CELL FRACTIONATION STUDIES ON THE GUINEA PIG PANCREAS

Redistribution of Exocrine Proteins during Tissue Homogenization

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ABSTRACT

A double-label protocol was used to estimate the extent of leakage and relocation artifacts that affect exocrine pancreatic proteins in cell fractionation experiments. Guinea pig pancreatic lobules were pulsed in vitro with a mixture of ^{14}C-amino acids to enable the lobules to produce and process endogenously labeled exocrine proteins. At the end of the pulse (10 min) or after an appropriate chase interval, the lobules were homogenized in 0.3 M sucrose to which a complete mixture of ^{3}H-labeled exocrine pancreatic proteins was added as an exogenous tracer. The distribution of both labels was studied in each cell fraction of interest at the level of TCA-insoluble proteins and individual exocrine proteins resolved by using a two-dimensional gel system. Based on the premises that the exogenous and endogenous label behave identically during homogenization-fractionation and that all endogenously labeled exocrine proteins found in the postmicrosomal supernate come from intracellular compartments ruptured during tissue homogenization, a series of equations was derived to quantitate leakage and adsorption and to define the ratio of endogenous label still in its primary location to total label (primary location index or PLI) for each cell fraction. Leakage was found to be uniform for all exocrine proteins, but unequal in extent from different cell compartments (condensing vacuoles > zymogen granules $\gg$ rough endoplasmic reticulum); it increased with exposure to shearing forces especially in the case of zymogen granules and condensing vacuoles, and was substantially reduced from rough microsomes by adding 10 mM KCl to the homogenization media. Relocation of exogenous label by adsorption to other subcellular components was extensive (~55%), uneven (free polysomes > rough microsomes $\gg$ smooth microsomes and zymogen granules), preferential (cationic proteins are massively adsorbed to ribosomes and membranes, resulting in a complementary enrichment of the postmicrosomal supernate with anionic exocrine proteins), and reversible (with successive 50-100 mM KCl washes). After correction for adsorption and leakage, the kinetics of intracellular transport derived from cell fractionation data were found to be nearly identical to those obtained from quantitative autoradiographic studies.

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KEY WORDS cell fractionation · leakage-adsorption in cell fractionation · intracellular transport · secretory proteins · two-dimensional gel analysis · nonspecific adsorption of proteins

In the past, cell fractionation procedures have been used either alone or in conjunction with autoradiography to establish the route and kinetics of intracellular transport of secretory proteins (identified primarily as pulse-labeled proteins) in the exocrine pancreas of the guinea pig. In general, the results obtained with these two different approaches point to the same conclusion: considered as a group, secretory proteins pass successively from the rough endoplasmic reticulum (ER)[1] to the Golgi region, to condensing vacuoles and to zymogen granules, where they are stored before release (upon hormonal or cholinergic stimulation) into the luminal compartment of the extracellular space (21, 10, 14). A more detailed comparison of the two approaches reveals, however, that the kinetic description obtained by assaying cell fraction is less sharply defined than that based on autoradiographic data (8–10 vs. 22, 23). Furthermore, in cell fractionation experiments, a substantial fraction of secretory proteins is recovered in the postmicrosomal supernate, and the origin of this fraction, in terms of in vivo intracellular compartmentation, is uncertain and controverted. It is assumed to be either the cell sol (16), or various compartments of the secretory pathway ruptured or otherwise damaged during tissue homogenization (21).

Cell fractions are liable to contamination at two different levels. The first or particulate level results from imperfect separation of subcellular components (organelles) and affects primarily cell fractions prepared by differential centrifugation. the extent of particulate contamination is usually estimated by electron microscopy and assays for biochemical markers (3, 4). The second or molecular level of contamination is caused by leakage from compartments damaged during tissue homogenization, followed by adsorption of leaked molecules to other sites. This type of contamination is likely to affect all cell fractionation procedures in current use.

Although the existence of molecular contamination has been recognized for some time (24, 25), the degree of leakage of soluble components from ruptured compartments and the extent of redistribution of leaked molecules to other subcellular components (and hence, upon homogenate fractionation, to other cell fractions) are still unknown. The apparent discrepancy between cell fractionation and autoradiographic data in relation to rough ER drainage suggests, however, that the distribution of secretory proteins among cell fractions is significantly affected by such artifacts.

In the work reported here, we set out to quantitate leakage and adsorption of secretory proteins from, and to, cell fractions of interest. The results obtained indicate that it is possible to bring the cell fractionation procedure to a level of resolution considerably higher than currently attained, if adequate correction for leakage-adsorption artifacts can be achieved. Furthermore, correction for leakage and adsorption can be applied on a protein-by-protein basis by using, for the analysis of cell fractions, a two-dimensional gel separation technique recently developed for the study of complex mixtures of soluble proteins (17).

MATERIALS AND METHODS

Pulse-Chase Procedure (Endogenous Label)

Guinea pigs (Rockefeller University stock) fed ad lib. and weighing 400–500 g were used. The wet tissue weight of the pancreas in these animals was ~1 g. Pancreatic lobules were prepared according to the procedure of Scheele and Palade (18). All incubations were carried out in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 1 mg/ml of glucose and amino acids as indicated, and exposed to a 95% O_2, 5% CO_2 gas phase. Units of one-half pancreas were used for incubation and cell fractionation studies and, where necessary, lobules from several guinea pigs were pooled. Pancreatic proteins were pulse-labeled for 5 or 10 min using a mixture of 15 ^{14}C-amino acids (Schwarz algal profile), 125 μCi per half gland. To increase the rate of incorporation, the pulse medium (10 ml) contained small supplementary quantities of amino acids as follows: 0.1% of the physiological concentration of 20 L-amino acids (6), and 0.01 mM L-glutamine, L-asparagine, L-cysteine, L-tryptophan, and L-methionine (e.g., the amino acids missing from the algal hydrolysate). In the guinea pig exocrine pancreas, 95% of the radiolabeled amino acids incorporated during a 5–10-min pulse appears in secre-

[1] *Abbreviations used in this paper*: ER, endoplasmic reticulum; PL, primary location; PLI, primary location index (the ratio of endogenous label in its primary location to total endogenous label in a given cell fraction); PMS, postmicrosomal supernate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

tory protein; 5% appears in nonsecretory protein (cf. footnote 7). The chase medium (to which the lobules were transferred for various time periods after the pulse) contained physiological levels of the 20 L-amino acids, as specified by Eagle (6).

Preparation of ^{3}H-Labeled Secretory Proteins

Pancreatic lobules from one gland were incubated for 4 h in 10 ml of medium containing 1.0 mCi of a mixture of 15 ^{3}H-amino acids (Schwarz algal profile) supplemented with amino acids as specified above, 2 μg/ml of soybean trypsin inhibitor, and 10^{-5} M carbamylcholine. During this time and under carbamylcholine stimulation, the majority of the proteins biosynthetically labeled by the tissue (~80%) were discharged into the incubation medium. At the end of the incubation, the medium was recovered by decantation, cleared of debris by a brief centrifugation (3×10^6 g_{av}-min at 3°C), stabilized (against zymogen activation) by the addition of 1 mM diisopropyl fluorophosphate, 1 mM benzamidine, and 20 μg/ml of soybean trypsin inhibitor, and stored in 1.5-ml Eppendorf plastic vials at −80°C after rapid freezing in liquid N_2. The mixture of proteins discharged in the incubation medium was analyzed by the two-dimensional gel procedure of Scheele (17) and found to contain the 19 characteristic exocrine proteins produced by the guinea pig pancreas, all biosynthetically labeled. The mixture was stable at −80°C for at least 1 yr; it showed no sign of activation of its zymogens.

The incubation medium was desalted by dialysis against 0.3 M sucrose (i.e., the solution used for tissue homogenization) using an Amicon PM-10 ultrafilter (Amicon Corp., Lexington, Mass.) The radioactivity distribution among the secretory proteins of the mixture (determined by two-dimensional gel analysis) did not change as a result of the desalting procedure. Desalted secretory proteins were used as tracer or exogenous label in cell fractionation experiments designed to estimate the extent of adsorption and leakage artifacts.

Cell Fractionation

Lobules were washed at 4°C with 0.3 M sucrose, then minced finely with scissors and suspended in 0.3 M sucrose to a final tissue concentration of 1/10 (weight/volume), unless otherwise indicated. Groups of lobules were homogenized with a Brendler tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa.; size A, 0.10–0.15-mm clearance) driven by a motor at 3,000 rpm. An uninterrupted series of three strokes (once up and down over ~6 s/stroke) was used to prepare the homogenate. To avoid vagaries introduced by tissue and cell debris in the assays of replicate samples of whole homogenates, a postnuclear supernate was usually prepared by centrifugation at 6,000 g_{av}-min and used as a starting preparation for subsequent fractionation as well as a basis for calculating recoveries. Besides giving more reproducible data, the procedure is justified because the events studied are essentially cytoplasmic, and because (with the reservations mentioned above) the radioactivity distribution among exocrine proteins was found to be the same in the original homogenate and its postnuclear supernate.

The procedures used for cell fractionation by differential centrifugation were those given by Tartakoff and Jamieson (25), except for the microsomal subfractionation procedure, which was modified as follows. To avoid damage to microsomal vesicles caused by their resuspension from pellets, 3.5 ml of a postmitochondrial supernate was directly introduced into a "sandwich gradient" of the following composition: (from bottom to top) 1.5 ml of 2.25 M sucrose, 1 ml of 1.30 M sucrose, 6 ml of the postmitochondrial supernate adjusted to 1.25 M sucrose, 1 ml of 1.20 M sucrose, and ~2.5 ml of 0.30 M sucrose (to the top of the tube). The gradient was spun overnight (18 h) at 190,000 g_{av} at 4°C with a SB 283 rotor in an International ultracentrifuge, model 6B (Damon/IEC Div., Needham Heights, Mass.). Smooth microsomes were collected at the 0.3-1.2 M interface, rough microsomes at the 1.3-2.25 M interface, and free ribosomes were pelleted at the bottom of the tube. After collection, aliquots of the microsomal subfractions were washed by dilution in 5 ml of 0.3 M sucrose (containing, in some experiments, KCl at appropriate concentrations) and recovered by centrifugation at 10×10^6 g_{av}-min. An equivalent of the usual postmicrosomal supernate was obtained by centrifuging either 1 ml of a postmitochondrial supernate for 3×10^6 g_{av}-min, or 5 ml of the same supernate for 6×10^6 g_{av}-min. When starting from the same homogenate, the distribution of radioactivity between final supernate and pellets was the same in the two cases.

Abbreviated Cell Fractionation Procedure

Rapid assessment of leakage and relocation by adsorption was accomplished by centrifuging at 6×10^6 g_{av}-min 1-ml aliquots of homogenates prepared from lobules doubly labeled according to the protocol to be described under Results. Under these conditions, the homogenate was fractioned into a pellet containing all subcellular particulates and a supernate equivalent to a postmicrosomal supernate in the usual fractionation procedures. 100-μl samples of the original homogenate, and the ensuing supernate and pellet fractions, diluted or resuspended in 1.0 ml of 25 mM Tris-HCl (pH 9.2) containing 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), were assayed for radioactivity by the TCA precipitation method of Mans and Novelli (12). The recovery of label in the postmicrosomal supernate obtained by this abbreviated procedure was comparable to that achieved in the preceding section (Cell Fractionation).

Two-Dimensional Gel Analysis of Cell Fractions

Fractions were solubilized in 1% Triton X-100 in 25 mM Tris-HCl (pH 9.2), to maximize membrane solubilization without altering the isoelectric points of secretory proteins (cf. reference 17). Samples for analysis contained an aliquot of the solubilized fraction, 5 mg/ml of cytochrome *c* as visual marker, and Ampholine (LKB-Produkter, Bromma, Sweden), pH range 3.5-10, in proportions of 35, 5, and 3 μl, respectively. When necessary, 10 μl (~30 μg) of nonradioactive pancreatic (guinea pig) exocrine proteins was added to the sample to insure that all secretory proteins could be identified in two-dimensional gels by Coomassie Blue staining (ICI United States, Inc., Wilmington, Del.). Samples were loaded in aliquots of either 40 μl (to Whatman GF/A glass fiber loading squares, 0.9 × 0.9 cm [Whatman, Inc., Clifton, N.J.]) or 80 μl (to Whatman GF/B loading squares, 0.9 × 0.9 cm), and their proteins were separated by the two-dimensional gel procedure of Scheele (17), except that the strips cut from isoelectric focusing gels were applied directly to the second-dimension gels without an interposed "bridge" of polyacrylamide. This procedure was originally designed to resolve discharged pancreatic exocrine proteins; but with the modifications mentioned above in the preparations of the samples, it proved useful in analyzing the exocrine proteins of individual cell fractions. The corresponding gels were analyzed for radioactivity either qualitatively by autoradiography, or quantitatively by cutting and subsequently counting Coomassie Blue-stained spots (17) in a liquid scintillation spectrometer. For all fractions, 95-98% of the radioactivity of the loaded sample was recovered in the spots of the second electrophoretogram. The exception was the microsomal fraction at the end of a 10-min pulse; in its case, the corresponding figure was ~70%, presumably because of the presence of labeled but unfinished polypeptide chains still attached to ribosomes.

Calculation of Leakage and Adsorption Using the Double-Label Protocol

The calculations for leakage and adsorption were based on the following premises: (*a*) endogenously labeled secretory proteins are restricted in vivo to the intracisternal space of the secretory compartments of the exocrine cell and to glandular lumina; (*b*) all endogenously labeled secretory proteins in the postmicrosomal supernate represent proteins leaked from secretory compartmants; and (*c*) during tissue homogenization, exogenously labeled secretory proteins added as a tracer and endogenously labeled proteins that leak from intracellular compartments mix fully and subsequently adsorb at the same rate and with the same affinity to available surfaces.

The basic relationships defined by these premises can be expressed as:

$$\frac{Ex_s^*}{Ex_a^*} = \frac{En_s^*}{En_a^*}, \tag{1}$$

where Ex^* and En^* represent exogenous and endogenous radioactive label, respectively; s, soluble radioactivity recovered in the postmicrosomal supernate; and a, adsorbed radioactivity on particulate cell fractions. Since Ex_s^*, Ex_a^*, and En_s^* can be measured, En_a^* can be calculated from Eq. 1.

Total leakage (L) from ruptured or damaged compartments can be expressed as

$$En_L^* = En_s^* + En_a^*, \tag{2}$$

or as (substituting En_a^* from Eq. 1

$$En_L^* = En_s^* + \frac{En_s^* Ex_a^*}{Ex_s^*}. \tag{3}$$

The extent of leakage as a percent of total En^* (En_t^*) is given by

$$\%En_L^* = \frac{En_s^* Ex_t^*}{En_t^* Ex_s^*} \times 100, \tag{4}$$

an equation derived from Eq. 3 by rearrangement and substitution (Ex_t^* for $Ex_s^* + Ex_a^*$).

With Eq. 4, the extent of total leakage can be easily calculated by using the double-label protocol and the abbreviated cell fractionation procedure described in the fourth section of Materials and Methods.

Leakage from a given cell fraction can be calculated only on the assumption that the corresponding cell compartment is the unique source of endogenous label. Available autoradiographic evidence indicates that the assumption is valid (to a reasonable degree) for specific cell compartments at well-defined time intervals in a pulse-chase labeling experiment (9, 10). In such cases, Eq. 4 can be used to calculate percent leakage from a specific cell compartment, or cell fraction.

Endogenously labeled proteins artifactually adsorbed to an individual cell fraction ($En_{a_f}^*$) can be calculated from:

$$En_{a_f}^* = \frac{En_s^* Ex_{a_f}^*}{Ex_s^*}, \tag{5}$$

where $Ex_{a_f}^*$ is the amount of exogenous label adsorbed to the fraction of interest.

Total adsorption of endogenous label to all particulate fractions can be calculated either by (*a*) summation of the adsorbed endogenous radioactivity to individually isolated cell fractions or (*b*) measurement of the endogenous radioactivity adsorbed to a combined particulate pellet.

The amount of endogenous label which is still present in its primary location in a given cell fraction (or, in the particular case of the pancreas, in the content of the fraction's vesicular components [c_f]) is the difference between total (t_f) and adsorbed (a_f) endogenous labels

in the corresponding cell fraction.

The ratio of endogenous radioactivity still in its primary location to total endogenous radioactivity can be used to assess the extent of relocation artifacts affecting a given cell fraction. For convenience, this ratio will be referred hereafter as the primary location index (PL index or PLI).

$$\text{PLI} = \frac{En_{c_f}^*}{En_{t_f}^*} = \frac{En_{t_f}^* - En_{a_f}^*}{En_{t_f}^*} \quad (6)$$

A high PLI (approaching 1) describes a cell fraction in which most of the endogenous label is still in its primary location; a low PLI (approaching zero) indicates that most of the endogenous radioactivity of the fraction is relocated by adsorption (secondary location) upon leakage from the same or another cell fraction.

Biochemical Assays

Protein was determined by the procedure of Lowry et al. (11), and ribonucleic acid was assayed by the Fleck-Munro (7) modification of the Schmidt-Thannhauser method (20).

RESULTS

Adsorption of Tracer Secretory Proteins to Subcellular Components during Tissue Homogenization

Adequacy of ^{3}H-Secretory Proteins as Tracer

To trace the adsorption of proteins assumed to have leaked from membrane-bounded compartments during tissue homogenization, we introduced tracer amounts of ^{3}H-secretory proteins into the 0.3 M sucrose solution used for homogenizing pancreatic lobules pulse-labeled in vitro for 10 min with mixed ^{14}C-amino acids and then chased for 10 min as indicated in detail under Materials and Methods. The 10-min chase was used to allow the termination of the radioactive polypeptide chains initiated and elongated during the 10-min pulse. We then followed the distribution of TCA-insoluble ^{3}H radioactivity in the postmicrosomal supernate and each of the sedimentable fractions (debris and nuclei, zymogen granules, mitochondria, rough microsomes, and smooth microsomes) derived from the homogenate to quantitate the extent of adsorption of the tracer to the particulates of each fraction. Figs. 1 and 2 demonstrate both qualitatively (Fig. 1) and quantitatively (Fig. 2) that the ^{3}H-secretory proteins used as exogenous label behave like endogenously labeled ^{14}C-secretory proteins, as judged by the results of the two-dimensional gel analysis. With the single exception of one form of proelastase (arrow in Fig. 1),[2] there is no detectable difference in isoelectric point and molecular weight between the corresponding proteins in the two sets. The slight variations in the ratios of individual secretory proteins in the two preparations shown in Fig. 2 can be ascribed in part to experimental error and in part to a certain degree of asynchrony[3] in the intracellular transport of these proteins.

Concentrations of Tracer Tolerated

The desalted ^{3}H-labeled exocrine proteins used as tracer did not appear to aggregate in 0.3 M sucrose because more than ~98% of the radioactivity remained nonsedimentable under the strongest centrifugal fields (6×10^6 g_{av}-min) used in relevant experiments. However, when the tracer-sucrose solution was used for tissue homogenization, more than half of the radioactivity became sedimentable and was recovered in a common particulate fraction of the ensuing homogenate. We assumed that the change reflects the adsorption of tracer molecules to the particulates of the homogenate. Fig. 3 shows that over two orders of magnitude of tracer input, the percent of adsorbed exogenous label remains constant at ~55%, presumably because saturation of the sites involved in adsorption has not yet been reached. All subsequent experiments were carried out in the lower range of the curve in Fig. 3 at an input concentration below 10% the concentration of endogenously labeled exocrine proteins.

Principal Sites of Adsorption

Similar mixing experients have been performed with the goal of establishing the subcellular components (organelles) to which extensive adsorption occurs. From Table I it is evident that the degree of contamination of both smooth and rough microsomal fractions (expressed as ^{3}H radioactivity per milligram of protein in each fraction) considerably exceeds that of the zymogen granule fraction. In part, such results would be anticipated from the surface to protein content ratio and from the surface to surface ratios (1) of

[2] Conversion from one form of proelastase to a second form, differing in both size and charge, occurs intracellularly over a period of hours. (G. Scheele. Unpublished observations.)

[3] Unpublished observations of G. Scheele, and A. Tartakoff and G. Scheele.

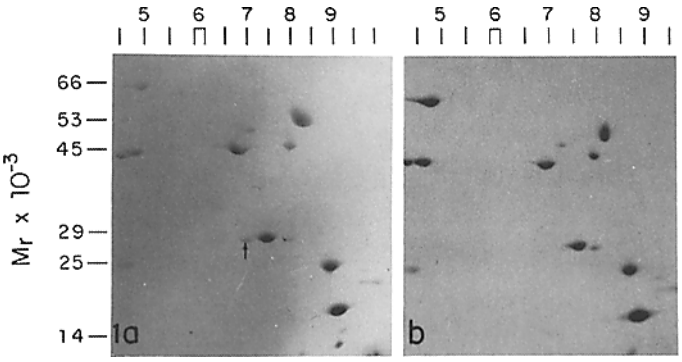


FIGURE 1 Comparison of the two-dimensional gel patterns of: (*a*) discharged exocrine proteins, and (*b*) intracellular exocrine (pulse-labeled) proteins. Fig. 1 *a* gives the staining pattern (with Coomassie Brilliant Blue) of a typical sample of exocrine proteins labeled with ^{3}H-amino acids and discharged from pancreatic lobules into the incubation medium during 4 h of carbamylcholine stimulation; Fig. 1 *b* is an autoradiograph of pulse-labeled proteins (10-min pulse followed by 10-min chase) detected in a sample of homogenate prepared from pancreatic lobules labeled in vitro with ^{14}C-amino acids. In a separate experiment in which the discharged secretory proteins were labeled with ^{3}H-amino acids, there was perfect coincidence of stained and fluorographic spots. The upper abscissae give the pH range (the bracket at pH 6 indicates the sample loading position), and the common ordinate indicates molecular weights. For the explanation of the small arrow, see the first section of Results and footnote 2.

the main components represented in the fractions of interest. Table I gives also recovery figures in a set of cell fractions of interest and shows that ~28% of the exogenous tracer appears in the rough microsomal fraction as a result of adsorption. The corresponding figures for smooth microsomal and zymogen granule fractions are considerably lower: ~6 and ~4%, respectively.

PROTEIN-BY-PROTEIN EVALUATION OF ADSORPTION

A two-dimensional gel analysis of the mixture of tracer exocrine proteins recovered in the postmicrosomal supernate (Fig. 4 *A*) shows that it is heavily enriched in acidic species, and that the relationship between isoelectric point and degree of enrichment is approximately linear, crossing the no-enrichment-line (given as 1) near pH 7.5.

The endogenously labeled exocrine proteins recovered in the postmicrosomal supernate show a strikingly identical pattern of distribution, with the same enrichment in acidic proteins and the same dependence on isoelectric point. This finding strongly supports our starting premises which assume identical behavior of exocrine proteins irrespective of their source, added as tracer to the homogenate, or released to the cell sol from intracellular compartments ruptured during tissue homogenization.

Fig. 4 *A* also shows that this distribution pattern is not tissue-specific. When ^{3}H-pancreatic secretory proteins were added to the sucrose solution in which guinea pig liver was homogenized, the distribution of radiolabeled secretory protein recovered in the liver postmicrosomal supernate was similar to that recovered in the pancreatic postmicrosomal supernate. The recovery of amylase in the pancreatic postmicrosomal supernate was greater than would be expected from its net charge (pI = 8.4). The reasons for this unexpected distribution are unknown, but the even greater recovery of amylase in the liver postmicrosomal supernate suggests that enzyme-substrate interactions of amylase with soluble (nonsedimentable) glycogen may account for the findings.[4] The striking underrepresentation of proelastase in the liver postmicrosomal supernate indicates the specific binding of this protein to particulates contained in the liver homgenate.

Fig. 4 *B* shows the inverse relationship for four sedimentable cell fractions, namely, free ribosomes, rough microsomes, smooth microsomes, and zymogen granules. In each of these fractions, the acidic proteins of the exogenous tracer are underrepresented, whereas its basic proteins are relatively enriched by comparison with their distribution in the postnuclear supernate (or discharged secretion). This relationship is most pronounced for free ribosomes, less for rough microsomes,

[4] In pancreatic homogenates, soluble glycogen is expected to originate from adipocytes.

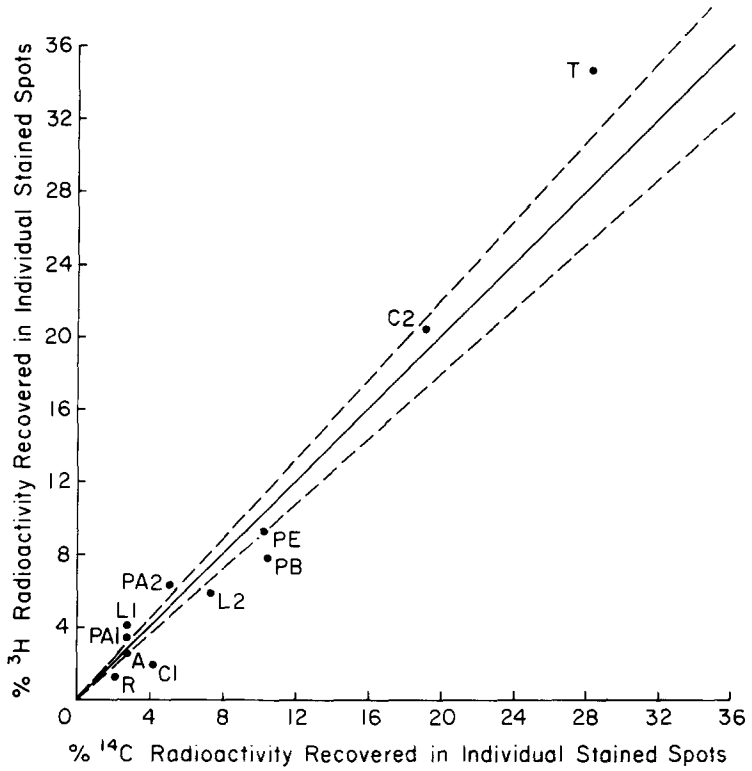


FIGURE 2 Quantitative comparison of radioactivity incorporated in discharged secretory proteins and in intracellular secretory proteins. Discharged secretory proteins were a cumulative sample collected after 4 h of continuous labeling with mixed ^{3}H-amino acids and continuous stimulation with carbamylcholine. The pulse-labeled sample was the same as in Fig. 1. Both samples were submitted to two-dimensional gel analysis, and the contribution of individual spots was expressed as percent of the total radioactivity recovered in each gel in the common set of stained spots which represents the exocrine proteins of the pancreas. The dotted lines indicate the limits of 10% experimental error. Individual secretory proteins are identified by the same letters as in Fig. 4, except for *R*, which indicates ribonuclease and *C1* and *C2* which mark two different forms of chymotrypsinogen. Representative data for a series of three experiments.

and least for smooth microsomes and zymogen granules. Lipase 2 shows distinctively higher affinities for smooth microsomes than for any other fraction.

The extent of adsorption is large enough and selective enough to be evident at the level of mass distribution of individual proteins in cell fractions prepared from lobules homogenized without the addition of any exogenous tracer. Fig. 5 compares the stained (Coomassie Brilliant Blue) two-dimensional gels of a zymogen granule fraction, a postmicrosomal supernatant fraction, and a rough microsomal fraction. Again, rough microsomes are heavily enriched in basic secretory proteins, whereas the postmicrosomal supernate is enriched in acidic secretory proteins.

It should be noted that in the electrophoretograms in Fig. 5, the nonsecretory proteins of the pancreatic tissue are hardly visible. The reasons for their apparent absence are several. Individually, they are expected to be present in much lower concentrations (perhaps 10–100 times lower) than individual exocrine proteins and, hence, not to be detectable unless the gels are overloaded. In fact, a few spots corresponding to nonsecretory proteins appear on the electrophoretogram of the postmicrosomal supernate. Moreover, some of the nonsecretory proteins are not soluble enough to penetrate the first gel. For instance, most ribosomal proteins and at least some membrane proteins remain on the loading square from which they can be extracted by 1% sodium dodecyl sulfate (SDS) (G. Scheele, unpublished observations). As already mentioned, how-

ever, practically all the exogenous or endogenous radioactivity loaded on the first gel is recovered in the spots corresponding to exocrine proteins on the second gel.

KCl Wash of Microsomal Subfractions

EFFECT ON RADIOACTIVITY CONTENT: Tracer secretory proteins adsorbed to rough microsomes and smooth microsomes can be partially removed by KCl treatment. Fig. 6 shows the effect of washing isolated rough microsomes with 0.3 M sucrose containing KCl at increasing concentrations. The rough microsomes were derived from lobules pulse-labeled for 10 min with ^{14}C-amino acids and then homogenized in the presence of ^{3}H-secretory proteins. 200-μl aliquots of rough microsomes (isolated by the sandwich gradient procedure) were resuspended in 2.0 ml of 0.3 M sucrose containing the indicated KCl concentrations and immediately sedimented at 1.2×10^{7} g_{av}-min. Clearly, the bulk (~85%) of the artifactually associated exogenous label can be removed with 50 mM KCl, whereas with KCl at the same concentration much less (~24%) endogenous ^{14}C label is lost. All or the major part of the latter probably represents endogenous label relocated by adsorption. Washing rough microsomes with LiCl, KBr, and KSCN (at comparable concentrations) gave no better results than washing with KCl. The removal of exogenous label by KCl treatment is extensive but incomplete, since ~15% of the ^{3}H radioactivity remains associated with the rough microsomes. The residual ^{3}H-proteins do not seem to be "sequestered" within microsomal vesicles (as a result of the latter's repeated rupture and sealing during tissue homogenization), since tracer molecules added before or after homogenization were removed by KCl to the same extent. Washing the rough microsomes with NaCl instead of KCl resulted in extensive but less complete removal of adsorbed exogenous label (Fig. 6), especially at concentrations below 100 mM.

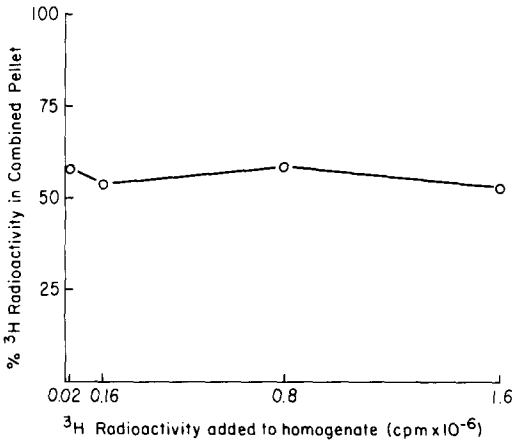


FIGURE 3 Distribution of ^{3}H-labeled secretory proteins (exogenous tracer) between postmicrosomal supernate and particulate cell fractions as a function of quantity of the tracer used. Increasing amounts of ^{3}H-labeled secretory protein were added to the sucrose used for the homogenization of unlabeled pancreatic lobules. The postnuclear supernates were centrifuged to collect a postmicrosomal supernate and a single pellet containing zymogen granules, mitochondria, and microsomes (cf. fourth section of Materials and Methods). The radioactivity recovered in these fractions is expressed as percent of input radioactivity. The highest amount of tracer added is estimated at 40% the amount of endogenous secretory proteins.

TABLE I

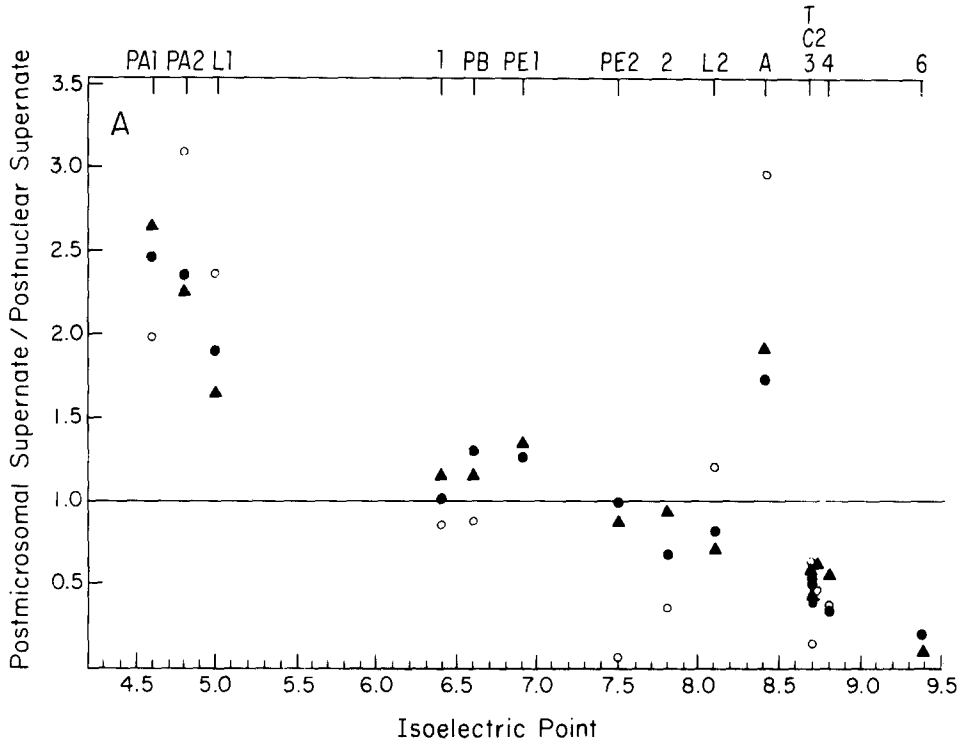
Distribution of Tracer Radioactivity among Relevant Cell Fractions

Cell fraction	^{3}H dpm x 10^{-5}/mg protein	^{3}H-labeled protein*
		%
Zymogen granules	0.4	3.8
Smooth microsomes	4.0	5.5
Rough microsomes	6.2	27.9
Postmicrosomal supernate	10.0	43.6
Homogenate	5.0	–

* The difference between the recovery given in this table (82%) and full recovery is due to adsorption of ^{3}H tracer to mitochondrial and nuclear fractions. Similar results were obtained in five other experiments.

A more efficient reduction of adsorbed exogenous label can be obtained by homogenizing the tissue in KCl-containing sucrose solutions[5] followed by KCl washing of the fractions as shown by the results of the following experiment. Lobules obtained from a whole pancreas were pulse-labeled (10 min) in vitro with ^{14}C-amino acids and then divided into two sets each representing a half pancreas. One set was homogenized in 0.3 M sucrose and the other in 0.3 M sucrose-100 mM KCl, with exogenous tracer added in both cases. Rough microsomes were harvested from each set using the sandwich gradient procedure (with or

[5] As will be shown later on, the addition of KCl to the homogenizing solution decreases leakage in addition to reducing adsorption.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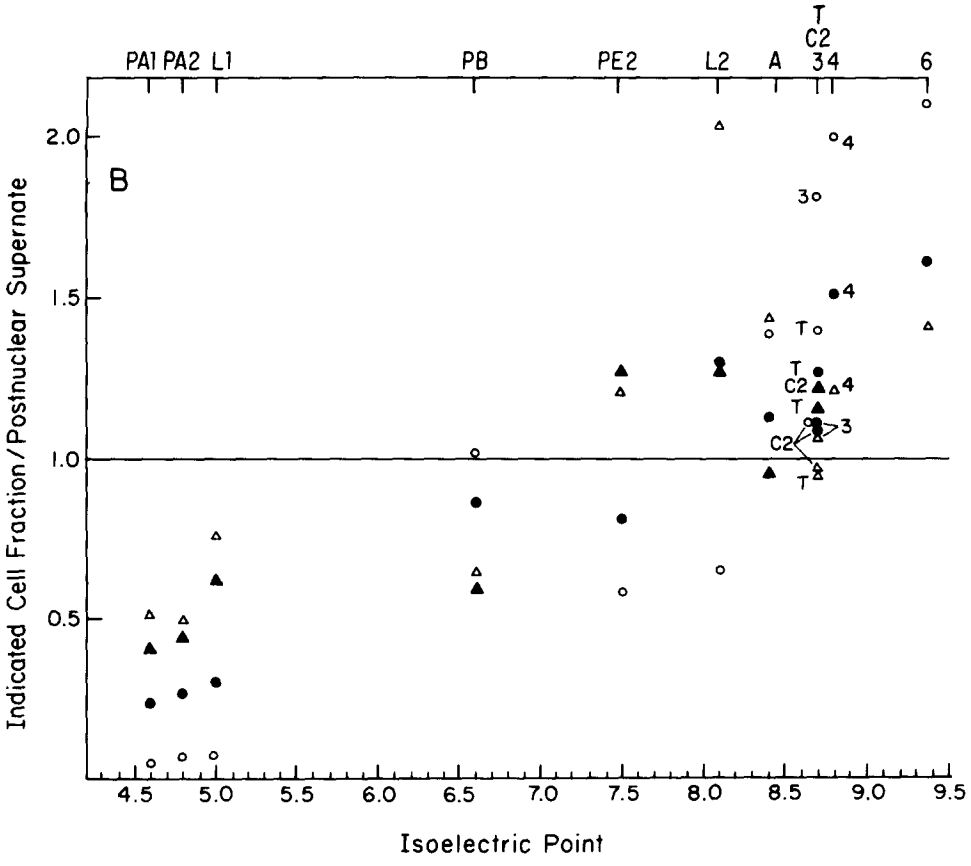
Isoelectric Point


PA1 PA2 L1
PB
PE2
L2
A
T C2 3 4
6
B
Indicated Cell Fraction / Postnuclear Supernate
2.0
1.5
1.0
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4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5
Isoelectric Point

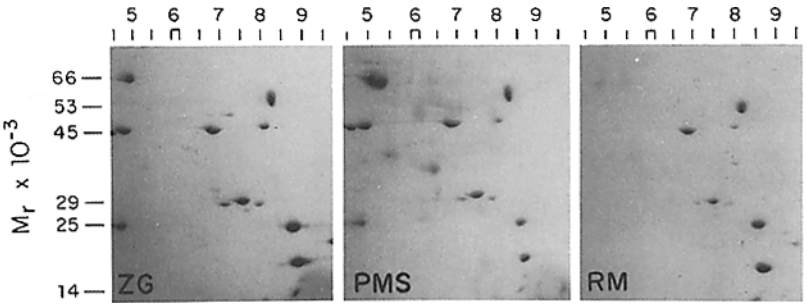

FIGURE 5 Differential adsorption of unlabeled endogenous secretory protein seen at the level of stained (Coomassie Brilliant Blue) two-dimensional gels. Three subcellular fractions, zymogen granules (*ZG*), postmicrosomal supernate (PMS), and rough microsomes (*RM*), were taken from a typical cell fractionation experiment in the absence of added tracer molecules. The upper abscissae give the pH range (the bracket at pH 6 indicates the sample loading position); the common ordinate indicates molecular weights.

without 100 mM KCl in the gradient, respectively). The microsomes harvested from tissue homogenized and fractionated in 0.3 M sucrose contained 44,000 ^{3}H-cpm and 20,000 ^{14}C-cpm, and—based on an En_s^*/Ex_s^* of 0.14 (^{14}C/^{3}H ratio in the postmicrosomal supernate)—showed a PLI of 0.69. Washing these microsomes with 100 mM KCl (as described above) removed 82.5% of ^{3}H tracer molecules but only ~10% of the ^{14}C endogenous label, raising the PLI to 0.94. Rough microsomes harvested from tissue homogenized in 0.3 M sucrose-100 mM KCl contained 3,000 ^{3}H-cpm and 7,000 ^{14}C-cpm, and showed a PLI of 0.88 (based on an En_s^*/Ex_s^* of 0.28). A 100 mM KCl wash removed ~85% of the remaining tracer molecules, but only ~6% of the endogenous label, thereby increasing the PLI of the fraction to 0.98.

The difference in PL indices (calculated before washing), 0.69 in the first case and 0.88 in the second case, indicated that in the presence of KCl in the homogenizing medium the adsorption of exogenous label is markedly reduced (by ~80%). Furthermore, a single KCl wash increases the PL index from 0.69 to 0.94 in the first case, and from 0.88 to 0.98 in the second case. A PL index approaching 1.0 indicates that nearly all contaminating (adsorbed) molecules have been removed. The decrease in ^{14}C radioactivity in rough microsomes prepared from sucrose-KCl homogenates reflects in part efficient removal of adsorbed endogenous label and in part, losses of rough microsomes to heavier cell fractions (zymogen granules and mitochondria) on account of aggregation and apparently less extensive fragmentation of the rough ER.

FIGURE 4 Differential adsorption of exogenous ^{3}H-labeled exocrine proteins on subcellular fractions. Individual fractions from a typical cell fractionation experiment were analyzed by two-dimensional gel electrophoresis and the radioactivity distribution among spots expressed relative to the radioactivity distribution in the postnuclear supernate. The lower abscissa indicates the isoelectric point of individual proteins. The identity of these proteins is given on the upper abscissa as follows: *PA*, procarboxypeptidase A; *L*, lipase; *PB*, procarboxypeptidase B; *PE*, proelastase; *A*, amylase; *T*, trypsinogen; *C*, chymotrypsinogen. Spots 1, 2, and 6 are secretory proteins to which enzymic activities have not yet been assigned. Spots 3 and/or 4 are ribonuclease. Other numbers refer to individual forms of enzymes or proenzymes as given in Scheele (17). Fig. 4*A* compares individual exocrine proteins in the postmicrosomal supernate (PMS) to the postnuclear supernate. The symbols are as follows: (●) ^{3}H-pancreatic exocrine proteins (exogenous label) in pancreatic PMS; (▲) ^{14}C-pancreatic exocrine proteins (endogenous label, 10-min pulse, 10-min chase) in pancreatic PMS; (○) ^{3}H-pancreatic exocrine proteins (exogenous label) in liver PMS. Fig. 4*B* compares the distribution of individual proteins of the exogenous tracer in the postnuclear supernate and in four pelletable fractions derived therefrom. The symbols represent the following cell fractions: (○) free ribosomes; (●) rough microsomes; (△) smooth microsomes; (▲) zymogen granules. Fig. 4*B* does not include data for three minor spots, namely 1, 2, and PE1. Representative data for a series of four experiments.

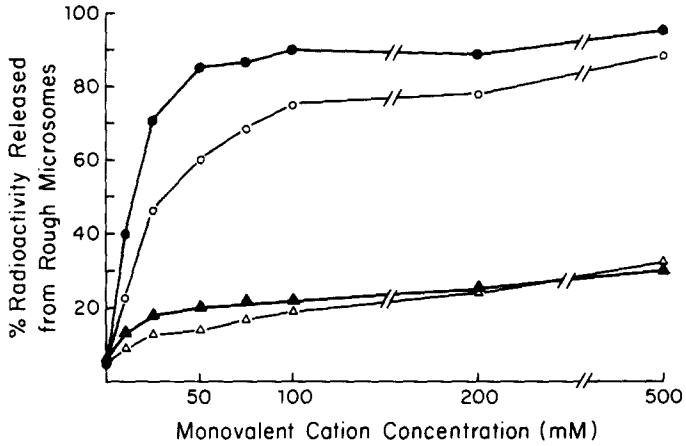

FIGURE 6 Release of exogenously and endogenously labeled exocrine proteins from isolated rough microsomes by KCl or NaCl treatment. Lobules were pulse-labeled for 10 min with a mixture of ^{14}C-amino acids and homogenized at the end of the pulse in 0.3 M sucrose containing ^{3}H-labeled exocrine proteins. Rough microsomes were isolated by using a sandwich gradient (cf. third section of Materials and Methods). Aliquots of this fraction were then washed by suspension and subsequent resedimentation in 2.0 ml of 0.3 M sucrose containing the indicated KCl or NaCl concentrations. Representative data from a series of six experiments in which the efficiency of removal of exogenous label by 50 mM KCl varied from 70 to 85%. Circles indicate removal of ^{3}H-labeled proteins; triangles indicate ^{14}C-labeled proteins. Closed symbols indicate KCl wash; open symbols, NaCl wash.

EFFECTS ON RNA DISTRIBUTION: As shown by the data in Table II, RNA recovery in a total microsomal fraction decreases by ~60% when this fraction is isolated from pancreatic tissue homogenized in 0.3 M sucrose-0.1 M KCl (instead of 0.3 M sucrose only). The amount lost appears primarily in the zymogen granule and mitochondrial fractions, recoveries in the nuclear fraction and postmicrosomal supernate being much less affected. In the presence of 10 mM KCl in the homogenizing sucrose solution, redistribution effects are less drastic: RNA recovery in the microsomal fraction is reduced by ~10% only, yet the RNA content of the zymogen granule fraction doubles.

TABLE II

Percent Distribution of RNA among Cell Fractions as a Function of KCl Present in the 0.3-M Sucrose Homogenization Solution

Cell fraction	KCl concentration (mM)		
	0	10	100
	%		
Nuclear	13.9	17.1	14.3
Zymogen granule	5.1	10.0	22.4
Mitochondrial	7.4	8.6	25.3
Microsomal (rough and smooth)	61.5	55.7	26.4
Postmicrosomal supernate	12.2	8.6	11.6

EFFECTS ON EXOCRINE PROTEIN CONTENT: Since the results already presented indicate that the majority of the adsorbed exocrine proteins is associated with the rough microsomal fraction, and since pulse-labeled radioactivity is expected to represent only a relatively small number of molecules in the tissue, we sought to determine the extent of molecular contamination of the fraction in terms of relative amounts of exocrine proteins recovered therein. To this intent, aliquots of rough microsomes derived from pulse-labeled (10 min) pancreatic lobules and harvested from a sandwich gradient were resuspended in 2.0 ml of 0.3 M sucrose, with or without 0.1 M KCl, and resedimented at 1.2 × 10^{7} g_{av}-min. Aliquots of the resuspended microsomes and of the supernates and pellets obtained upon their centrifugation were adjusted to 1% Triton-X 100 and 25 mM Tris, pH 9.2, to lyse all membranes, and each of these preparations was assayed as done in reference 18 for the following actual or potential enzyme activities: amylase, trypsinogen, chymotrypsinogen, and procarboxypeptidase B. Washing the microsomes with 0.3 M sucrose released 8.3-9.4% of the actual or potential enzymic activities of each of the four proteins mentioned above, whereas washing with 0.3 M

TABLE III

Removal of Pulse-Labeled Radioactivity, Potential Enzyme Activity, and Protein from Isolated Rough Microsomes by a 100-mM KCl Wash

	Prewash	Postwash	% Removed
Radioactivity (0-min chase)	13×10^5 cpm	11.4×10^5 cpm	12
Potential enzyme activity:			
Trypsinogen	2,794 U	308 U	89
Chymotrypsinogen	652 U	68 U	90
Protein	0.88 mg	0.62 mg	29

sucrose-0.1 M KCl proved much more effective, the corresponding figures ranging from 84 to 89%. Since complete removal of all adsorbed proteins is not achieved with a single KCl wash, it is evident that—at least in the case of the basic and neutral enzymes and proenzymes assayed in this experiment—the great majority (>90%) of the secretory proteins associated with isolated rough microsomes represents molecules relocated from other sources. This figures should be taken, however, as a rather rough estimate since our present means of analysis do not allow a precise assessment of the stability of the microsomes and of the extraction of their contents through the various steps of the cell fractionation procedure.

The massive loss in secretory enzyme activities recorded above should be contrasted with the much more limited losses in endogenous radioactivity ascribable to newly synthesized secretory proteins still associated with the rough microsomes. Table III gives the results of an experiment in which rough microsomes prepared from a 0.3-M sucrose solution were washed with 0.3 M sucrose-0.1 M KCl and assayed for losses in radioactivity, potential enzyme activity and protein. The data show that the KCl wash resulted in losses on the order of ~90% for the two secretory proteins, and ~30% for total protein with only a 12% loss in endogenous radioactivity.

An independent estimate of the amount of exocrine proteins relocated by adsorption on rough microsomes could be obtained if: (*a*) the specific radioactivity of the exogenous tracer were known; (*b*) the specific endogenous radioactivity of an alkaline extract of the zymogen granule fraction were determined after an appropriate chase period (e.g., 120 min); and (*c*) the exogenous and endogenous radioactivity of a rough microsomal fraction isolated at the same time as the zymogen granule fraction were converted into protein mass. In an experiment in which $a = 1.7 \times 10^6$ cpm/mg of protein, and $b = 6.2 \times 10^5$ cpm/mg of protein, *c* was found to amount to 0.93 and 1.80 mg of protein for the exogenous and endogenous label, respectively, for a total amount of 7.7 mg of rough microsomal protein.

Leakage of Radiolabeled Secretory Proteins during Tissue Homogenization

The extent of leakage of endogenous label from various intracellular compartments depends in the first place on the intensity of shearing forces to which the tissue is subjected during homogenization. This relationship is convincingly demonstrated by the results of the experiment in Fig. 7, which show that the fraction of endogenous label recovered in the postmicrosomal supernate increases with the vigor of shearing or—in practical terms—with the number of "strokes" used to disintegrate the tissue (the "stroke" is defined in Materials and Methods, third section). Fig. 7 also shows that this fraction is smallest immediately after the pulse, when practically all the endogenous label is in rough microsomes. It becomes larger after a 120-min chase, when most of the label is already in zymogen granules, and it reaches its highest values after a 60-min chase, when a sizable fraction of the newly synthesized secretory proteins is still in the condensing vacuoles of the Golgi complex. If three strokes are used to homogenize the tissue, 14.5% of the endogenous label is recovered in the postmicrosomal supernate immediately after the pulse, and 20.5 and 24% after a 2- and a 1-h chase, respectively. The corresponding figures increase to 18.5, 34, and 41% of the total endogenous label if 25 strokes are used for disrupting the tissue. These results indicate that zymogen granules are more fragile than microsomes, the condensing vacuoles being the most fragile of all the compartments of the secretory pathway. Using a single homogenization stroke, the shearing forces were further reduced by varying the revolutions per minute of

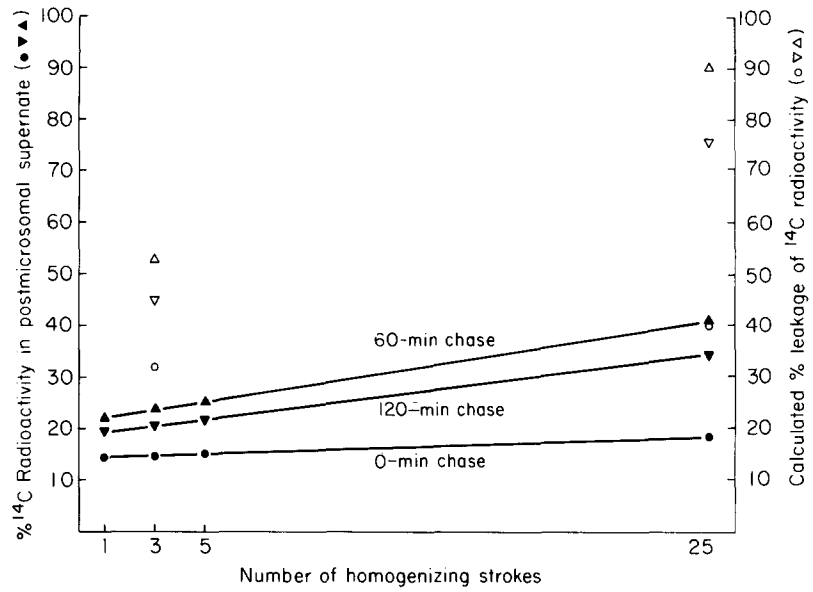

FIGURE 7 Appearance of pulse-labeled proteins in the postmicrosomal supernatant fraction as a function of chase time and number of homogenizing strokes (closed symbols). After a 10-min pulse and the indicated period of chase, the lobules were fractionated into a postmicrosomal supernate and a combined particle pellet. TCA-precipitable radioactivity recovered in the PMS is expressed as percent of homogenate radioactivity. Open symbols indicate percent leakage of ^{14}C radioactivity (calculated as given in the sixth section of Materials and Methods) at 3 and 25 homogenizing strokes. Representative data from a series of three experiments.

the motor-driven pestle of the tissue homogenizer. While leakage of endogenous label at the end of the pulse (i.e., primarily from rough microsomes) was little affected by this variable, leakage at the end of a 120-min chase (i.e., from zymogen granules) was significantly reduced: by ~21% upon reduction from 3,000 to 2,000 rpm, and by ~29% upon reduction from 3,000 to 1,000 rpm.

If we assume that the entire amount of endogenously labeled exocrine proteins found in the postmicrosomal supernate represents leakage (following rupture, damage, or lysis of the compartment of origin) minus adsorption to other sites, Eq. 4 can be used to calculate the extent of leakage from the main component(s) of each cell fraction. When three strokes are used for homogenization, the leakage from rough microsomes (0-h chase) amounts to 32% of the initial content of the tissue's endogenous label,[6] the corresponding figures being 45 and 52.5% for zymogen granules (120-min chase) and condensing vacuoles (60-min chase), respectively. These figures increase to 40.5, 76, and 91%, respectively, when 25 strokes are used to homogenize the tissue.

Were the leakage from intracellular compartments uniform, i.e., without preferential retention of some proteins, then the distribution of endogenously labeled secretory proteins in the postmicrosomal supernate should be the same as that of exogenously labeled tracer. Fig. 4*A* shows that this is the case within the limits of detection of our experimental procedures.

The extent of leakage of endogenous label from intracellular compartments can be varied reproducibly by appropriate changes in the experimental protocol. Two examples (already discussed) are the vigor of tissue disruption and the location of the pulse-labeled proteins at the time of homogenization. Other factors are the temperature and the tissue/medium ratio. The extent of leakage is less when the tissue is homogenized at 3°C than at 23°C by 10-25%, it is also less when the ratio of homogenizing medium (volume) to tissue (weight) increases as shown by the following experiment: 200-mg aliquots of the same set of pancreatic lobules pulse-labeled in vitro were homogenized

[6] $En_s^*/En_t^* = 0.14$ is taken from the ordinate in Fig. 7; $Ex_t^*/Ex_s^* = 2.29$, the inverse of 0.43, is taken from Table I; thus $\% En_L^* = 32\%$ (see Eq. 4).

(at 3°C) in increasing volumes, i.e., 0.5, 1.0, 2.0, and 5.0 ml of 0.3 M sucrose containing the usual ^{3}H tracer. The leakage of pulse-labeled proteins decreased with increasing volumes from 46 to 36, 31, and finally 28% of the initial amounts of label assumed to be present in particulate fractions. Similar results were seen when tissue was homogenized at 23° or 37°C.

Effects of KCl on Leakage

The results of the experiments in Fig. 8 indicate that leakage of endogenously labeled proteins is greatly reduced if, at the end of a 10-min labeling pulse, the tissue is homogenized in 0.3 M sucrose containing small concentrations of KCl. At 10 mM KCl, leakage is reduced to 42%, and at 100 mM, to 33% of the values found in control fractions isolated from tissue homogenized in 0.3 M sucrose only. At 10 mM KCl the reduction ranged from 40 to 62% in six experiments. A similarly large effect is not detected, however, when after an appropriate chase the labeled proteins are in condensing vacuoles or in zymogen granules. This finding suggests that the effect is exerted primarily during the fragmentation of the rough ER and the resealing and stabilization of the ensuing microsomal vesicles. As already mentioned in the fifth section of Results, the addition of KCl to the homogenizing medium also reduces the adsorption of exogenous label (as well as of released endogenous label) to subcellular particles, but higher concentrations are required to achieve the latter goal: whereas 10 mM KCl is sufficient to reduce leakage, 50 mM KCl is needed for an effective reduction of adsorption.

The addition of KCl to the homogenization medium leads, therefore, to a general reduction in the molecular contamination of cell fractions by a decreased leakage of secretory proteins as well as by a reduction of the latter's relocation by adsorption to other particulates. But, as already mentioned, this favorable effect is lost in part by an increase in particulate contamination of all cell fractions and by a decrease in the recovery of rough microsomes. As shown in Table III, with 10 mM KCl in the homogenizing medium, RNA recovery in a total microsomal fraction is reduced to ~90% of the control value, with a concomitant and commensurate increase in the RNA content of the corresponding mitochondrial and zymogen granule fractions. The separation of rough microsomes from smooth microsomes is only marginally affected by adding KCl to the sandwich sucrose gradient used for this purpose.

The Use of the Double-Label Protocol for Correcting Data Obtained in Pulse-Chase Experiments

A quantitative estimate of the PL index of subcellular fractions is possible if lobules labeled in vitro with ^{14}C-amino acids are homogenized and processed through cell fractionation in the presence of ^{3}H-secretory proteins added as an exogenous tracer to the homogenization medium. With this double-label protocol, the PL index of each fraction of interest can be calculated as given under the sixth section of Materials and Methods. Fig. 9 gives PLI changes for two cell fractions, rough microsomes and zymogen granules, as a function of chase time. Rough microsomes isolated immediately after a 5-min pulse have a relatively high PLI, whereas those isolated after a 15-, 30-, 45-, or 120-min chase have lower indices that decrease with time under chase. The PL indices of zymogen granules fractions prepared after a 40–120-min chase are very high, as expected; but the PLI of the same fraction isolated at the end of the pulse is also relatively high (though quite variable from one experiment to another). A high PLI appears as contrary to expectations since at that time zymogen granules contain practically no endogenously labeled secretory proteins (cf. reference 9). This finding can be explained by the fact that PLI calculations do not detect particulate contaminants in isolated fractions. Zymogen granule fractions are contaminated by rough microsomes which at the end of the pulse contain a significant amount of endogenous label unaccounted for by adsorption from the postmicrosomal supernate.

Fig. 10*a* and *b* give the kinetics of intracellular transport of pulse-labeled proteins before (Fig. 10*a*) and after (Fig. 10*b*) correction for adsorption of endogenous label calculated as given under the sixth section of Materials and Methods. The correction sharpens the kinetics of drainage of the ER and brings the data in closer approximation to those obtained by quantitative autoradiography (9). The actual kinetics of drainage of the rough ER and overall intracellular transport are probably even sharper than indicated by the corrected values. The factors which are still distorting and blunting the kinetics are: (*a*) the difficulty in obtaining a sharply defined pulse when a mixture

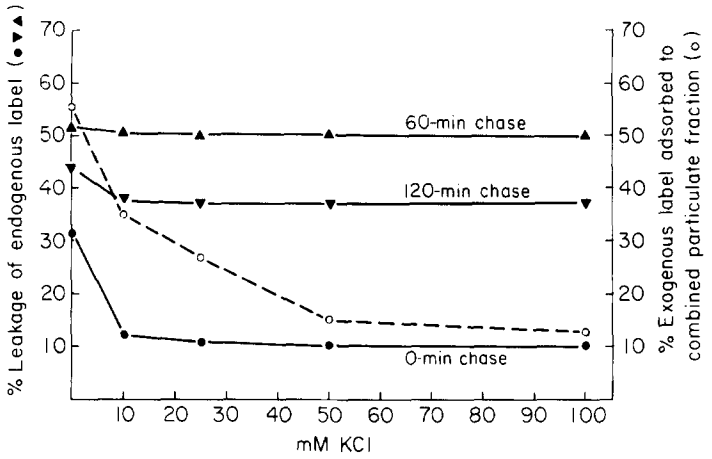

FIGURE 8 Percent leakage of ^{14}C pulse-labeled proteins (endogenous label) and percent adsorption of ^{3}H-labeled tracer proteins (exogenous label) as a function of KCl concentration in the homogenizing medium. Leakage is presented for three different chase times, and is calculated according to the formula given in the sixth section of Materials and Methods. It assumes that all ^{14}C-proteins present in the PMS leaked from membrane-bounded compartments during tissue homogenization. For example, in the case of the 0-min chase, the values for En_s^*/En_t^* were: 0.167, 0.095, 0.091, 0.098, 0.087; and those for Ex_t^*/Ex_s^* were: 1.82, 1.53, 1.45, 1.27, 1.15 for 0, 10, 25, 50, and 100 mM KCl, respectively.

of 15 amino acids is used for endogenous labeling; (*b*) the incorporation of a minor but not precisely defined amount of radioactivity in nonsecretory proteins;[7] (*c*) the possibility that drainage of the rough ER is not equally efficient in all cells; (*d*) the possibility that transport from the rough ER to the Golgi complex is retarded in the case of some proteins;[3] and (*e*) the lack of a correction for leakage of the data in Fig. 10*b*. In Fig. 10*c*, the data for two of the fractions in Fig. 10*b*, namely the rough microsomal fraction and the zymogen granule fraction, are corrected for leakage as well as for relocation by adsorption. With this additional correction, a picture very similar to that obtained by quantitative autoradiography emerges: ~80% of the endogenously labeled proteins is in the rough microsomes (i.e., in the rough ER) at the end of a 10-min pulse, and in the zymogen granule fraction (i.e., in zymogen granules) at the end of a 120-min chase. The apparent underrecovery of endogenously labeled proteins is explained, in large measure, by losses of microsomes as well as zymogen granules to other cell fractions, primarily the mitochondrial fraction.

Fig. 11 presents in diagrammatic form the data obtained on the adsorption of exogenously labeled exocrine proteins used as a tracer, as well as on the leakage and relocation by adsorption of endogenously labeled exocrine proteins.

A justifiable objection to the protocol of our mixing experiments is that the ^{3}H-labeled secretory proteins added to the homogenization medium may perturb cell fractionation in some still unknown fashion. To answer this objection, we have performed a limited number of experiments in which a first pulse with ^{3}H-amino acids was followed by a chase long enough (120 min) to allow the extensive concentration of the labeled proteins in zymogen granules, and then by a second pulse of ^{14}C-amino acids to label a second wave of secretory proteins in the rough ER. In such experiments, the PL index of rough microsomal fractions isolated at the end of the pulse (or shortly after) could be calculated. The values obtained were similar to those arrived at by using an exogenous ^{3}H tracer as in the orginal mixing protocol.

[7] Counting (after careful and comprehensive excision) of all exocrine protein spots from two-dimensional gels used to separate the proteins of postmicrosomal fractions indicates that after a 40-min chase, ~91% of the radioactivity of the preparation is recovered in exocrine proteins. Under the same pulse-chase conditions, the corresponding figure for the rough microsomes is ~85%; hence, ~15% represents microsomal nonsecretory proteins. Similar evaluation of the original homogenate by (*a*) comprehensive two-dimensional gel analysis and (*b*) evaluation of the radiolabeled proteins remaining on the glass fiber loading square by comprehensive one-dimensional SDS gel analysis indicates that 95% of the incorporated radiolabeled amino acids appear in discrete secretory proteins.

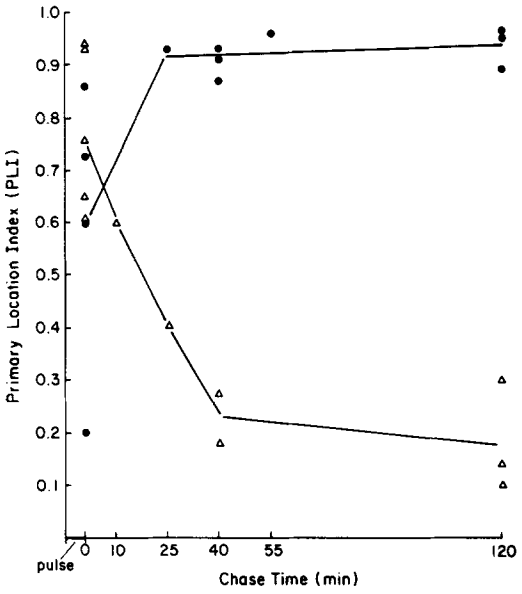

FIGURE 9 Estimation of the PL index of zymogen granules and rough microsomes using the double-label protocol in typical pulse-chase experiments extending to 2 h. At the indicated time points, tissue was submitted to cell fractionation, and PL indices for the indicated fractions ([●] zymogen granules, [△] rough microsomes) were calculated according to the PLI formula given in the sixth section of Materials and Methods.

DISCUSSION

In the past, cell fractionation procedures have been evaluated primarily for their efficiency in resolving a tissue homogenate into distinct populations of subcellular components, the general aim being to approach homogeneity in each cell fraction or—at least—to limit, to a known and tolerable extent, the frequency of particulate contaminants in each fraction. The means used in monotoring the results have been qualitative (15) or quantitative (27) electron microscopy, and more often biochemical assays for marker components (3, 4).

The existence of molecular (as distinct from particulate) contamination of cell fractions has been known for some time (24, 25), but no systematic attempts have been made in the past to assess its extent. Molecular contamintion is probably a frequent, if not general, problem in cell fractionation caused primarily (but not exclusively) by (*a*) disruption of compartmental membrane barriers, (*b*) imposition of altered environmental conditions which allow a series of proteins to gain access to membrane surfaces they never reach in vivo, and (*c*) a drastic alteration in the general ionic conditions (strength, species, and concentrations) to which intracellular organelles are exposed. These events are inherently con-

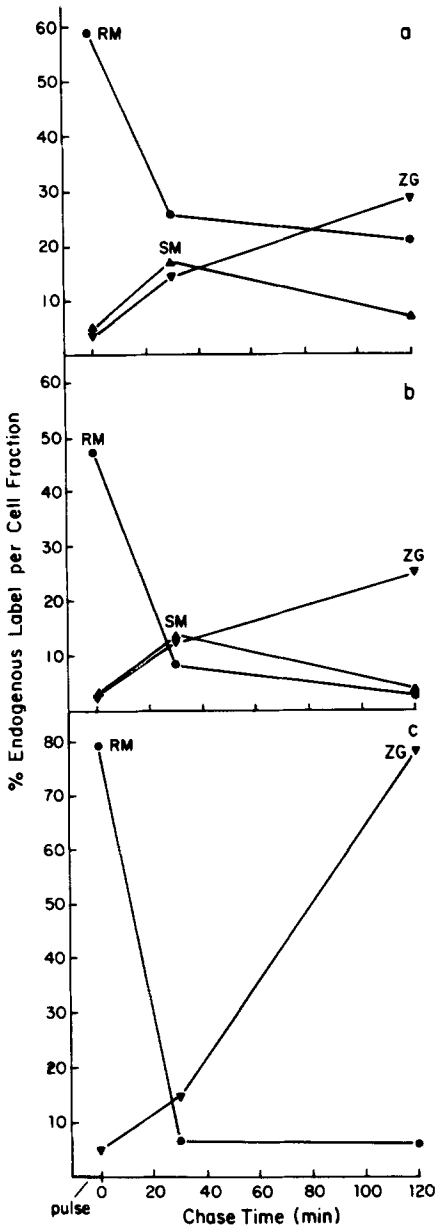

FIGURE 10 Intracellular transport kinetics for pulse-labeled proteins in the exocrine pancreas presented as raw data (uncorrected) (Fig. 10*a*) and after correction for adsorption (Fig. 10*b*), and adsorption and leakage (Fig. 10*c*). A curve for the smooth microsomal fraction cannot be given in Fig. 10*c* because direct, pertinent data on leakage from this fraction are not available at present. Representative data for a series of three experiments.

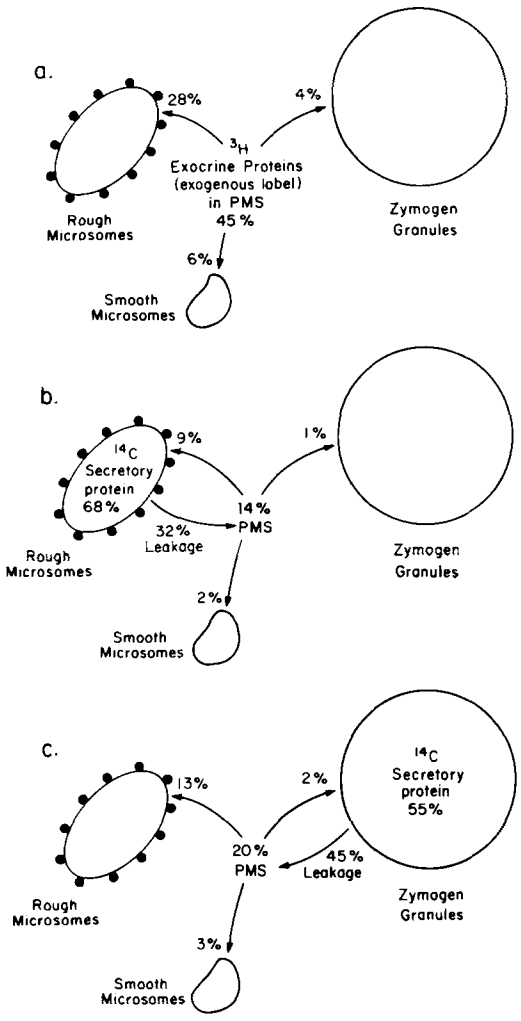

FIGURE 11 Diagrammatic representation of leakage and relocation by adsorption of exocrine proteins during the homogenization-fractionation of guinea pig pancreatic lobules. (*a*) Distribution of exogenously labeled exocrine proteins added to the homogenizing solution. (*b*) Leakage from ER fragments (rough microsomes) followed by relocation on the components of all cell fractions derived from the secretory pathway. (*c*) Leakage from zymogen granules followed by relocation as in Fig. 11*b*. In all cases, each cell fraction is represented by its major component. Data for nuclear and mitochondrial fractions are not included; this explains apparent underrecoveries. Leakage and readsorption artifacts are expected to affect all the components of a given cell fraction including its particulate contaminants.

nected with tissue homogenization. In the experiments reported, we have studied only a limited aspect of this general problem, namely the leakage and relocation of soluble secretory proteins caused by the homogenization of pancreatic tissue, which in practical terms can be equated to the disruption of pancreatic exocrine cells, because other cell types represent <10% of the total pancreatic cell population. We have chosen to analyze this system for the following reasons: (*a*) the relatively high rate at which pancreatic exocrine proteins are synthesized facilitates the quantitative assessment of leakage and relocation; (*b*) at the time we started, there was still a rather large discrepancy between autoradiographic and cell fractionation data as far as kinetics of intracellular transport are concerned; and (*c*) there is still no final agreement concerning the intracellular pathway followed by secretory proteins (cf. reference 16). Leakage and relocation of secretory proteins may have considerable bearing on the last two points.

We have started from the premise that all the exocrine proteins found in the postmicrosomal supernate represent leakage from the compartments of the secretory pathway incurred during tissue homogenization, and we have used extensively an experimental protocol in which pancreatic lobules labeled in vitro with ^{14}C-amino acids—and chased thereafter for appropriate times—were homogenized in the presence of ^{3}H-labeled exocrine proteins added as exogenous tracer to the homogenization media. The distribution of the two labels was studied in parallel in the cell fractions prepared from such homogenates, and the data obtained were used to calculate the extent of leakage from the compartments of interest, as well as the extent of relocation of the leaked proteins by adsorption to different particulates (see Eqs. 1–6).

The general picture that emerges from this inquiry indicates that leakage is extensive, uniform for all secretory proteins, and unequal from one compartment to another. All the secretory proteins leak to the same extent and, in terms of endogenous label, calculated leakage amounts to ~32% of the content of the rough microsomes, ~53% of that of the condensing vacuoles, and ~45% of that of the zymogen granules at the time of maximal labeling for each of the corresponding compartments. Under our "standard" homogenization conditions (three strokes), 14–24% of the total exocrine proteins of the tissue are found in the postmicrosomal supernate. The contribution of the different secretory compartments to the pool of secretory proteins in the postmicrosomal supernate can be roughly[8] estimated using enzyme

[8] The estimates are based on data concerning the

distribution data available in the literature (cf. reference 21). According to such estimates, more than ~90% is contributed by condensing vacuoles and zymogen granules and less than ~10% by ER (rough microsomes). An undetermined (but probably small) fraction must come from the acinar lumina as a result of the slow continuous discharge of zymogen granules ("hunger secretion").

In addition to the intracellular location of the secretory proteins, a number of other conditions were found to influence the extent of leakage, the most important among them being the shearing forces to which the tissue is subjected during homogenization, and the ionic environment in which tissue disruption is performed. Our data show that leakage increases as the exposure to shearing forces increases, and that the crucial event for all compartments is the first homogenizing stroke. In the case of ER, this first stroke causes a loss of ~30% of the endogenous label, and this figure is only moderately increased by repeated exposure to shearing forces. Extensive ER fragmentation is unavoidable on account of the geometry of the system and probably occurs during the first stroke, at which time leakage through transient discontinuities in the ER membrane is expected. The small size and physical characteristics of the ensuing rough microsomes may explain their relative resistance to repeated shearing. Condensing vacuoles and zymogen granules are more sensitive and respond gradually to increased intensity (revolutions per minute) and exposure (number of strokes) to shearing (cf. Fig. 7). These compartments exist in the cell as isolated units and their physical characteristics (size, rigidity, and fragility) may explain their more pronounced and better-correlated response to shearing.

The other important point that emerges from our findings is that the relocation of leaked secretory proteins by adsorption to the surfaces of different subcellular components is extensive (55% of the exogenous tracer), unequal (from compartment to compartment), preferential (for certain secretory proteins), and controlled primarily by ionic interactions. In relative terms, relocation is maximal on free polysomes, very extensive on rough microsomes, and relatively limited on smooth microsomes (Golgi elements), condensing vacuoles, and zymogen granules. We have used Scheele's two-dimensional gel system (17) (which has proved successful in the evaluations of soluble proteins present in cell fractions) to analyze adsorption at the level of individual secretory proteins. Those cationic in nature were found to adsorb preferentially to polysomes and rough microsomes, presumably by interacting with acidic groups on ribosomes and membranes. Accordingly, relocation results in a relative enrichment of cationic proteins in particulate fractions and a complementary enrichment of anionic proteins in the postmicrosomal supernate, irrespective of the origin of the exocrine proteins; i.e., added exogenous tracer or leaked endogenous product. In the case of the rough microsomes, relocation is so extensive that on the basis of enzymic activities, it accounts for more than ~90% of the neutral and basic secretory proteins found in the fraction. Other conditions found to affect leakage are the temperature at the time of homogenization and the dilution of the homogenate: leakage is higher at 25°C than at 4°C, and decreases with increased dilution. In these respects, conditions currently used (homogenization at approximately 4°C in a 1:10 tissue:diluent ratio) appear to be satisfactory

Leakage from the rough ER can be reduced by 40–62% if KCl is introduced in small concentrations (10 mM) into the homogenizing medium, and adsorbed proteins are efficiently removed from rough microsomes by washing the latter with KCl solutions (50 and 100 mM). The inclusion of KCl in the homogenizing medium and the KCl washing of the ensuing fractions can eliminate most of the leakage-relocation artifacts affecting the rough microsomal fraction, but the effect on other fractions (smooth microsomes, zymogen granules) is expected to be much more limited (cf. Fig. 8). In addition, the yield of rough microsomes decreases when KCl is present in the homogenization medium. The decrease is limited at concentrations that effectively reduce leakage (10 mM), but becomes severe at concentrations that minimize adsorption (50–100 mM). Concomitantly, the contamination of the zymogen granule fractions by rough microsomes increases. Notwithstanding these limitations, it would be highly desirable to try to define ionic, shearing, and other conditions that could minimize leakage and relocation of exocrine proteins without severe reduction in par-

distribution of RNase activity in reference 21 and on leakage and readsorption calculated from the data in Figs. 7 and 3 of this paper. Precise figures cannot be obtained since the homogenization-fractionation procedures used in reference 21 were different from those used in this work.

ticulate yield.

An explanation different from the one presented and discussed above has been advanced by Rothman (16) according to whom secretory proteins are present in vivo in equilibrium (across intracellular membranes) between secretory compartments and the cell sol. In Rothman's interpretation, some or all of the secretory proteins in the postmicrosomal fraction originate from the cell sol which is their physiological location. Against this hypothesis are the following findings: losses of endogenously labeled secretory proteins from the various compartments of the secretory pathway increase with the intensity and extent of shearing during homogenization; they decrease under ionic conditions that approach those prevailing in vivo and they also decrease with increasing homogenate dilution (an opposite effect would be expected on the basis of Rothman's postulated equilibrium). Rothman's hypothesis is also rendered unlikely by recent results which indicate that secretory proteins must be vectorially transported across the membrane of the ER (presumably as polypeptide chains in their primary extended conformation) to gain access to the cisternal space of the ER for a series of co-translational and post-translational modifications (removal of the signal sequence [5, 19], glycosylation [26], hydroxylation of amino acid residues [2, 13]) by which they are processed towards their final size and tertiary structure (cf. reference 14).

In the experiments reported, we have shown that leakage of exocrine proteins can be reduced substantially by decreasing the extent and intensity of the shearing forces involved in tissue homogenization. In particular, we have succeeded in reducing leakage from the rough ER to less than ~10% of the total endogenous radioactivity found in the pancreatic tissue at the end of a 10-min labeling pulse by homogenizing the lobules in sucrose containing low concentrations (10 mM) of KCl. Admittedly, we have not succeeded in completely eliminating leakage, but we have established a strong correlation between extent of shearing during homogenization and extent of this artifact. In fact, it is highly unlikely that leakage can be entirely prevented, given the geometry of the secretory compartments (especially the rough ER), and the essentially disruptive nature of the homogenization procedure. On the basis of the trend established by our data, we can conclude that in vivo the cell sol is either free of exocrine proteins or contains a negligible fraction (much less than 10%) of their total intracellular amounts. We can also conclude that most, if not all, of the secretory proteins found in the postmicrosomal supernate result from uniform leakage during tissue homogenization followed by preferential adsorption of cationic proteins to the surface of various subcellular components. This is clearly demonstrated by the remarkable parallelism in the distribution of individual exocrine proteins in the final supernate, irrespective of their origin or type of labeling (exogenous or endogenous). These conclusions amount in fact to a validation of our starting premises.

As a by-product of these investigations, we have defined some of the biological parameters of nonspecific adsorption of soluble proteins to particulate cell fractions. As already mentioned, this phenomenon, which is based primarily on charge interactions, results in the binding of cationic proteins to negative charges on ribosomes and membranes. Although direct binding measurements have not been made, strength of binding appears to correlate with the isoelectric point of the adsorbed molecules in the pH range 4.6–9.6. Binding of these cationic proteins shows no tissue specificity and no saturation notwithstanding the relatively high concentrations so far tested. Relatively low concentrations of KCl (50–100 mM) proved successful in removing these proteins from their putative adsorptive sites. A single wash with KCl removes ~80–90% of the adsorbed protein, and a further wash removes an additional 8–9%. Ionic conditions which prevent or counteract the adsorption of these molecules approximate those found in the intracellular space and suggests, that, under physiological conditions, adsorption of this type is minimal. As already mentioned, NaCl at equal concentrations is less effective than KCl in removing adsorbed exocrine proteins. Binding that does not exhibit these characteristics is probably specific. Our studies provide two such examples. Lipase 2 has considerably higher affinity for smooth microsomes than would be expected from its isoelectric point (cf. Fig. 4*B*) and, as judged by its net charge, amylase is overrepresented in the postmicrosomal supernate. These findings probably reflect enzyme-substrate interactions, the first to membrane lipid in smooth microsomes and the second to soluble glycogen.

The reliability of the correction based on our calculations for PL index is shown by the success of the 100-mM KCl wash in removing amounts of adsorbed secretory proteins roughly comparable

(first wash) and nearly identical (second wash) to those calculated from the PL index. Since the PL index can detect only the moledular contamination of a given cell fraction and is insensitive to particulate contamination, evaluation of the degree of particulate heterogeneity of the cell fractions of interest is still necessary and can be carried out by morphological survey or by using biochemical markers.

Finally, correction of our cell fractionation data for leakage and relocation by adsorption gives drainage kinetics for the endoplasmic reticulum and general kinetics for intracellular transport comparable to those obtained by quantitative autoradiography. The major point on which autoradiography retains an advantage is the ability to distinguish between grains over condensing vacuoles and zymogen granules. This distinction is not possible by cell fraction analysis since condensing vacuoles are recovered in zymogen granule fractions, whereas in quantitative autoradiographic analysis they are considered separately or included in the Golgi complex. This operational difference explains the lack of a smooth microsome peak (i.e., a Golgi equivalent peak) well above the zymogen granule curve in Fig. 10*b*.

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