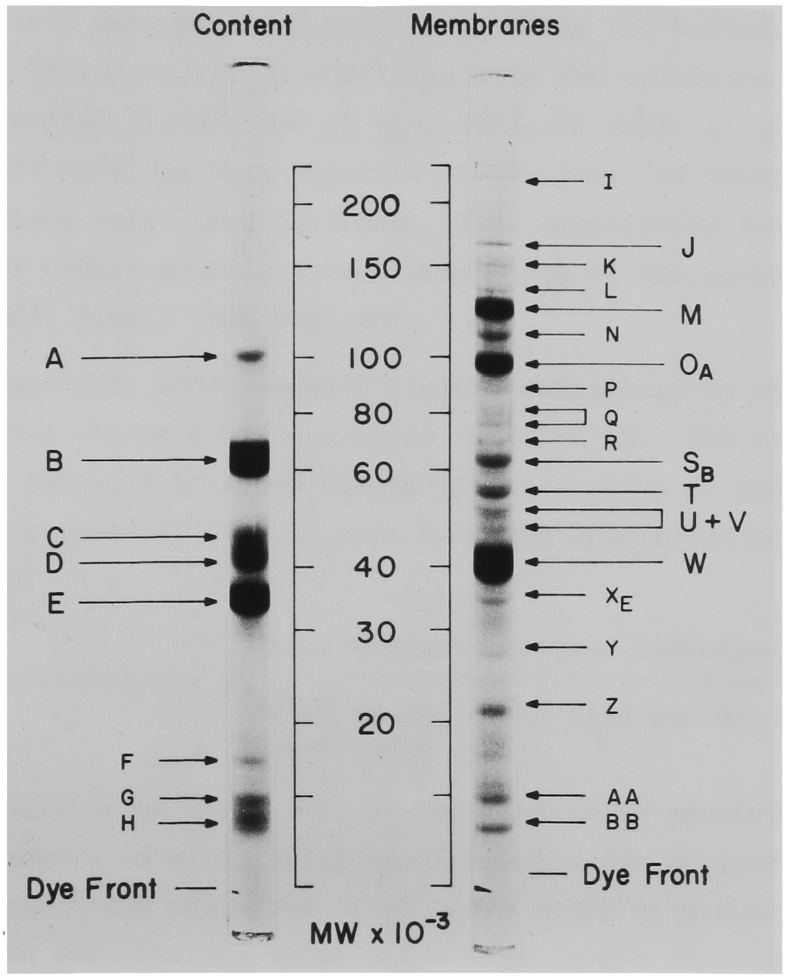


Fig. 30. The nomenclature used to identify all content (granule extract) and membrane polypeptide species visualized as Coomassie Blue-stained bands. Bands which were cut from the gels for protein hydrolysis and determination of specific radioactivity are indicated by the offset, large letters. For some letter identifications in the gel of the membrane subfraction, subscripts have been included to indicate polypeptides that co-migrate with, or represent, residual content contaminants.

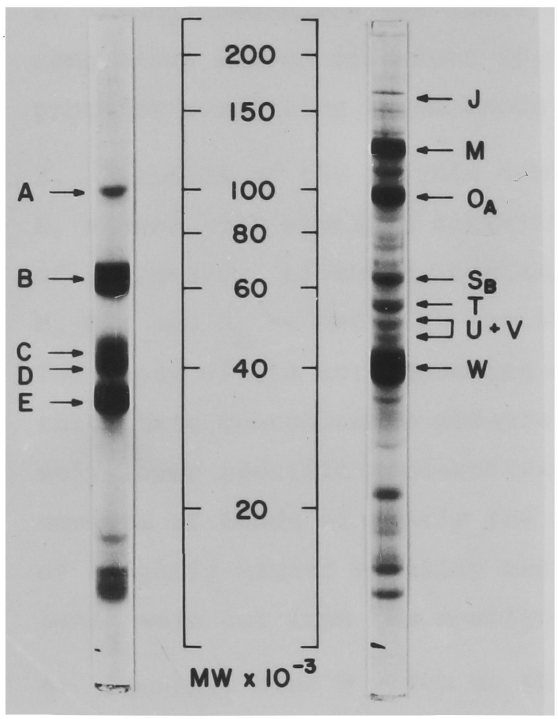
polypeptides, and furthermore, a comparison of the specific activities of content bands with corresponding values found for membrane contaminants could indicate whether the membrane contaminants co-migrate during electrophoresis with (and thus obscure) bona fide membrane bands. Amount of protein in each case was determined by quantitating amino acid analyses of appropriate protein hydrolysates.

The hydrolysates (after removal of aliquots for radioactivity determination) were resolved on a single long ion exchange column (Felix and Terkelson, 1973) outfitted with the sensitive fluorescamine detection system (Udenfriend et al., 1972a,b; Stein et al., 1973). Reproducible anaylses were obtained at the level of tenths of nmoles of the various amino acid residues. This sensitivity facilitated analyses of rather minor gel band components of the membrane profiles, for instance, Band J (see Fig. 30).

The specific radioactivity figures calculated on the basis of the data thus obtained are presented in Table IX. The accompanying Fig. 31 is included to allow visual identification of the bands. The specific radioactivity of each band was calculated according to the formula:

$$\text{Specific radioactivity} = \frac{\text{Total CPM per Gel Band Hydrolysate}}{\text{Total nmoles amino acid per Gel Band Hydrolysate}}$$

The calculated value could thus be expected to be sensitive to: a) volume errors in withdrawing small samples for determining radioactivity especially where the total amino acids is a small sum, b) errors in quantitating amino acids when levels of individual residues are on the order of 0.1 nmole, c) variabilities of precursor (amino acid) specific radioactivities in the medium and in the intracellular pools, d) variations of frequencies of individual amino acid residues in different polypeptides, and e) the extent of amino acid interconversion within the cell. The degree to which these variables affect the interpretation of the results will be discussed in Appen-



Gel Band Specific Radioactivities

Band Source	Gel Band	Specific Activity cpm/nmole
Content	A	146
	B	162
	C	248
	D	134
	E	142
Contaminant	M	226
	O_A	128
	S_B	104
Membrane	J	74
	T	20
	U + V	42
	W	206

Fig. 31 and Table IX. A composite of: 1) gel profiles of content (granule extract) and membranes with accompanying indication of the bands dissected, hydrolyzed, and subjected to specific radioactivity determination, and 2) the specific radioactivity data obtained by radioactivity determination and amino acid analysis of each of the bands.

dix IIb which indicates in general, that the specific radioactivity differences observed for these experiments should be considered real.

From Table IX, it is evident that:

1. Four of the five membrane polypeptide bands analyzed -- J, T, U and V -- showed specific radioactivities lower than those of content bands ranging from 1/2 down to approximately 1/8 to 1/10 of the specific radioactivity of the latter. Thus, these membrane proteins do not appear to be synthesized pari passu with secretory content.

2. Among themselves the individual bands identified as membrane components showed different specific radioactivities suggesting that proteins comprising these bands are synthesized at different rates.

3. Proteins of the granule content, represented by bands A through E, showed high specific activities and thus comparatively high rates of synthesis. Likewise, contaminant bands in the membrane profile -- M, O_A, and S_B -- had high specific radioactivities but not as high as for those of the corresponding content bands. Thus it is possible that these contaminants obscure bona fide membrane bands of considerably lower specific radioactivity in the gel profile or that small amounts of bands of nearly the same mobility (for example, the band of slightly higher mobility near S_B) were included when contaminant bands were cut from the membrane gel for analysis.

4. Finally, band W which on the basis of amino acid analyses (Table XII in Appendix II) and Coomassie Blue staining properties (W stains blue while C plus D stains pink) has a distribution apparently unique to membranes (i.e., it is distinct from either band C or band D of the content which have the same mobility) required special consideration. Its specific radioactivity was as high as that of the whole complement of granule content bands and immediately raised the question of whether it represented a tenaciously adsorbed content contaminant, or, more interestingly, a true membrane component with a high rate of synthesis. In the first alternative, it must be postulated that component W is present in the extracted content, total

granules, and duct collection in amounts too small to be detected in normally loaded gels, which seems unlikely. In the second alternative, this rapidly synthesized component may act as a membrane modifier related to the ultimate differentiation of granule membranes from precursor Golgi membranes. This component could be responsible for controlling the specific interaction of granule membranes and apical plasmalemma involved in secretion. An answer to this intriguing possibility awaits further investigation.

Summary of Specific Radioactivity Determinations for Membrane and Content

With the hindsight provided by the specific radioactivity analysis at individual band level, the difficulties encountered in comparing average specific radioactivities of membrane versus content proteins can be better understood. In addition to factors already discussed (the magnitude of the correction for contaminants and its sensitivity to error), the data were affected by the high rate of synthesis of band W which tended to equalize the specific radioactivity values. The more thorough analysis at the level of individual gel bands raises the following two points: 1) with regard to the experimental approach to this problem, it emphasizes quite clearly that considerations at an average level can be misleading and are useful only after unambiguous identification of which bands truly represent membrane polypeptides and after insuring that the magnitude of contaminant correction of membrane specific radioactivity is small; 2) with regard to the data at individual band level, there exist apparently heterogeneous synthetic rates for granule membrane proteins which are, with the one significant exception of band W, well below the rates of synthesis of content proteins. Thus working primarily at the more refined level (individual band analysis), a conclusion has been reached that is opposite to that of Amsterdam *et al*. (1971) which suggested on the basis of uncorrected average specific radioactivities that rat parotid secretion granule membranes and content are synthesized concomitantly.

An Example of Amino Acid Analysis and Specific Radioactivity Determination

Amino acid analyses and specific radioactivity determinations for the metachromatic-staining gel band A of granule extract and its corresponding contaminant of the gel profile of membranes, band O_A, are presented in Table X. The calculated specific radioactivity (based on total cpm in the hydrolysate per total nmoles of amino acid in the hydrolysate) of O_A was somewhat lower (128 CPM/nmole) than that of A (146 CPM/nmole). Also, there exist a few notable discrepancies in residue frequencies between the two analyses, for instance, aspartic acid, valine, and phenylalanine. Both of these features suggested that there might have been a minor membrane band of low specific radioactivity which, having a mobility quite similar to that of O_A, was included with the latter during processing for specific radioactivity determination.

Analyses of bands A and O_A were chosen for presentation for two reasons: 1) their highly specialized amino acid compositions indicating large amounts of glutamic acid, proline, and glycine are very similar to those of very acidic proteins of unknown enzymatic activity or function collected from human parotid saliva reported by Hay (1967), Oppenheim *et al*. (1971) and Bennick and Connell (1971), thus lending (by composition only) an identity to an additional granule content species; 2) the amino acid analysis of granule membranes of the rat parotid reported by Amsterdam *et al*. (1971) -- the same membranes used for their calculation of the uncorrected average membrane specific radioactivity -- showed outstanding amounts of glutamic acid, proline and glycine. Thus their conclusion that rat parotid granule membranes and content are synthesized concomitantly could reflect, in part, adsorption to membranes of proteins similar to those comprising rabbit granule bands identified as M and O, both of which are high in these three amino acid residues and both of which have high specific radioactivities.

Table X

Amino Acid Composition and

Specific Radioactivity of Bands A and O_A

	Band A nmoles/100 nmoles	Band O_A nmoles/100 nmoles
Aspartic Acid	8.6	4.6
Threonine	2.4	3.0
Serine	6.9	7.2
Glutamic Acid	26.9	25.7
Proline	14.8	12.9
Glycine	27.6	26.0
Alanine	3.5	4.1
Valine	1.0	4.1
Methionine	1.1	0.6
Isoleucine	1.1	1.0
Leucine	1.4	2.4
Tyrosine	0.9	0.9
Phenylalanine	0.2	1.1
Lysine	0.9	2.2
Histidine	(Hidden in NH_3 peak)	0.8
Arginine	2.8	3.2
Specific Radioactivity	146 $\frac{cpm}{nmole}$	128 $\frac{cpm}{nmole}$

IV. DISCUSSION

DISCUSSION

The Choice of the Rabbit Parotid Gland as an Exocrine System for Study

The extensive study of the exocrine pancreas of the guinea pig has produced a substantial body of information concerning the mechanisms by which cells specialized for the export of proteins synthesize, process, and release their products for use in other organs of the body in this and presumably other species (Palade, 1959; Palade et al., 1962; Caro and Palade, 1964; Jamieson, 1972). The various steps comprising the overall process in the exocrine pancreas have been identified as: a) synthesis of proteins destined for export on polyribosomes attached to an organized intracellular network of membrane-bounded spaces, the rough endoplasmic reticulum; b) segregation of these newly synthesized proteins from the cytoplasm proper within the cisternal space of this network; c) transport from this cisternal compartment presumably within a vesicular shuttle to the Golgi complex where the proteins are modified (for example, by glycosylation); d) concentration for storage within membrane-bounded vesicles (secretion granules); and e) release of the stored protein by exocytosis into the acinar lumen. Similar steps have been postulated and in many cases a number of them have been found to be common not only for the generality of glandular cells associated with the digestive tract, but also for a large number of endocrine glands (liver, adeno- and neurohypophysis, adrenal medulla, thyroid) and "unicellular glands" (plasmocytes, mast cells, and possibly fibroblasts) which contribute to the composition of the blood plasma and interstitial fluid, as well as for other mammalian cell types with other functions (exorbital lacrimal gland, spermatocytes, oocytes, and ovarian follicular cells) (Beams and Kessel, 1968).

Within this general framework a detailed study of the secretion

granule membrane is of particular importance since this membrane may participate in the modification and concentration of the secretory products and especially since it is definitely involved in the recognition of, and interaction with, the luminal membrane, the result of which is discharge of the secretory product by exocytosis. Equally important is the fate of this membrane post-discharge. For this type of study the pancreas does not appear to be the system of choice since membranes in general and secretion granule membranes in particular appear to be altered and possibly degraded as a result of tissue homogenization and fractionation procedures. The lipolytic activity of the packaged pancreatic secretory content has been shown to alter -- physically and chemically -- native zymogen granule membranes through accumulation of large amounts of free fatty acid derived from both exogenous lipids (lipid droplets in fat cells present in the stroma of the tissue) and, more importantly from the phospholipids of the granule membranes themselves (Meldolesi et al., 1971a,b). Furthermore, the large complement of activatable protease concentrated within zymogen granules represents a potential source of degradation of membrane polypeptides during prolonged experimental procedures.

The parotid salivary gland was chosen as a system for investigation since, it was expected to be a source of less degraded or chemically altered membranes on account of what was known about the nature of its secretion. These assumptions were confirmed in the study at hand: little if any lipase activity could be demonstrated in lysates of purified secretion granules, and general protease activity appeared to be negligible at neutral pH. In addition, the parotid secretory granules of the rabbit contain no endogenous peroxidase. Hence, precautions to prevent peroxidation of membrane lipids (Amsterdam et al., 1971), another possible membrane chemical artifact, were not necessary. Finally, the parotid glands of the rabbit provide a substantial amount of tissue per animal with a highly uniform population of secretory cells packed with secretion granules, which are useful features for the logistics of this type of experiments.

A Lobule System Used for In Vitro Incubation

An *in vitro* incubation system represents a satisfactory alternative for *in vivo* experimentation if it is capable of maintaining for sufficiently long periods of time (several hours) structural integrity and basic functions -- in the case of glandular tissues, the ability to synthesize secretory proteins and to discharge them in response to external stimuli. Should these requirements be met, the *in vitro* system can be used to significant advantage since it allows superior control of the exposure of the tissue to chemical substituents and since tissue from a single animal is often sufficient to provide samples for a whole experiment covering several timepoints or other variables. This latter feature is extremely useful since it eliminates individual variation among animals.

The *in vitro* incubation system for rabbit parotid tissue that has been developed is capable of supporting incorporation of leucine into protein and maintaining structural integrity for up to 9-10 hr. The tissue further shows sensitive response to sympathomimetic stimuli. It releases ~90% of a representative secretory protein, α-amylase, over a 2 hr period, thus indicating that the tissue is capable of maintaining *in vitro* an energy pool sufficient to meet the demands of massive exocytosis.

The new system represents an improvement over the thin slices previously used for the guinea pig pancreas (Jamieson, 1966; Jamieson and Palade, 1967a,b). The dissection of individual parotid lobules reduces the damage to acinar cells incurred during the slicing procedure, and the use of a tissue culture medium, i.e., Nutrient Mixture F12, instead the amino acid-supplemented Krebs-Ringer-bicarbonate medium extends the period of useful incubation at least 3-4 hr past that achieved with guinea pig pancreatic slices (Jamieson and Palade, 1971).

A significant advantage of the *in vitro* incubation system over its *in vivo* correspondent is the ability to expose the tissue very

briefly to a radioactive precursor, for example, by substituting [^{3}H]-leucine for ^{1}H-leucine in the medium for a well-defined time (pulse) followed by rapid and efficient dilution of unincorporated label (chase). In the case at hand, it was possible to a) assess the relative rates of incorporation of labeled leucine into secretory proteins and proteins retained for intracellular use, b) follow autoradiographically the pathway and kinetics of intracellular transport of proteins destined for secretion, and c) estimate the thoroughness with which the various intracellular compartments constituting the transport pathway are drained.

Relative Rates of Synthesis of Exportable and Non-Exportable Proteins

For rabbit parotid lobules, a minimum of two-thirds of the pulse-labeled protein of the tissue could be discharged from the tissue by adrenergic stimulation initiated after a period of chase incubation of sufficient duration to label secretion granules. Assay of amylase activity discharged from the same lobules indicated release of 95% of the lobule-associated activity. Considered together, the extent of release of amylase activity and protein radioactivity would suggest that about 70-75% of the incorporated labeled protein precursor was destined for export. Thus synthetic rates for secretory proteins were roughly three-fold higher than those for proteins retained for intracellular use. Although the differences in synthetic rates for secretory and non-secretory proteins were not as dramatic as those demonstrated by Siekevitz and Palade (1960) in studies of the specific radioactivities of purified chymotrypsinogen versus bulk non-exportable proteins for the exocrine pancreas of the guinea pig, they were sufficient to make feasible the identification in parotid acinar cells of the intracellular transport pathway for secretory proteins by autoradiography since under these conditions the incorporation of [^{3}H]-leucine into non-exportable proteins would constitute a reasonably low autoradiographic background.

The lower rate of incorporation into exportable protein relative to that of non-exportable protein in comparison to the situation found in the exocrine pancreas may be correlated with the very large population of secretory granules kept in intracellular storage even in animals fed *ad libitum*. The glandular cell appears to meet massive demands for secretory products by either maintaining a large intracellular storage (parotid acinar cell) or by maintaining a high rate of synthesis of exportable proteins (pancreatic exocrine cell).

Autoradiographic Studies of the Intracellular Transport Pathway

Autoradiographic studies of the processing of pulse-labeled secretory protein showed that the general pathway identified in the guinea pig exocrine pancreas (Caro and Palade, 1964; Jamieson and Palade, 1967a) was also operative in parotid acinar cells except for certain distinguishing features. 1) Although the processing of newly synthesized proteins from their sites of synthesis to the Golgi complex was very similar in the *in vitro* incubated guinea pig pancreas and rabbit parotid, the concentrative process which precedes intracellular storage was found to be greatly prolonged in the acinar cell of the rabbit parotid to the point where distinct granule maturation steps became evident both morphologically and kinetically. The identified immature granule is distinguished by being topographically located in the vicinity of the Golgi region, removed from the apically accumulated granular mass, and by possessing a substructure which is likely to reflect uneven concentration of secretory protein. Similar granules with a heterogeneous content have been described by Parks (1961) in studies of the parotid acinar cells of the rat. The orderliness of packing is quite striking, and the ability to recognize immature secretion granules in isolated granule fractions shows that packing is not sensitive to the variables involved in the fractionation procedure. Thus, by virtue of the prolonged maturation of its secretion granules the parotid potentially represents a useful system for the detailed study of the packaging process. 2) The morphology of the RER-Golgi connection in the parotid acinar cell is not well

defined. Neither is the pathway that newly synthesized protein follows within the Golgi complex. Concentration of these proteins is initially detected in distended cisternae on the exit side of the stacks rather than in detached and distinct condensing vacuoles as in the case of the pancreatic exocrine cell of the guinea pig (Jamieson and Palade, 1967a, b). Moreover, multiple concentrative sites are observed within the same stacks (Fig. 14). 3) Newly labeled secretion granules fail to randomize within each cell's population of accumulated granules. This is at variance with the situation found in the guinea pig pancreas where labeled zymogen granules were randomized (and in many cases found apposed to apical lumina) after two hours post-pulse incubation (Jamieson and Palade, 1967a). In the rabbit parotid, it appears as if granule movement is restricted possibly by tight packing of the granular population.

Quantitation of the autoradiographic findings shown in Table IV and Fig. 18 indicates that pulse-labeled, newly synthesized secretory proteins move as a distinguishable wave against a reasonably constant autoradiographic background totaling about 20-25% of the grains and found primarily in the cytoplasmic region occupied by the RER. This background is considered to represent, by and large, proteins retained for intracellular use -- in good agreement with the assessment of the percentage incorporation of labeled precursor into protein which could not be discharged from acinar cells by stimulation (Fig. 10). Quantitative considerations further indicated that the drainage of labeled protein from the RER compartment was quite rapid, approaching the background level by 26-36 min post pulse. Without having fractionated parotid acinar cells to obtain cell particulates representing the RER (rough microsomes), it is not possible to assess precisely the extent of drainage of incorporated label from this compartment. The Golgi complex taken as a whole, however, drains initially quite rapidly and certainly quite efficiently as evidenced by its labeling to the extent of only 3% of the total grains at late chase timepoints. Likewise, immature granules are efficiently converted to mature granules, 3% of

the autoradiographic grains overlying the immature form at 356 min post pulse.

The accumulation of labeled secretory proteins against a concentration gradient in the Golgi complex (the cellular concentrative site) and the maintenance of this concentrated state throughout the rest of the phases of intracellular transport with no evidence of "back-diffusion" (i.e., significant reappearance of label in regions occupied by the RER) suggest that the process of intracellular transport is unidirectional (vectorial). Other aspects of intracellular transport (e.g., vectorial discharge of newly synthesized secretory protein from attached polyribosomes and segregation within the cisternal space (Redman et al., 1966; Redman and Sabatini, 1966; Sabatini and Blobel, 1970), existence of energy dependent steps at the levels of transport from RER to Golgi and release of packaged granule content by exocytosis (Jamieson and Palade, 1968, 1971a), and absence of reversed transport (diffusion?) in the absence of energy (Jamieson and Palade, 1968) have not been investigated. For the time being, it is assumed that in all these respects the situation is comparable to that found in the exocrine pancreas of the guinea pig.

The Isolation of Parotid Secretion Granules

Since no data were available concerning the density of secretion granules of the rabbit parotid as well as their permeability to the suspension media, the parameters of the granule isolation procedure were determined empirically. The technique is based essentially on differential velocity sedimentation (theoretically discussed by Svedberg and Peterson (1940) and de Duve et al. (1959)) with three modifications: 1) a cushion of high molarity sucrose (through which granules will not penetrate) was loaded in the bottoms of tubes for centrifugations designed to pellet secretion granules. In this manner close contact and aggregation between granules and other cellular organelles as well as damage to granules incurred by resuspension of tightly packed pellets were largely avoided; 2) an intervening layer of

sucrose was introduced above the cushion in the second centrifugation to minimize contamination of the sedimented fraction by particulates smaller than granules; 3) a final purification step which makes use of high density sucrose media served to remove residual membranous debris by flotation and to wash simultaneously the pelleting granules.

The results of the procedure were monitored by morphology (for homogeneity) and by enzymatic assay (for recovery and extent of contamination). No attempts were made to attain full recovery; a fraction representing 15% of the total tissue-associated amylase was considered sufficient to warrant its use in subsequent experiments. The high purity of the granules can be ascribed in part to their large size and unusually high density.

Lysis of Secretion Granules

As discussed in detail in the Results, the procedure developed for lysing the secretion granules of the rabbit parotid exploited granule lability in hypotonic media rather than slightly alkaline pH (7.6-8.0), known to be very effective in the lysis of zymogen granules (Greene et al., 1963; Hokin, 1955; Meldolesi et al., 1971) since in phosphate buffer the granules remained relatively stable when pH was adjusted upwards to mild alkalinity. The use of an Amicon ultrafiltration cell proved advantageous since tonicity could be reduced much more rapidly than by standard dialysis, yet the decrease in sucrose concentration could be made gradual enough, to avoid wholesale fragmentation of the granule membrane. Such fragmentation occurred if granules were transferred directly from the high molarity sucrose in which they were isolated into very hypotonic lysis medium.

It is not known under what conditions the ghosts of lysed secretion granules reseal. If it is assumed that they remain unsealed up to the last step of the separation procedure, contamination by entrapment of content can be estimated at less than 0.01% taking into account all dilution-, concentration-, and washing steps involved in the process.

The granule membrane subfraction obtained in this manner is unusual in that it is not a population of thoroughly extracted spherical ghosts, as anticipated, but rather it appears to be a homogeneous population of collapsed and infolded vesicles. The fine fibrillar material seen protruding from the inner aspects forms a quasi continuous layer in regions where the vesicles are flattened. This suggests that the special form of the granule ghosts is due to the interaction of the fibrillar material lining the apposed parts of the granule membrane. Considering the extent to which granule content species (especially the proteins containing exceptional amounts of glutamic acid, proline, and glycine) remain adsorbed to granule membranes post-lysis, it can be speculated that the fibrillar adhesive material represents selectively retained content species.

The Nature of the Secretion Granule Extract

Polyacrylamide gel electrophoresis of secretion granule extract and the released secretion collected by cannulation of parotid ducts has revealed that the spectrum of polypeptides destined for export in the rabbit parotid is considerably more complex than generally assumed in the literature or suggested by the somewhat limited enzymatic characterization carried out in this study. Several major bands spanning the molecular weight range of 12000-130000 daltons are visualized by staining with Coomassie Blue; however, the number does not approach the 20-40 bands visualized by Meyer and Lamberts (1968) for human parotid saliva subjected to electrophoresis in gels not containing detergents. Amylase has been identified and used throughout the present studies as an enzyme characteristic of secretion granules, and it has been localized to the region of estimated molecular weight 55000-60000 in SDS polyacrylamide gels. The nucleases, DNase and RNase, although easily assayable in granule lysates appear to represent only minor components of the parotid secretion based on calculations which assume that their specific activities are equal to those of crystallized pancreatic standards. According to these calculations the nucleases cannot represent major bands of the electrophoretic pattern. Thus at

least six major content species resolved on the basis of molecular weight remain unidentified in terms of activity and function. They might represent digestive enzymes (or zymogens) for different substrates like the kallikrein described in human parotid saliva (Bennick and Connell, 1971) or proteins with or without enzymatic activity acting as antibacterial agents. Lysozyme might be present as it is in other secretions, especially since peroxidase is absent (Klebanoff, 1965). Another possibility to be investigated in the future concerns the presence of secretory immunoglobulins.

Four of the polypeptides present in total granules and in the collected secretion of the rabbit parotid are represented in polyacrylamide gels as bands which stain metachromatically with Coomassie Blue. They have been identified as bands M, N, A, and C in order of increasing electrophoretic mobility. Three of these bands M, A, and C have been subjected to amino acid analysis and found to resemble collagen in their biased amino acid compositions: glutamic acid, proline, and glycine were present in amounts representing 45-86% of the total residues.* As mentioned in the Results, proteins of similar composition have been identified and purified from human parotid saliva by Oppenheim et al. (1971) and found to be susceptible to degradation by bacterial collagenase.

There is, however, a large discrepancy in molecular weights determined or estimated for these proteins in the two species studied thus far. The proteins of human parotid saliva were found to have molecular weights of 12,000 daltons or smaller using analytical ultracentrifugation (Oppenheim et al., 1971; Bennick and Connell, 1971). In addition, they contain little or no bound carbohydrate. The proteins of the rabbit parotid secretion were found by SDS gel electrophoresis to have molecular weights ranging from 40,000 -

*In all amino acid analyses carried out in this work, hydroxyproline, which elutes from the ion exchange column just ahead of aspartic acid, was not determined. A method for its determination in amino acid analysis using the fluorescamine procedure is soon to be published (Felix and Terkelson, in press).

130,000 daltons, and a positive PAS reaction, suggested that they are glycoproteins. If they are glycoproteins, they would be expected to show an anomalously low mobility during gel electrophoresis in the presence of SDS (Segrest et al., 1971; Glossmann and Neville, 1971). However, it remains to be determined by further study if the observed molecular weight differences are influenced by extent of glycosylation, the action of specific proteases, or whether they actually reflect species differences in molecular size.

As yet no function has been identified for these unusual secretory proteins although Hay (1966) has proposed, based on the demonstration of a strong adsorption of the proteins to both hydroxyapatite and dental enamel, that they may be involved in tooth mineralization. Certainly for the rabbit parotid their tenacious adsorptive properties have posed a serious problem to studies requiring a thorough separation of secretion granules into membranes and content. The inability to remove the metachromatic species from the membrane even by competition with high concentrations of NaCl or NaBr has complicated the characterization of secretion granule membranes and has made studies of the average biosynthetic rates of granule membranes versus packaged content unreliable because of the magnitude of the necessary correction for adsorbed content contaminating the membrane. However, in the future it may be possible to remove more thoroughly the adsorbed granule content, in particular the proteins rich in glutamic acid, proline, and glycine by exploiting their tenacious binding to hydroxyapatite or by inserting a low pH step to try to reduce net charge on these molecules, which in human parotid saliva have an isoelectric point between pH 4 and 5.

Amylase, already recognized as a major component of the granule content, has been isolated from rabbit parotid and studied in considerable detail by Malacinski and Rutter (1969). They found it to be a single molecular species not divisible into subunits by criteria which rely on molecular charge and molecular size (cellulose acetate zone electrophoresis, polyacrylamide gel electrophoresis at pH 4.5 in

the absence of detergents, and analytical ultracentrifugation in the presence of 6 M guanidine-HCl); in the present study a single species was identified on the basis of molecular weight (SDS gel electrophoresis after reduction of disulfide bonds). Hence, these observations suggest that the existence of isozymes or multiple gene products for parotid amylase from the rabbit is not likely in contrast to human parotid amylase (Keller et al., 1971) and a variety of pancreatic amylases (Keller et al., 1971; Malacinski and Rutter, 1969; Sanders and Rutter, 1972). The glycoprotein nature of amylases (in many cases related to the occurrence of multiple enzymatic forms) is quite variable. Rhesus monkey and baboon parotid amylases contain no demonstrable carbohydrate (Keller et al., 1971) in contradistinction to human parotid amylases and rat, porcine, and human pancreatic amylases which are all glycoproteins (Keller et al., 1971; Sanders and Rutter, 1972). For the rat parotid enzyme the reports are conflicting: Robinovitch (personal communication to Keller et al.) found little or no evidence for bound carbohydrate while Sanders and Rutter (1972) reported low concentrations. The rabbit parotid amylase does not give a positive PAS reaction in gels. This cannot be considered as more than suggestive evidence; before any conclusion about the presence and extent of glycosylation of secretory proteins, amylase included, can be reached, bound carbohydrate must be carefully quantitated for each molecular species.

Peripheral to the main theme of this thesis but providing a partial evaluation and justification for the use of the very sensitive amino acid analyzer equipped with a fluorometric detection system is a comparison of the amino acid composition of amylase obtained by hydrolyzing and analyzing a segment of polyacrylamide gel containing the band identified as amylase with the composition determined by Malacinski and Rutter (1969) for purified rabbit parotid amylase. The comparison is presented in Table XI.

The agreement of the two analyses (with the exception of glutamic acid) is quite satisfactory and stresses the potential, already dis-

Table XI

Amino Acid Composition of Rabbit Parotid Amylase

Amino Acid Residue	Gel Band		Purified Amylase [a]
	nmoles	nmoles residue / 100 nmoles	nmoles residue / 100 nmoles
Aspartic Acid	2.11	15.8	14.6
Threonine	0.69	5.2	5.2
Serine	0.94	7.0	7.4
Glutamic Acid	1.48	11.1	6.9
Proline	0.36	2.7	4.0
Glycine	1.64	12.3	11.4
Alanine	0.80	6.0	5.4
1/2 Cystine	n.d.	n.d.	2.4
Valine	1.05	7.9	8.4
Methionine	0.20	1.5	1.5
Isoleucine	0.66	4.9	5.5
Leucine	0.73	5.5	5.1
Tyrosine	0.55	4.1	4.3
Phenylalanine	0.61	4.6	4.8
Lysine	0.61	4.6	4.2
Histidine	0.28	2.1	1.9
Arginine	0.62	4.7	5.3
Tryptophan	n.d.	n.d.	1.7

n.d. = not determined.

[a] Malacinski and Rutter (1969).

cussed at length by Stein et al. (1973) for accurately analyzing very small amounts of protein by the fluorescamine procedure.

The Polypeptide Spectrum of Secretion Granule Membranes

Polyacrylamide gel electrophoresis of membrane subfractions dissolved in SDS has shown that the polypeptides of this subfraction, presumably the membrane polypeptides, can be resolved into at least twenty bands (neglecting content contaminants) of distinct molecular weight. What is most striking about this electrophoretic pattern is that a single band of estimated molecular weight 40000 predominates. Other polypeptide bands recognized as membrane components are by and large of molecular weight greater than 40000.

Comparison of the gel electrophoretic pattern obtained for the membranes of the secretion granules of the rabbit parotid with the patterns recorded for granule membranes in other systems (e.g., zymogen granules of the exocrine pancreas (MacDonald and Ronzio, 1972; Meldolesi and Cova, 1972) and chromaffin granules of the adrenal medulla (Winkler et al., 1970) seems to indicate that membranes specialized for storage of exportable products have a relatively simple composition, that is, are resolved into a limited number of Coomassie Blue-stainable bands and contain a few polypeptide species which constitute a large fraction of the total protein as estimated from stain distribution in the entire profile. This situation should be contrasted with that found for other intracellular membranes for which the number of resolvable bands is considerably higher and in which no majority components have been detected (Meldolesi and Cova, 1972; Glossmann and Neville, 1971; Kreibich and Sabatini, 1974).

Available evidence suggests that secretion granule membranes are functionally specialized to recognize cellular membrane on the apical front of the cell and to interact with it in exocytosis. If there exist no other functions performed by this membrane then the presence of a few majority components becomes understandable. The majority components so far recorded in the membranes of secretion

granules are, however, of different molecular weights in different cell types and possibly in different species: 40000 for parotid; 74000-85000 for pancreas (MacDonald and Ronzio, 1972).

Several of the polypeptide bands in the gel electropherogram of the granule membrane fraction reacted positively with the PAS procedure suggesting the presence of bound polysaccharide. At least three of the reactive species can be recognized as content bands contaminating the membrane gel pattern -- M, O_A, and X_E -- by correlating electropherograms in gradient- and 11% non-gradient gels. The rest presumably represents true membrane glycoproteins. As already mentioned, it should be understood that the characterization of the carbohydrate-containing species requires more stringent criteria. In the future it would be of interest to determine by appropriate means (surface-labeling techniques) if the glycoproteins of the granule membrane are asymmetrically distributed as is the case for cell membranes in other systems (Steck et al., 1971; Segrest et al., 1973; Hubbard and Cohn, 1972; Bretscher, 1971a,b,c).

Assessment of the Separation of Granule Extract from Granule Membranes

As indicated in the Results, a comprehensive determination of the extent of contamination of granule membranes by residual secretory species is best carried out by establishing a reference system which should be qualitatively and quantitatively an accurate index of all content species released under physiological conditions from the granules. For these experiments the reference system consisted of parotid secretion collected by cannulation of parotid ducts. Unlysed secretion granules can be used as an additional convenient reference since they are expected to contain the polypeptides of the granule membrane in negligible or undetectable amounts and since they constitute a control for any alteration of the polypeptide composition the duct collection may incur during desalting and lyophilization prior to electrophoresis. The two reference systems were defined in

terms of bands resolved by gel electrophoresis since in the absence of known functions for the secretory proteins of the parotid, activity assays could not be considered. With this approach the metachromatically staining bands of low mobility which were very prominent in the electrophoretic pattern for granule membranes were immediately recognized as being adsorbed proteins which under physiologic conditions are released from secretion granules and, in fact, represent a substantial fraction (as roughly estimated from relative staining intensities) of the secretory protein packaged within the granules.

The mixing experiments using radioactive granule extract were useful for sorting out from a complex electrophoretic profile, in an exact manner, which polypeptides of the membrane subfraction co-migrate with material recognized as residual contaminating content. In retrospect, the use of radioactive secretion collected from in vitro incubated lobules, in place of radioactive granule extract, would have been advantageous for these mixing experiments since, in this way, losses of content proteins, especially of metachromatically staining species, by adsorption to granule membranes and presumably to the walls of the Amicon ultrafiltration chamber succeeding granule lysis could have been avoided. Moreover, the main reference system, i.e., the secretion collected by cannulation of parotid ducts, could be checked by comparison with secretory proteins discharged from lobules stimulated in vitro since potential modification of the secretion by cells lining the major ducts of the gland could be avoided.

In summary, although the lack of identification of assayable enzymes in parotid secretion of the rabbit necessitates devising alternative means for achieving comprehensive assessment of residual content contaminants of granule membranes, the analysis at the level of recognizing contaminants as stained gel bands is advantageous since: 1) it allows identification of contaminants endowed, as well as not endowed, with enzymatic activity, and 2) it defines the level below which enzymatic activities of the residual content must be reduced to qualify as negligible contaminants. In the study at hand, the amylase

that remains in the membrane subfraction after granule lysis and membrane isolation (0.03-0.07% of the total granule amylase) represents one of the prominent bands in the stained profile of the membranes.

Studies performed using three other glandular systems (rat parotid, guinea pig exocrine pancreas, and bovine adrenal medulla) seem to indicate that complete separation of granule membrane from packaged content by non-physiologic means has not been attempted in rigorous fashion; adsorption of content to the granule membranes post-lysis appears to be a common problem.

As already discussed in the Results the membranes of secretion granules of the rat parotid (Amsterdam et al., 1971) are probably contaminated to a considerable extent by adsorbed polypeptides analogous to the metachromatically staining species observed for the granule membranes of the rabbit parotid, since amino acid analysis of hydrolysates of granule membranes of rats indicate outstanding amounts of glutamic acid, proline, and glycine.

The membrane ghosts prepared by mild alkaline extraction of pancreatic zymogen granules of the guinea pig were found by enzymatic assays to contain low levels (0.1-0.2% of the total granule complement) of a number of residual enzymes and zymogens. However, gel electropherograms of granule membrane subfractions prepared from [^{3}H]leucine pulse-labeled and chase-incubated slices showed that nearly all radioactivity could be identified as residual contamination by content polypeptides based on comparison with gels of the alkaline granule extract. (Meldolesi, 1974).

On the other hand, contrary to the general assumption that membrane polypeptides should be different from those traditionally identified as content polypeptides, it has been suggested that the membranes of the chromaffin granules of the bovine adrenal medulla actually contain as part of their structure a protein which simultaneously exists in soluble form packaged within the granule: dopamine β-hydroxylase (Winkler et al., 1970; Viveros et al., 1969). Hörtnagl

et al. (1972) have concluded, based on electrophoretic mobility, comparative amino acid analysis, and immunological cross-reaction, that the soluble dopamine β-hydroxylase is the same protein as that termed chromomembrin A, one of the major "membrane-associated" polypeptides. After demonstrating, in the work at hand, the tenacious sticking to parotid secretion granule membranes of selected secretory proteins, in particular the metachromatically staining species, it is tempting to suggest that the situation with dopamine β-hydroxylase is similar, especially since means other than repeated hypotonic washing were not attempted to test the nature of the association of the residual enzyme with the membrane. However, caution must be exercised in concluding that insoluble dopamine β-hydroxylase is an adsorption artifact because chromaffin granule membranes as isolated by Winkler et al. (1970) have a protein: lipid weight ratio of 0.45 mg protein: mg phospholipid, which, as discussed by Korn (1969), is already lower than that of most biological membranes (0.7-4.0 mg:mg).* Thus, any further removal of dopamine β-hydroxylase (chromomembrin A), which is one of the major polypeptides of the chromaffin granule membrane subfraction would decrease the protein: lipid weight ratio to levels not encountered thus far for biological membranes. Whether such reservations affect any of the polypeptides found in the membrane subfraction of secretion granules of the rabbit parotid remains to be determined by further investigation.

Quantitation of Protein Using Different Assays

The lobule system, the secretion granule fractionation scheme, and the subfractionation of granules to obtain membranes and content are the elements required to undertake an analysis of the biosynthetic rates of proteinaceous components of the secretion granule membranes

*The ratio does not appear to be artificially lowered as a result of endogenous lipolytic activity as is the case in the pancreas (cf. Meldolesi et al. (1971)) even though these membranes contain large amounts of lysolecithin relative to other membranes of the cell (Blaschko et al., 1967).

and their packaged content. The final phase of this thesis presents the initial results of just such an analysis in which biosynthetic rates were estimated by the determination of the specific radioactivities of individual polypeptides resolved by polyacrylamide gel electrophoresis from membrane and granule extract (content) subfractions derived ultimately from lobules subjected to *in vitro* labeling with a mixture of [^{14}C]amino acids. Since specific radioactivities are normalized to the amount of assayable protein, the results are critically dependent on the reliability with which total protein is determined in each sample. Two conventional protein assays -- that of Lowry *et al.* (1951) and the microbiuret assay -- were found to be unsatisfactory for this purpose because they were susceptible to variations in individual residue frequencies and lacked sufficient sensitivity, respectively. Hence, quantitating all amino acids in protein hydrolysates was considered to be the assay of choice because: 1) it could be performed on aliquots taken from the identical hydrolysate used to determine radioactivity, thus eliminating variations introduced by non-parallel processing of individual samples; 2) it accounts for all residues on a common basis and is thus insensitive to variations in individual residue frequencies; and 3) it is extremely sensitive, especially where individual amino acids are reacted for detection with fluorescamine, being capable of accurately quantitating very small amounts of protein, thus obviating the need to procure large samples for analysis.

The specific radioactivities have been expressed in the Results as $\frac{\text{total CPM}}{\text{total nmoles amino acid}}$, both numerator and denominator representing values for 50 µl aliquots of hydrolysate reconstituted after flash evaporation. The denominator was intentionally left in the form of nmoles rather than converting to a weight quantity (ng amino acid or ng protein) because it represents the desired normalization, i.e., to the total number of constituent molecules which contain radioactivity.

Rates of Biosynthesis of Polypeptide Components of Granule Membranes and Secretory Content

Exocytosis, the mechanism by which cells specialized for the synthesis of proteins and other macromolecules destined for export achieve a controlled extracellular release of these products, entails the intriguing feature that the cell is capable of conserving, at least temporarily, the membrane that has already been used to package and release the exportable products. Upon fusion of apical cellular and secretion granule membranes, packaged content is released while the granule membrane remains in continuity with the cell surface. In fact, under conditions where discharge has continuously been stimulated, the apical cell membrane becomes dramatically expanded as a result of the newly introduced granular membranes. With time following massive release of secretion, the apical plasmalemma returns to its original size; however, the mechanism by which cells achieve this surface reduction, presumably by reinternalization, remains unknown. Intracellularly, concomitant with the reduction in apical cellular surface area, the Golgi complex increases in volume and is often seen as an especially impressive array of stacked flattened cisternae (Jamieson and Palade, 1971; Hopkins and Farquhar, 1973; Castle et al., unpublished) suggesting that previously used granule membrane might be reinternalized possibly as closed vesicles which move to the Golgi complex where they contribute to the enlargement of the cisternal stacks. At this site the membrane would be available for reutilization in the formation of storage granules containing newly processed content. If membrane conservation does indeed function in this manner then one would expect that the granule membrane would have a lifetime which spans a number of generations of secretory content for which function dictates lack of conservation.

However, alternatives have been proposed to the recycling scheme which entail either partial or complete degradation of the membrane reinternalized following exocytosis. For instance, it has been postulated that the recovered membrane enters the lysosomal compartment (Amsterdam et al., 1971). It is not specified, however, whether this

entry is comparable to the fusion achieved between phagocytic vacuoles and primary or secondary lysosomes in polymorphonuclear leucocytes, macrophages, L-cells, and other cell types (Cohn, 1968) or implicates the formation of autophagic vacuoles. Should degradation of the membrane take place following each wave of induced exocytosis, then the turnover of the polypeptides of the membrane of the secretion granules should more closely approximate the turnover of the polypeptides of secretory content.

Basically, three different types of studies are useful for providing the necessary data to determine which alternative, conservation and reutilization or degradation and resynthesis, applies.

1) Reinternalization of membrane can be demonstrated morphologically by labeling either the extracellular medium or the newly expanded cellular surface after inducing exocytosis and subsequently following the uptake of the marker. With this approach, the reinternalization of membrane has been convincingly demonstrated for neuromuscular junctions (Ceccarelli et al., 1973; Heuser and Reese, 1973) although, even in this case the experiments have not convincingly proved recycling. As far as is known, conclusive results on reinternalization and recycling through the Golgi complex have not been obtained in glandular cells. This type of study might best be achieved using glandular tissue that has been dissociated into functional single cells. The risk involved in experiments of this type is the possibility of inducing an altered intracellular pathway by interaction of the membrane with a foreign substance (cf. Heuser and Reese, 1972, 1973).

2) The rates of biosynthesis of granule membrane proteins and of secretory proteins could be compared since the alternatives, conservation and reutilization or degradation and resynthesis of granule membrane proteins post-exocytosis would require rates of accumulation of new membrane components much lower or comparable, respectively, to the rates of appearance of newly synthesized secretory proteins.

Three groups have followed this second approach which assumes that relative incorporation rates of radioactivity reflect biosynthetic rates. As mentioned in the Results, Amsterdam et al. (1971) concluded that membranes and content of the secretion granules of the rat parotid are synthesized concomitantly based on determination of average specific radioactivities for the whole mixture of the content- and of the membrane-subfraction, the latter being uncorrected for adsorbed contaminants. On this basis, they assumed that complete degradation and resynthesis of the secretory granule membrane (rather than its recycling) occurs after exocytosis. On the other hand, Winkler et al. (1972) studying chromaffin granules of the bovine adrenal medulla and Meldolesi (personal communication) studying zymogen granules of the guinea pig exocrine pancreas detected low levels of labeling of polypeptides of granule membranes relative to those of the granule content. Moreover, Meldolesi corrected the specific radioactivity of membrane proteins for adsorbed content by using a formula similar to that presented in Appendix I and concluded that membrane polypeptides on the average are synthesized much more slowly than are content polypeptides. Thus these data would support the alternative of conservation and possible reutilization of secretion granule membrane.

3) The third approach relies on a combined examination of the long term- versus short term conservation of radioactive protein by cells labeled in vivo. In this manner, the disappearance of labeled polypeptides associated at one time with either granule membranes or content, which, in most cases, follows first order kinetics, can be followed to estimate protein half-lives. If the same protein precursor used for long-term labeling is administered as a short-term label (marked with a different radioactive isotope) shortly before sacrificing the animal, then the examination of isotope ratios for the incorporated radioactive amino acid allows an assessment of the relative turnover of the proteins under study. Arias et al. (1969), Bock et al. (1971), Dehlinger and Schimke (1971), and Kuriyama (1972) have used this approach to examine the turnover of components of

microsomal and plasma membranes, while Meldolesi (1974) applied these techniques to study turnover of pancreatic zymogen granule membranes and secretory content. In the latter study, the high ratio of short term: long term labeled precursor incorporation for packaged secretory content and the low ratio for membranes (on the average, less than one-fourth that of the content) showed that the average membrane polypeptide turns over more slowly than does the average content polypeptide, which supports the alternative that membranes are conserved post-exocytosis and makes possible their reutilization for packaging new content.

In summary, the investigation of membrane reutilization following exocytosis by glandular cells requires three types of studies which taken together should constitute a tenable evaluation of the extent of the recycling process. 1) The morphological demonstration of reinternalization and of its pathway serves to place restrictions on postulates concerning the involvement of lysosomes or the nature of any excess membrane or membrane component reservoirs which could represent steps that intervene before reutilization. 2) The examination of protein biosynthetic rates for membrane and content polypeptides treated individually or as unresolved mixtures can indicate if these proteins are synthesized at similar or different rates. 3) The double labeling experiments under certain circumstances can give more reliable data especially for proteins characterized by a slow turnover.

For secretion granule subfractions of the rabbit parotid initial efforts were directed toward a study of type 2 above, i.e., a comparison of rates of biosynthesis of membrane and content polypeptides. As radioactive protein precursor a mixture of fifteen [^{14}C]amino acids was chosen in hopes that incorporation could be maximized and widely enough distributed among a variety of residues to insure a reasonable labeling of all proteins irrespective of their turnover and amino acid composition.

Conclusions and Implications for Biosynthetic Rates of Membrane- and Content Polypeptides of Secretion Granules of the Rabbit Parotid

The data obtained from the study of the biosynthetic rates of the polypeptides of the membrane and content of secretion granules of the rabbit parotid appear to indicate that newly synthesized secretory content becomes packaged within membranes which are neither completely newly synthesized nor entirely pre-existing entities. Rather the situation is intermediate. Several polypeptides identified as belonging to secretion granule membranes are synthesized at individually different rates which are, in general, lower than those of the polypeptides of the exportable content. However, a polypeptide of estimated molecular weight 40000 daltons which predominates in the staining profile of the electropherogram of granule membranes, and which is likely to be truly membrane associated*, has a specific radioactivity comparable to that of the spectrum of content species, hence, a synthesis which parallels that of the content. Putting these features into structural terms, it appears that those membrane-associated polypeptides which have biosynthetic rates considerably slower than the content might represent components of a pre-existing membrane framework (part of the Golgi complex?) or a framework newly assembled from pre-existing components destined to undergo major modification by insertion of a polypeptide of molecular weight 40,000 daltons concomitant with some stage of the packaging of the content. The polypeptide represented by band W then assumes the possible role of a membrane modifier. If specific interaction and fusion between granule membrane and apical cell membrane represent the major, possibly unique, functions of the secretion granule membranes, then the polypeptide comprising gel band W could contribute in some way to these processes.

The intermediate situation, described above, where a selected major membrane component is synthesized quite rapidly, at the same rate

*It should be recalled that this result stands pending a rigorous demonstration that band W represents polypeptide belonging to the membrane.

as content, while the rest of the membrane components are synthesized relatively slowly poses an interesting problem if one considers the implications of these findings for granule membrane recycling and reutilization. If the once-used granule membrane is reinternalized and returned in vesicular form to the Golgi complex for reutilization in packaging newly synthesized secretory products, as the low specific radioactivities (hence, low rates of synthesis) of polypeptides comprising bands J, T, U, V suggest, then it is necessary to identify the mechanism by which the polypeptide of band W, characterized by a high specific radioactivity and presumably high turnover, is removed from the membrane framework during recycling in an operation which may amount to a "dedifferentiation" of highly specialized membrane. Two possibilities can be considered: a) some sort of transient segregation and highly specific, degradation within the lysosomal system of the cell, or b) a specific dissociation from the membrane possibly by sloughing off into the acinar lumen perhaps as a prerequisite to the reinternalization process. As mentioned previously, the capacity to follow reinternalization morphologically will likely make evaluation of the first possibility straightforward; however, the latter mechanism might be quite difficult to assess and would certainly complicate the definition of a membrane protein by introducing the condition of temporal association.

To summarize, several polypeptides which are considered to be truly associated with the granule membranes have heterogeneous rates of biosynthesis which range from 1/2 down to 1/8 - 1/10 those of exportable secretory proteins, suggesting that protein destined for extracellular release is packaged in membranes whose proteins are not newly synthesized but rather predate the content, constituting some sort of intracellular reserve, speculated (on morphologic grounds) to be associated with stacked cisternae of the Golgi complex. However, a predominant polypeptide species of the granule membrane subfraction has a rate of synthesis comparable to that of the proteins

of the secretory content. If further investigation succeeds in demonstrating a true membrane association for this polypeptide, then this species deserves consideration as a possible modifier of a membrane of more general utility (Golgi membrane?) into a highly specialized storage and shuttle system (secretion granule membrane).

As already mentioned, it remains to be shown, by making use of appropriate membrane markers, what is the intracellular pathway followed by, and the eventual fate of, the reinternalized granule membranes. As far as the reutilization issue is concerned, turnover experiments, especially experiments in which massive discharge is interposed between long- and short-term labeling, may help in demonstrating whether or not the set of slowly synthesized membrane polypeptides (J, T, U, V) reappear in the same relative amounts and with the same relative radioactivity in the membranes of secretion granules produced by the cell after a nearly complete discharge of the type elicited by isoproterenol. It should be pointed out, however, that such an experiment cannot conclusively discriminate between reutilization of disassembled membrane proteins and recycling of assembled membranes. To decide this final issue, mass markers of the membrane as a structure would be necessary. The most interesting observation arising from this work remains, however, the detection of a rapidly synthesized membrane component (band W). It is felt that future work should be directed toward a better characterization of the nature and functional role of this component.

V. APPENDICES

APPENDIX Ia

The General Equation for the Correction of Membrane Specific Radioactivity for Adsorbed Content

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\frac{\text{Membrane Subfraction CPM}}{\text{Membrane Subfraction Protein}} - \frac{\text{Adsorbed Content CPM}}{\text{Adsorbed Content Protein}}}{\frac{\text{Membrane Subfraction Protein}}{\text{Membrane Subfraction Protein}} - \frac{\text{Adsorbed Content Protein}}{\text{Membrane Subfraction Protein}}} .$$

Multiply the right side of the equation by 1 in the form:

$$\frac{\frac{1}{\text{Membrane Subfraction Protein}}}{\frac{1}{\text{Membrane Subfraction Protein}}} .$$

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\frac{\text{Membrane Subfraction CPM}}{\text{Membrane Subfraction Protein}} - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein}}}{1 - \frac{\text{Adsorbed Content Protein}}{\text{Membrane Subfraction Protein}}} .$$

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\text{Membrane Subfraction Specific Radioactivity} - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein}}}{1 - \frac{\text{Adsorbed Content Protein}}{\text{Membrane Subfraction Protein}}} .$$

Assume that: $\frac{\text{Granule Extract CPM}}{\text{Granule Extract Protein}} = \frac{\text{Adsorbed Content CPM}}{\text{Adsorbed Content Protein}}$, which holds in the strict sense only if adsorbed content bands do not obscure bonafide membrane bands.

Rearranging this last expression:

$$\frac{\text{Adsorbed Content}}{\text{Protein}} = \frac{\text{Adsorbed Content}}{\text{CPM}} \times \frac{1}{\text{Granule Extract Specific Radioactivity}} .$$

Therefore, by substitution:

$$\text{Corrected Membrane Specific Radioactivity} = \frac{\text{Membrane Subfraction Specific Radioactivity} - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein}}}{1 - \frac{\text{Adsorbed Content CPM}}{\text{Membrane Subfraction Protein} \times \text{Specific Radioactivity Granule Extract}}} .$$

All of the quantities required for the equation in this final form can be determined from:

a) Total Radioactivity data for the membrane subfraction and for the granule extract (content subfraction).

b) Total Protein data (from quantitating amino acid analyses) for both the membrane subfraction and the granule extract.

c) Quantitation, in a gel profile, of radioactivity ascribable to residual adsorbed content.

APPENDIX Ib

Calculation of the Corrected Membrane Specific Radioactivity for Comparison to the Specific Radioactivity of Granule Extract in Two Different Experiments

Experiment 1 (Corresponding to the membrane profile seen in Fig. 29)

a) Uncorrected specific radioactivity of the membrane subfraction was 59.7 CPM/nmole total amino acid, where the total membrane protein was equal to 13.8 nmoles total amino acid.

b) Granule extract specific radioactivity was 62.7 CPM/nmole total amino acid.

c) After electrophoresis of the membrane subfraction, 90% of the radioactivity initially added to the gel was recovered in gel slices.

From quantitation of radioactivity in the slices,

$$\frac{\text{CPM identifiable as content contaminating granule membrane}}{\text{CPM total membrane subfraction}} = 0.60.$$

The total radioactivity present in 50 µl of the acid hydrolysate of the membrane subfraction was 822 CPM.

Therefore, 822 x 0.60 = 493 CPM which represents content contaminant.

Making use of the equation developed in Appendix Ia:

$$\text{Corrected Membrane Specific Radioactivity} = \frac{59.7 \text{ CPM/nmole} - \frac{493 \text{ CPM}}{13.8 \text{ nmoles}}}{1 - \frac{493 \text{ CPM}}{13.8 \text{ nmoles} \times 62.7 \text{ CPM/nmole}}}$$

Corrected membrane specific radioactivity = 55.8 CPM/nmole.

The corrected membrane specific radioactivity, 55.8 CPM/nmole total amino acid, is practically identical to that of the granule extract, 62.7 CPM/nmole total amino acid, for this experiment. Furthermore, from Fig. 29, the residual content contaminants plus the major band (W) assumed to represent membrane polypeptide account for 90% of the radioactivity of the membrane subfraction.

Experiment 2

a) Uncorrected specific radioactivity of the membrane subfraction was 19.3 CPM/nmole total amino acid, where the total membrane protein was equal to 28.1 nmoles total amino acid.

b) Granule extract specific radioactivity was 29.8 CPM/nmole total amino acid.

c) After electrophoresis of the membrane subfraction, 78% of the radioactivity initially added to the gel was recovered in gel slices. From quantitation of radioactivity in the slices,

$$\frac{\text{CPM identifiable as content contaminating granule membrane}}{\text{CPM total membrane subfraction}} = 0.53$$

The total radioactivity present in 50 µl of the acid hydrolysate of the membrane subfraction was 542 CPM.

Therefore, 542 x 0.53 = 287 CPM which represents content contaminant. Making use of the equation developed in Appendix Ia:

$$\text{Corrected Membrane Specific Radioactivity} = \frac{19.3 \text{ CPM/nmole} - \frac{287 \text{ CPM}}{28.1 \text{ nmoles}}}{1 - \frac{287 \text{ CPM}}{28.1 \text{ nmoles} \times 29.8 \text{ CPM/nmole}}}$$

Corrected membrane specific radioactivity = 13.8 CPM/nmole.

The corrected membrane specific radioactivity, 13.8 CPM/nmole total amino acid, is somewhat less than half that of the granule extract, 29.8 CPM/nmole amino acid, and is thus not consistent with the calculations of Experiment 1. Once again, however, the residual content contaminants plus the major band (W) assumed to represent membrane polypeptide account for 90% of the radioactivity of the membrane subfraction.

If the corrected membrane specific radioactivity for Experiment 2 is "recorrected", this time treating the major membrane polypeptide band (W) as a contaminant, then the resulting "recorrected" membrane specific radioactivity is 4.6 CPM/nmole total

amino acid, roughly one-seventh the content value, which suggests that membrane polypeptides, excluding one, are synthesized at considerably slower rates than content polypeptides.

In conclusion, the inconsistent results obtained for the relationship of the specific radioactivities of the membrane and granule extract subfractions from these two experiments indicate that this approach to the study of biosynthetic rates of membranes and content of the secretion granules is unreliable.

APPENDIX II

Consideration of Possible Effects of Variations in Amino Acid Frequency Among Proteins and of the Intracellular Protein Precursor Pool on the Specific Radioactivities of Individual Gel Bands

As indicated in the text and in Table IX the specific radioactivities for individual polypeptides are expressed as CPM per gel band/nmoles total amino acid per gel band. This formula was developed as a workable compromise during the experiments, although it is admittedly open to question. Originally it was estimated that under the experimental conditions given (use of a mixture of fifteen [^{14}C]amino acids at a total radioactivity concentration of 100 μCi/ml in the labeling medium), the amount of label introduced into the polypeptides of both content and membrane subfractions would be sufficient to permit specific radioactivity determination of individual amino acid residues in the protein hydrolysates (e.g., incorporated leucine CPM/nmole leucine, incorporated alanine CPM/nmole alanine, etc.). This approach would have avoided the necessity of considering the influence of the specific radioactivities of individual amino acids in the intracellular pool. In addition, it would have ruled out the effect of large variations in animo acid residue frequency on labeling of the various polypeptides since the label in different polypeptides could have been compared residue by residue. However, determination of the radioactivity present in individually collected amino acids (after reaction with fluorescamine) by liquid scintillation spectrometry proved unreliable on account of the variable levels of chemiluminescence produced by fluorescamine products present in amounts proportional to the quantity of each amino acid. The radioactivity values were variable and inconsistent and had to be recorded above an excessive background.

As shown in the text, the formula (total CPM per gel band/total

nmoles amino acid per gel band) gave differences in specific radioactivity for the bands representing polypeptides of the membrane and content subfractions. Before further interpretation, these data should be tested for their sensitivity to variations in amino acid composition of the bands analyzed and to differences in specific radioactivities of amino acids in the incubation medium. The necessary data are available in Tables XII and XIII. The questions to be considered are: 1) Can the observed differences in residue frequencies between individual bands account for the entire range of specific radioactivities for polypeptides of both membrane and granule extract subfractions? 2) Do these differences in specific radioactivity reflect variations in the specific radioactivity of amino acids in the intracellular pool which, in turn, may reflect either selective uptake of particular amino acids from the medium or large-scale metabolic interconversion? These questions are deemed relevant and critical since affirmative answers to either of them will support the opposite hypothesis, namely, that polypeptides of the membranes and the content of secretion granules are synthesized concomitantly.

To carry out these tests, the data (Tables XII & XIII) were treated in two ways:

A. Specific radioactivities were compared for pairs of gel bands — one from the membrane- and one from the granule extract profiles — that have quite similar amino acid compositions. For each comparison, the contributions to the specific radioactivity of residues considered to show significant discrepancy in frequency between the two bands were maximized by: 1) decreasing specific radioactivity of the extract band and 2) increasing specific radioactivity of the membrane band. This correction is intended to distort the data toward maximal convergence for content and membrane specific radioactivities.

B. Several assumptions were examined (since no data were obtained) concerning the relative concentrations of labeled precursor amino acids within the cell for their potential influence on the specific radioactivity values experimentally determined for individual polypeptides of

Table XII

Amino Acid Determinations in Hydrolysates of Gel Bands

	Gel Band					nmole/100 nmole								
	Extract					Contaminants			Membranes					
Residue	A	B	C	D	E	M	O_A	S_B	J	T	$T^{c\dagger}$	U+V	W	$W^{r\dagger}$
Aspartic Acid	8.6	15.8	17.0	19.5	12.1	2.2	4.6	13.9	18.6	6.7	8.9	7.7	10.7	10.8
Threonine	2.4	5.2	4.6	6.2	6.3	1.3	3.0	5.5	5.4	4.4	5.8	4.3	6.1	6.3
Serine	6.9	7.0	8.0	14.3	7.9	1.6	7.2	11.0	10.2	5.9	7.9	5.9	9.4	9.7
Glutamic Acid	26.9	11.1	18.8	20.1	12.0	31.6	25.7	12.4	14.8	9.6	12.8	10.0	15.0	14.8
Proline	14.8	2.7	20.9	7.0	5.4	20.0	12.9	4.1	3.8	4.0	5.4	5.3	4.8	5.5
Glycine	27.6	12.3	4.3	5.3	3.2	34.5	26.0	12.9	16.4	6.1	8.1	10.0	5.9	5.1
Alanine	3.5	6.0	4.7	3.9	11.6	1.0	4.1	8.2	7.1	9.3	12.3	8.1	6.0	5.8
Valine	1.0	7.9	1.4	1.3	8.0	0.3	4.1	7.2	3.9	7.2	9.6	7.1	6.7	6.4
Methionine	1.1	1.5	-	-	0.8	0.4	0.6	1.4	-	2.0	2.7	2.4	1.7	1.6
Isoleucine	1.1	4.9	0.9	1.7	4.5	0.5	1.0	5.2	3.8	3.4	4.5	4.6	4.1	3.9
Leucine	1.4	5.5	3.4	5.6	9.3	0.9	2.4	8.2	7.0	7.6	10.1	8.7	10.6	10.6
Tyrosine	0.9	4.1	3.6	3.6	5.5	0.7	0.9	3.8	1.4	1.6	2.1	2.6	5.6	5.6
Phenylalanine	0.2	4.6	0.5	1.4	3.9	0.5	1.1	4.1	2.4	2.8	3.7	3.9	2.6	2.4
Lysine	0.9	4.6	2.0	3.1	3.7	1.3	2.2		4.9	24.9	-	16.7	3.5	3.7
Histidine	-	2.1	9.8	6.4	1.5	0.1	0.8	2.4	-	-	-	-	3.6	3.2
Arginine	2.8	4.6	-	0.8	4.3	3.2	3.2	-	-	4.4	5.8	2.9	3.7	3.6
Protein $\frac{\text{nmole aa}}{50\ \mu l}$	1.9	13.3	1.4	2.5	11.4	5.8	6.5	2.1	1.1	2.8	2.1	4.0	4.2	9.4
S.R.† $\frac{\text{CPM}}{\text{nmole}}$	146	162	248	134	142	226	128	104	74	20	27	42	196	216

† S.R.= Specific radioactivity; T^c= T recalculated excluding anomalously high lysine; W^r= a duplicate of W from the same experiment but cut from a different gel.

The radioactivity data are averages of duplicate counts for which the 2 σ error ranged from ±3% to ±10%.

Pipetting reproducibility for aliquots removed for radioactivity determination: ±3%.

Accuracy of amino acid analysis: Variations for each residue between bands W and W^r: Average: 5% (range: 0-12%).

Experimentally observed accuracy of overall specific radioactivity determination: For band W compound to band W^r, ~10%.

All amino acid determinations were corrected by subtracting, for each amino acid, values obtained for a gel blank. For all residues the blank value was < .05 nmoles, except for serine and glutamic acid where it was .07 nmoles and for glycine (present in the electrophoresis buffer) where it was .45 nmoles.

Table XIII

Composition of the Labeling ("Pulse") Medium Used for Specific Radioactivity Experiments

Amino Acid (aa)	Non-Ess. or Ess.	μCi / mCi total label	% Total label	Total Amino Acid Conc. (mM) in Medium	% of Total aa in Medium	S.R.† in Medium DPM / nmole aa x 10^{-5}
Alanine	N.E.	100	10.0	.114	4.5	1.84
Arginine	E.	70	7.0	.333	13.0	0.44
Aspartic Acid	N.E.	90	9.0	.093	3.6	2.08
Glutamic Acid	N.E.	125	12.5	.099	3.9	2.64
Glycine	N.E.	40	4.0	.089	3.5	0.94
Histidine	E.	15	1.5	.105	4.1	0.30
Isoleucine	E.	50	5.0	.216	8.4	0.49
Leucine	E.	125	12.5	.240	9.4	1.09
Lysine	E.	60	6.0	.219	8.6	0.58
Phenylalanine	E.	75	7.5	.116	4.5	1.35
Proline	N.E.	50	5.0	.070	2.7	1.50
Serine	N.E.	40	4.0	.076	3.0	1.10
Threonine	E.	50	5.0	.224	8.8	0.47
Tyrosine	E.	40	4.0	.109	4.3	0.77
Valine	E.	70	7.0	.227	8.9	0.65
Asparagine				.050	2.0	
Glutamine				.050	2.0	
Cystine				.050	2.0	
Methionine				.050	2.0	
Tryptophan				.025	1.0	

†S.R. = Specific radioactivity

granule extract and membranes. Both treatments rely on the postulate that a common precursor pool supplies amino acids for biosynthesis of all granule membrane and content polypeptides.

Data Treatment A. Consideration of the effects of frequency variations in amino acid residues per band.

For this treatment it is assumed as a first approximation that the radioactivity present in each type of amino acid residue of a gel band hydrolysate is maximally represented either by 1) the percentage that the amino acid in question represents of the total radioactivity in the "pulse" medium (this value can be found in Table XIII), or by 2) the percentage that this amino acid represents of the total residues present in the hydrolysate (this value can be taken from Table XII, since amino acid frequencies are expressed as nmoles/100 nmoles total amino acid, hence, are percentages). The underlying assumption is that this treatment will cover in reasonable fashion the alternatives that the intracellular distribution of the precursor label among amino acids is or is not proportional to the distribution found in the medium. The correction is performed with whichever of the above percentages is larger.

The specific radioactivity of the gel band chosen from the membrane profile was corrected by subtracting only from the amino acid content (i.e., the denominator of the specific radioactivity) the appropriate maximum percentage of each variant residue.

The specific radioactivity of the gel band chosen from the granule extract profile was corrected by subtracting only from the radioactivity (i.e., the numerator of the specific radioactivity) the appropriate maximum percentage for each variant residue.

Comparative corrections of granule extract band E: membrane band T and of granule extract band B: membrane bands U+V are presented in Table XIV. Evidently the correction of specific radioactivities for

Table XIV

Band E vs. Band T

		Granule Extract E		Membrane T	
Specific Radioactivity before correction:		14200 CPM / 100 nmoles		2050 CPM / 100 nmoles	
Residue Showing Significant Difference* (Table	Residue % of Medium CPM (Table	nmoles 100 nmoles (Table	Maximum Correction CPM	nmoles 100 nmoles (Table	Maximum Correction nmoles
Lysine	6.0	3.7	-850(6.0%)	24.9	-24.9
Glycine	4.0	3.2	-570(4.0%)	8.1	- 8.1
Tyrosine	4.0	5.5	-780(5.5%)	2.1	- 4.0
			-2200		-37.0
Specific Radioactivity after correction:		(14200-2200)/100		2050/(100.0-37.0)	
		= 12000 CPM / 100 nmoles		= 3300 CPM / 100 nmoles	

* Significant difference: Residue has 2 times the frequency in one band compared to the other.

Band B vs. Bands U+V

		Granule Extract B		Membrane U+V	
Specific Radioactivity before correction:		16200 CPM / 100 nmoles		4200 CPM / 100 nmoles	
Residue Showing Significant Difference* (Table	Residue % of Medium CPM (Table	nmoles 100 nmoles (Table	Maximum Correction CPM	nmmoles 100 nmoles (Table	Maximum Correction nmoles
Aspartic Acid	9.0	15.8	-2560(15.8%)	7.7	- 9.0
Proline	5.0	2.7	- 810 (5.0%)	5.3	- 5.3
Tyrosine	4.0	4.1	- 665 (4.1%)	1.6	- 4.0
Lysine	6.0	4.6	- 970 (6.0%)	16.7	-16.7
Arginine	7.0	4.6	-1130 (7.0%)	2.9	- 7.0
			-5404		-42.0
Specific Radioactivity after correction:		(16200-5400)/100		4200/(100.0-42.0)	
		= 10800 CPM / 100 nmoles		= 7200 CPM / 100 nmoles	

* Significant difference: Residue has 1.5 times the frequency in one band compared to the other.

residues showing significant frequency differences* between bands cannot bring the values for granule extract and membrane polypeptides to convergence: 120 CPM/nmole vs. 33 CPM/nmole for E vs. T and 108 CPM/nmole vs. 72 CPM/nmole for B vs. U + V.

Data Treatment B. The effect of assumed variations in the intracellular amino acid precursor pool assumption on the data.

It is first assumed that specific radioactivities for amino acids in the intracellular pool reflect either equilibration or proportional uptake of all residues in the pulse medium and would thus be represented by the absolute values (DPM/nmole) listed in the final column of Table XIII or by values directly proportional to them†.

Residue frequencies in gel bands (nmoles residue/100 nmoles total amino acid) were accordingly multiplied by specific radioactivities for residues in the medium (DPM/nmole). The product (DPM/100 nmoles amino acid) when summed for all residues comprising a single band represents a hypothetical specific radioactivity which should be proportional to the actual specific radioactivity experimentally determined if: 1) the assumption about the nature of the precursor pool is correct, and 2) the polypeptides of the bands being compared are all synthesized concomitantly. The parallelism, or lack of it, between the calculated and experimental determinations for the specific radioactivities of the content species A, B, E, M, O_A (very likely to be synthesized concomitantly) can be used as a test for accepting or rejecting the possible validity of each assumption about the precursor pool. The calculated values, referred to as medium-compensated spe-

*Levels of significant frequency difference were chosen separately for each comparison of band pairs based on the difference of specific radioactivity (CPM per gel band/total nmoles amino acid per gel band) between each member of the pair as indicated in Table XIV.

†Examination of Table XIII shows that if intracellular pools reflect to a considerable degree the medium composition, then the maximum specific radioactivity difference attainable for concomitantly synthesized proteins would be 8-fold, corresponding to the difference in specific radioactivity given extreme compositions polyglutamic acid and polyhistidine.

cific radioactivities (Σ(Medium S.R. x Residue Frequency)), for the initial assumption, i.e., proportional uptake, are presented in Table XV. The values for bands A, B, E, M, O_A are all similar (range 130 - 160 x 10^5 DPM/100 nmole) as expected by their presumed parallel synthesis. If significance can be attached to the difference actually calculated, however, then values for A and O_A are somewhat high relative to values of B and E, on the one hand, and M, on the other, when compared to the experimentally observed trends. The comparison suggests that the assumption that the precursor pool reflects proportional amino acid uptake from the medium is reasonable only as a first approximation. In any event, without exception, calculation of the medium-compensated specific radioactivities for membrane polypeptides of bands J, T, U+V and W gave values of the same magnitude as for exportable proteins, obviously at variance (excepting band W) with the experimental findings. Thus, proportional amino acid uptake from the medium and concomitant synthesis of all membrane and granule extract polypeptides cannot obtain simultaneously.

Alternative assumptions about the nature of the intracellular precursor pool of amino acids are: a) essential amino acids have higher intracellular concentrations than do nonessential amino acids, b) the converse of assumption a, or c) some of the residues which are the result of extensive metabolic conversion (especially proline derived from glutamic acid) contribute to the radioactivity of the intracellular pool more than the other residues. This last assumption is suggested by correlations of experimentally determined specific radioactivities and amino acid frequencies of secretory proteins, in particular those presented in bands M, A, O_A. Calculations based on these assumptions* did not remove the differences between the specific radioactivities of membrane and content bands. Moreover, these assumptions

*The exact nature of the assumptions tested were:
Type a: If intracellular concentrations of essential amino acids are assumed to be higher than those concentrations of nonessential amino acids, recalculate the medium-compensated specific radioactivities assuming that intracellular specific radioactivities of essential amino acids are 1/2 or 1/5 their values in the medium whereas intracellular

Table XV

Medium-Compensated Specific Radioactivities of Gel Bands

Amino Acid	Med. S.R.* x10^{-5}	Gel Bands A	B	E	M	O_A	J	T	$T^{c\dagger}$	U+V	W	$W^{r\ddagger}$
Aspartic Acid	2.08	17.9	32.9	25.1	4.5	9.5	38.8	14.0	18.6	16.0	22.2	22.4
Threonine	0.47	1.1	2.4	3.0	0.6	1.4	2.6	2.1	2.7	2.0	2.9	3.0
Serine	1.10	7.6	7.7	8.7	1.7	7.9	11.2	6.5	8.7	6.5	10.3	10.7
Glutamic Acid	2.64	71.0	29.4	31.6	83.3	67.8	39.1	25.3	33.7	26.4	39.6	39.1
Proline	1.50	22.2	4.1	8.1	30.0	19.4	5.7	6.0	8.0	8.0	7.1	8.2
Glycine	0.94	25.9	11.6	3.0	32.4	24.4	15.4	5.7	7.6	9.4	5.6	4.8
Alanine	1.84	6.4	11.1	21.3	1.8	7.5	13.0	17.1	22.7	14.8	11.0	10.6
Valine	0.65	0.6	5.1	5.2	0.2	2.6	2.5	4.7	6.2	4.6	4.4	4.2
Methionine	-	-	-	-	-	-	-	-	-	-	-	-
Isoleucine	0.49	0.5	2.4	2.2	1.1	0.5	1.9	1.7	2.2	2.3	2.0	1.9
Leucine	1.09	1.6	6.0	10.1	1.0	2.6	7.6	8.3	11.0	9.5	11.6	11.6
Tyrosine	0.77	0.7	3.2	4.3	0.5	0.7	1.0	1.2	1.6	2.0	4.3	4.3
Phenylalanine	1.35	0.3	6.1	5.3	0.6	1.5	3.3	3.7	5.0	5.3	3.5	3.3
Lysine	0.58	0.5	2.7	2.1	0.7	1.3	2.8	14.5	-	9.7	2.0	2.1
Histidine	0.30	-	0.6	0.4	-	-	-	-	-	-	1.1	0.9
Arginine	0.44	1.2	2.0	1.9	1.4	1.3	-	1.9	2.6	1.3	1.6	1.6
Σ(Medium S.R. x Residue Frequency) x 10^{-5}		158	127	132	160	148	145	113	131	118	129	129
Gel Band S.R. CPM/nmole (From Table IX)		146	162	142	226	128	74	20	27	42	196	216

* Med. S.R. = Medium specific radioactivity

† T^c = recalculation ignoring lysine suspected to be anomalously high

‡ W^r = duplicate of band W

resulted in medium-compensated specific radioactivities for content species A, B, E, M, O_A which showed less satisfactory relative agreement (with regard to trends and magnitudes within this group of bands) than given by the initial assumption of proportional uptake of all labeled amino acids from the medium.

In conclusion, this appendix indicates that the experimentally observed differences in specific radioactivity among gel bands representing membrane and content polypeptides expressed as total radioactivity (distributed among fifteen amino acid residues) per total amino acid are unlikely to reflect unusual amino acid compositions for the polypeptides in question and intracellular accumulations of labeled precursors that are disproportionate to the composition of the extracellular medium. Hence, as a first approximation, they can be considered valid. The analysis has not been extended beyond two types of data treatment because considerations of this nature are only a temporary substitute for either the determination of specific radioactivities of polypeptides, residue by residue, as was initially intended or complete definition of the size and composition of the intracellular protein precursor pool.

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specific radioactivities of nonessential amino acids are represented by the values given for the medium.
Type b: If intracellular concentrations of nonessential amino acids are assumed to be higher than those concentrations of essential amino acids, then recalculate the medium compensated specific radioactivities according to the converse of a.
Type c: Glutamic acid, proline, and glycine specific radioactivities in the intracellular pools are 1/2 or 1/5 the values given for the medium while the intracellular specific radioactivities for all other amino acids are 1/10 the values of the medium.

VI. BIBLIOGRAPHY

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