

DYNAMICS OF CYTOPLASMIC MEMBRANES IN GUINEA PIG PANCREATIC ACINAR CELLS

I. Synthesis and Turnover of Membrane Proteins

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ABSTRACT

The rate of synthesis and the turnover of cytoplasmic membrane proteins were determined in the acinar cells of guinea pig pancreas with the aim of investigating the mechanisms by which the intracellular transport of secretion products occurs. These cells are highly specialized toward protein secretion.

By means of *in vitro* pulse-chase experiments and *in vivo* double-labeling experiments, using radioactive L-leucine as the tracer, it was found that the turnover of secretory proteins is much faster than that of all membranes involved in their transport (rough and smooth microsome and zymogen granule membranes). Sodium dodecyl sulfate-polyacrylamide disk gel electrophoresis of membrane proteins revealed that in each of these membranes there is a marked heterogeneity of turnover; generally the high molecular weight polypeptides have a shorter half-life than the low molecular weight polypeptides.

These data indicate that the membranes participating in the intracellular transport of secretory proteins are not synthesized concomitantly with the latter. Rather, they are probably reutilized in several successive secretory cycles.

The possible relevance of these findings to other secretory systems is discussed.

INTRODUCTION

It has been firmly established in a number of secretory systems that exportable proteins are vectorially transported within the cells along a discontinuous pathway, made functionally continuous by the interconnection of a series of distinct membrane-bounded compartments. This series involves the rough-surfaced endoplasmic reticulum (RER),¹

¹ *Abbreviations used in this paper:* GC, Golgi complex; i.p., intraperitoneal; PM, plasmalemma; RER, rough-surfaced endoplasmic reticulum; RM, rough microsomes; SDS, sodium dodecyl sulfate; SM, smooth microsomes; TCA, trichloroacetic acid; TH, total homogenate; TM, total microsomes; ZG, zymogen granules.

the Golgi complex (GC), and the secretory granules. The content of the latter is ultimately discharged into the extracellular space by exocytosis (1-10).

Although the route and timetable of the intracellular transport are known in several systems, the mechanisms involved in the transport from compartment to compartment are still unclear. Models suggested so far range between the following extremes. On the one hand it has been proposed that the participating membranes flow within the cell together with the secretion products (11-13). According to this interpretation, participating membranes would be synthesized as RER membranes

concomitantly with the secretion products, transformed into GC and secretion granule membranes, and degraded at the end of each secretory cycle. If this model were correct, one would expect (a) the structure and composition of participating membranes to change progressively, step by step, along the secretory pathway, and (b) the half-life of participating membranes, and especially that of the secretory granule membranes, to be comparable to that of the secretory products.

According to an alternative interpretation, the movement of secretion products would not imply the concomitant biogenetic flow of the membranes that bound the product, which could therefore be synthesized and turn over independently from one another. The vectorial transport of secretion products between cell compartments would be effected through nonrandom fusion and fission of their limiting membranes, which could be reutilized in multiple secretory cycles (2, 3, 14). This model does not place restrictions on the composition and turnover of the membranes involved.

Over the last few years we have carried out a detailed investigation of the composition of cytoplasmic membranes of the acinar cells of the guinea pig pancreas (15-17). This cell type has been chosen because fractions containing the different organelles involved in intracellular transport as well as membrane subfractions can be obtained in reasonably pure form by relatively mild procedures (18, 19). Our results do not appear consistent with the membrane flow model, since different membrane fractions were found to have specific patterns of enzymatic activities and specific lipid and protein composition (15-17). This conclusion is now strengthened by the results of the present study, in which the membranes involved in the intracellular transport of secretion products were labeled *in vitro* and *in vivo* and found to have independent biogenesis and slow and asynchronous turnover of their molecular components.

MATERIALS AND METHODS

Male albino guinea pigs (gift of Sigurtà Drug Co., Milan, Italy), weighing 400-450 g, were fasted for 24 h before sacrifice by a blow on the head followed by cardiosection. Pancreases were quickly excised and immersed in ice-cold 0.3 M sucrose or Krebs-Ringer bicarbonate solution.

In Vitro Studies

Pancreas tissue slices, obtained with a Stadie and Riggs hand microtome, were pulse labeled for 5 min

with L-[4,5-³H]leucine (0.75 μ M; sp act 32 mCi/ μ mol) in 5 ml of oxygenated Krebs-Ringer bicarbonate solution, pH 7.4, supplemented with a complete set of aminoacids and glucose, as described by Jamieson and Palade (2). Labeled slices were reincubated in 10 ml of chase medium (containing 2 mM non-radioactive L-leucine) for 0, 20, 80, or 150 min.

In Vivo Studies

In four preliminary experiments carried out to estimate both the rate of leucine incorporation into the protein of the different cell fractions and the evolution of the specific radioactivity with time, animals were injected intraperitoneally (i.p.) with L-[4,5-³H]leucine, 100 μ Ci, and sacrificed 8, 10, 13, and 18 h later. In double-label studies (two experiments) two 10 h starved animals were given 1 mCi of L-[4,5-³H]leucine (sp act 32 Ci/mmol), injected i.p. in four equal doses at 20-min intervals. Food was restored 13 h later. After either 4 or 6 days this schedule was repeated using a total of 300 μ Ci of L-[U-¹⁴C]leucine (sp act 260 μ Ci/ μ mol) per guinea pig and the animals were sacrificed 13 h later. In control experiments carried out to determine the limits of reliability of the double-label technique, two 10 h starved guinea pigs were given simultaneously 125 μ Ci of L-[U-¹⁴C]leucine and 240 μ Ci of L-[4,5-³H]leucine per guinea pig and sacrificed 13 h later.

Cell Fractionation

Pancreas homogenates were fractionated by differential and density gradient centrifugation as described in reference 18 to yield the following fractions: mitochondria; total, rough (RM = RER) and smooth (SM = mainly GC) microsomes, and zymogen granules (ZG). The latter three fractions were subfractionated to yield the corresponding membranes and soluble components. Since the procedures are described in detail in references 18 and 19, only a summary of them will be given here.

RM and SM were incubated first with 0.5 mM puromycin, 1 M KCl, 5 mM MgCl₂, pH 6. The resulting membranes were purified by flotation in a sucrose density gradient and then treated with 0.2 M NaHCO₃ buffer, pH 8. The treatment with KCl-puromycin releases the ribosomes as well as a large proportion of the secretory and adsorbed proteins (KCl-puromycin subfractions). The NaHCO₃ treatment extracts residual secretory proteins (NaHCO₃ extract) (19). Total RM- and SM-soluble subfractions were obtained by mixing, in the appropriate proportion, the corresponding NaHCO₃ extract and KCl-puromycin-released subfractions, after dilution and high speed centrifugation of the latter to remove the ribosomes.

ZG membranes were isolated by differential cen-

trifugation from ZG lysed by treatment with 0.2 M NaHCO₃ buffer, pH 8, and purified by flotation in a discontinuous sucrose density gradient (18). The ZG-soluble subfraction is virtually exclusively accounted for by secretory proteins (18).

As judged from analytical and enzymatic determinations, all membrane subfractions contain only trace amounts of nonmembrane contaminants (17-19).

Thin Layer Chromatography of Amino Acids

Samples of *in vivo* doubly labeled pancreatic homogenates, microsomes, and microsomal membranes were hydrolyzed in 6 N HCl in sealed ampoules under vacuum at 110°C for 18 h. Aliquots of the hydrolyzed samples were applied to precoated ion exchange thin layer plates (Ionex 25 SA Na⁺ from Macherey Nagel, Duren, Germany) which were developed in 0.4 M citrate buffer, pH 3.3 at 50°C as recommended by Devenji et al. (20). After staining with ninhydrin, the different aminoacids, identified with the help of reference standards, were scraped off the plate, suspended in 10 ml of Packard Instagel, and counted.

Gel Electrophoresis

10 and 10.5% high resolution sodium dodecyl sulfate (SDS)-polyacrylamide gels (0.5 or 0.9 cm in diameter, 12 or 20 cm in length) were prepared and run as described by Maizel (21), with a few modifications (17). After staining with Coomassie brilliant blue and scanning in a Joyce and Loeb Chromoscan MK₂ densitometer, they were usually cut in slices either 1.5 or 2.8 mm thick, with the aid of the apparatus described by Jandolo (22). The slices were dried in disposable plastic counting vials, dissolved by incubation in small volumes of 30% H₂O₂ (at 50°C for 2 h), mixed with 10 ml of Packard Instagel, and counted.

Radioactivity and Chemical Assays

In order to determine radioactivity in tissue homogenates and cell fractions, samples were precipitated at 4°C with 10% trichloroacetic acid (TCA), washed twice with 5% TCA, resuspended in 5% TCA at 90°C for 15 min, extracted twice with 3:1 ethanol-ether at 37°C for 1 h, and finally dissolved in 10 ml of Packard Instagel. This extraction scheme was followed because, particularly in the *in vivo* turnover experiments, a considerable amount of radioactivity was extracted by the hot TCA and, especially, by the ethanol-ether treatments. The lipid nature of the latter extract was established by thin layer chromatography. In the case of the membrane fractions labeled *in vitro* and *in vivo*, ~85 and ~70%, respec-

tively, of the radioactivity originally precipitated by TCA was recovered in the extracted samples.

All samples were counted in an Intertechnique SL 30 liquid scintillation spectrometer (Intertechnique, Plaisir, France). In single-label counting, the ³H efficiency was over 40%; in double-label counting, conditions were selected to have no spill of ³H counts into ¹⁴C channel; efficiency of ³H counts was ~28% and of ¹⁴C counts, ~60%. Spill of ¹⁴C counts into the ³H channel was always less than 8%. Background for ¹⁴C was 10 cpm, for ³H, 4 cpm. Corrections for quenching and spill were made by external standardization.

In control experiments (simultaneous injection of 125 μCi of L-[U-¹⁴C]leucine and 240 μCi of L-[4,5-³H]leucine) we found that the expected double-isotope ratio of 0.52 was found within a 10% accuracy in all samples containing a minimum of 20 dpm for ³H and 10 dpm for ¹⁴C, provided that the ¹⁴C:³H ratio was < 2, spill of ¹⁴C in the ³H was < 6% and counting was carried out for 100 min. Therefore, the gel slices of membranes were always counted for 100 min.

Furthermore, in each experiment, duplicate gels of RM and SM membranes were analyzed. With ZG membranes, since the material was sufficient for only one gel, samples were counted three times for 100 min. Results presented are the averages of the three determinations which agreed within 8%.

Protein was determined according to Lowry et al. (23) on TCA precipitates dissolved in 1 N NaOH, using crystalline bovine serum albumin as standard.

Materials

L-[4,5-³H]Leucine (sp act 32 mCi/μmol) and L-[U-¹⁴C]leucine (sp act 260 μCi/μmol) were purchased from NEN, Langen, Germany. The sources of the other materials used are given in reference 17.

RESULTS

In Vitro Experiments

In order to investigate the biogenetic relationships of the different cytoplasmic membranes involved in secretion, pancreatic tissue slices were pulse labeled for 5 min with L-[4,5-³H]leucine and then incubated in chase medium up to 150 min. In agreement with the previous data by Jamieson and Palade (2, 3) we found that at the end of the pulse a very high specific radioactivity was associated with the RM, while the SM had ~66% of the specific radioactivity of their rough counterpart and the ZG were nearly unlabeled. During chase the radioactivity decreased in microsomes and increased in ZG so that after 150 min the specific

radioactivity in the latter fraction was sixfold higher than in RM and SM (Table I). When RM, SM, and ZG were processed in order to yield the corresponding membranes, most of the radioactivity was recovered in the subfractions known to contain secretory proteins (the NaHCO₃ extract, which is accounted for nearly exclusively by secretion products, and the KCl-puromycin-released subfraction, where secretory proteins are recovered together with ribosomes and adsorbed proteins) (Table I). A considerable specific radioactivity was found in both the RM and SM microsomal membranes at the end of the pulse (~30–40% of that found in intact RM and SM) (Table I and Fig. 1). In RM membranes the radioactivity was clearly reduced after 20 min of chase incubation. Between 20 and 80 min there was an additional decrease, with little further change at the last time-point. An analogous but less marked decrease was found in SM membranes, whereas a completely different curve was found in ZG membranes. These had low specific activity at the end of the pulse.

During chase incubation the labeling increased progressively, so that after 150 min the protein specific radioactivity of ZG membranes exceeded that of microsomal membranes (Fig. 1).

However, when the proteins of the membrane fractions were resolved by SDS-polyacrylamide disk gel electrophoresis, it became clear that a large proportion of their radioactivity was contributed by highly labeled secretory proteins. This conclusion is at variance with that reported in a previous publication, in which, however, the distribution of the radioactivity in gels of membrane proteins was not studied (24).

In the present experiments 10% polyacrylamide gels, 5 mm in diameter and 12 cm in length, were used. Representative examples of the results obtained are shown in Figs. 2, 3, and 4 B. A limited number of radioactivity peaks were found in the gels of both microsomal membrane subfractions, and these accounted for most of the radioactivity present at the end of the pulse (Figs. 2 and 3 B). These peaks were identified as secretory proteins

TABLE I
Specific Radioactivity of RM, SM, ZG, and Their Subfractions Obtained from Guinea Pig Pancreatic Slices Pulse Labeled for 5 Min with L-[4,5-³H]Leucine and Incubated in Chase Medium for 0 or 150 Min*

Cell fraction	dpm/mg protein		Cell fraction	dpm/mg protein		Cell fraction	dpm/mg protein	
	0 min in chase	150 min in chase		0 min in chase	150 min in chase		0 min in chase	150 min in chase
RM	157,143	45,679	SM	105,000	50,617	ZG	7,160	298,074
RM; NaHCO ₃ - extract	355,185	62,395	SM; NaHCO ₃ - extract	241,975	84,593	ZG; NaHCO ₃ - extract	4,642	395,062
RM; KCl- puromycin released	205,348	62,321	SM; KCl- puromycin released	162,716	67,210			
RM mem- branes	48,062	23,284	SM mem- branes	42,002	30,025	ZG mem- branes	12,637	37,037
Corrected‡ RM mem- branes	~18,000		Corrected‡ SM mem- branes	~23,500		Corrected‡ ZG mem- branes	~13,000	

* Values given are the averages of three consistent experiments.

‡ Correction of the specific activity of the membrane subfractions is based on the following assumptions: (a) the NaHCO₃-extracts are exclusively accounted for by secretory proteins (as suggested by the evidence reported in references 17 and 19), (b) the labeling of true membrane proteins remains unchanged over 150 min of chase incubation (Figs. 2, 3, and 4 B), and (c) the amount of contaminating secretory protein is the same at both timepoints considered. Calculations were made by solving the following equations for each organelle (RM, SM, and ZG): $\frac{(100-x)}{100}y + \frac{xa}{100} = b$ and $\frac{(100-x)}{100}y + \frac{xa'}{100} = b'$, where x = percent contamination by secretory protein in the membrane subfraction, y = corrected specific activity of the membrane subfraction, a and a' = specific activity of secretory protein (NaHCO₃-subfractions) after 0 and 150 min chase, respectively, b and b' = specific activity of the membrane subfraction at the same timepoints.

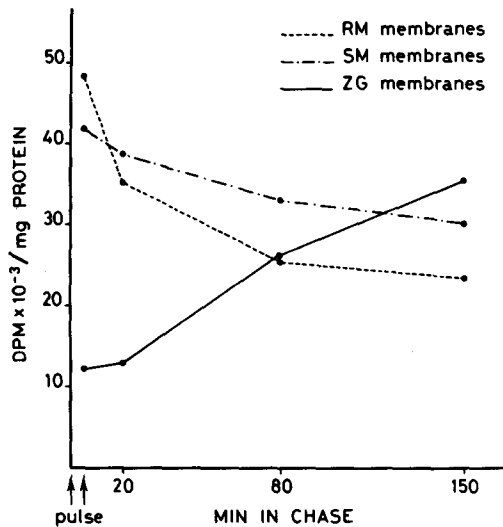


FIGURE 1 Specific protein radioactivity of membrane fractions isolated from pancreatic slices pulse labeled with L-[4,5-³H]leucine for 5 min and then incubated up to 150 min in chase medium. Values given are averages of either three (0 and 150 min chase) or two (20 and 80 min in chase) consistent experiments. Analogous data were obtained previously using L-[U-¹⁴C]leucine as tracer (24).

for the following reasons: (a) their migration rates were identical with those of highly labeled peaks present in RM- and SM-soluble fractions (Figs. 2 and 3 A), (b) in the membrane gels they did not systematically coincide with major absorption bands (Figs. 2 and 3 B), and (c) after 150 min of chase their specific activity decreased in parallel with that of secretory protein peaks, most likely as a consequence of transport of secretion products from microsomes to ZG (2, 3). In the remaining portions of the microsomal membrane gels, where only true membrane proteins appear to be recovered, labeling was low and remained essentially unchanged during chase incubation.

In gels of ZG membranes peaks of radioactivity with migration rates coinciding with secretory proteins were absent at the end of the pulse, but appeared after chase (Figs. 4 A and B). True ZG membrane proteins, separated in the remaining portions of the gel, had low specific activity as at the end of the pulse.

Since the concentration of leucine is approximately the same in secretory and membrane proteins,² the data obtained by gel electrophoresis indi-

² Concentration of leucine in RM, SM, and ZG mem-

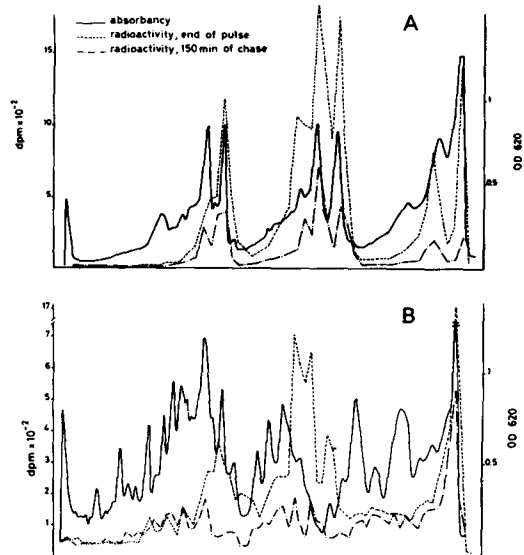


FIGURE 2 Electrophoretic patterns of radioactivity and optical density of RM-soluble (A) and membrane (B) fractions isolated from pancreas tissue slices pulse labeled in vitro with L-[4,5-³H]leucine. 10% high resolution SDS-polyacrylamide gels (5 mm in diameter, 12 cm in length) were loaded with $\sim 100 \mu\text{g}$ of protein of the fractions isolated either at the end of the pulse or after 150 min of chase. In each panel the radioactivity data obtained by separate gels at the two timepoints are superimposed. Since the gel scans of the samples isolated at the two timepoints did not differ significantly, only that of the end of the pulse sample is shown. Electrophoretic migration from left to right. Specimen:record ratio = 1:3.

cate that the rate of synthesis of membrane proteins is very slow relative to secretory proteins. In order to get a rough estimate of the true specific activity of membrane proteins, the contribution by secretory proteins was calculated by solving the equation described in Table I. As can be seen, the corrected specific activities are $\frac{1}{15}$ – $\frac{1}{30}$ of those found in secretory proteins. The amounts of contaminating secretory proteins can be calculated as 7.5–8.5% for microsomal membrane and $\sim 6.2\%$ for ZG membrane fractions.³

branes was 10.02, 11.92, and 11.22%, respectively (17); in soluble proteins extracted from ZG, 10.01%. The amino acid analyses were kindly performed by Dr. S. Saracchi at the Istituto di Scienze Agrarie, University of Milan.

³ It should be noted that these estimates indicate that the degree of contamination previously calculated for these fractions by well-established analytical and

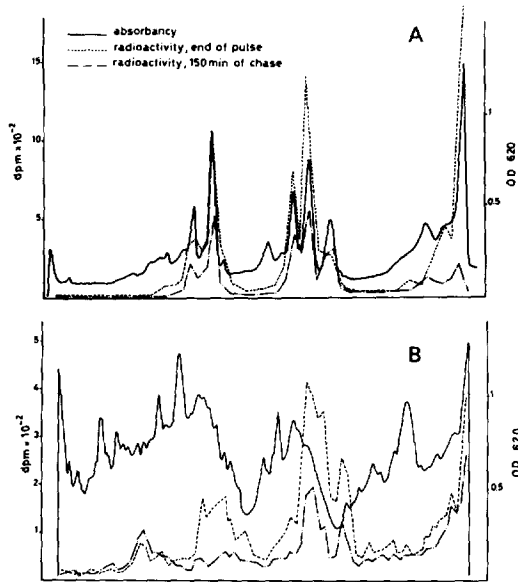


FIGURE 3 Electrophoretic patterns of radioactivity and optical density of SM-soluble (A) and membrane (B) fractions isolated from pancreas tissue slices pulse labeled in vitro with L-[4,5-³H]leucine. Experimental conditions as in Fig. 2.

In Vivo Experiments

Because of the low rate of labeling of membrane proteins by comparison with secretory proteins, *in vitro* experiments proved inadequate to yield detailed information about the turnover of the proteins of the different cytoplasmic membranes. We switched therefore to *in vivo* experiments, which can be conducted on a much longer time scale.

Since the work of Schimke and his associates, it is known that relative turnover of proteins can be adequately determined *in vivo* by a simple double-label technique, in which animals sequentially receive the same amino acid labeled with different isotopes (25, 26). As employed here, L-[4,5-³H]leucine was injected either 4½ or 6½ days and L-[U-¹⁴C]leucine 13 h before the sacrifice. Thus, the ¹⁴C and ³H radioactivities represent 13 h and 4½- or 6½-day timepoints, respectively, and decay

enzymological criteria (17-19) was largely inaccurate. This finding raises the question of the general reliability of the methods currently used for assessing the purity of cell fractions and indicates that, at least in the cases where it is critical to the results, this problem should be approached by more than one type of experiment.

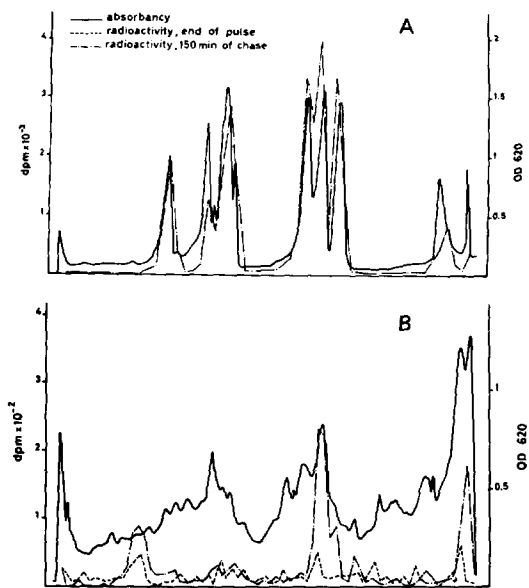


FIGURE 4 Electrophoretic patterns of radioactivity and optical density of ZG-soluble (A) and membrane (B) fractions isolated from pancreas tissue slices pulse labeled in vitro with L-[4,5-³H]leucine. Experimental conditions as in Fig. 2 with the following exceptions: (a) due to low specific activity of the ZG-soluble proteins at the end of the pulse, only the distribution of radioactivity at the end of the chase was determined. Also the gel scan refers to the latter timepoint. (b) Membrane gels were loaded with ~80 µg of protein.

kinetics can be deduced from these measurements. Proteins of high turnover will have high ¹⁴C:³H ratios and vice versa (25, 26). Isotope injections were separated by rather long intervals in order to obtain a sizable decrease of the ³H radioactivity associated with the slowly turning over membrane proteins. A number of preliminary experiments were carried out to establish the appropriate conditions, since it is known that the technique used yields correct results only when the following requirements are met (25, 26): (a) at the time the animals are killed the labeled proteins should be in a process of isotopic decay. Fig. 5 shows that, at least as a first approximation, this seems to be the case for total microsomal (TM) and ZG membranes 13 h after the injection of labeled leucine. It is likely that the individual membrane proteins have analogous behavior.⁴ (b) Labeled leucine

⁴ This assumption may not be completely valid, since for different proteins the time needed to reach maximum specific activity is a function of their specific rate constants of degradation and is therefore variable.

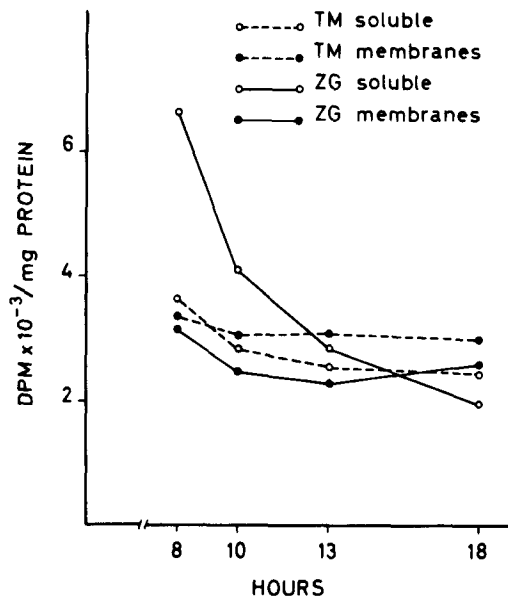


FIGURE 5 Specific protein radioactivity of guinea pig pancreas membrane and soluble fractions. L-[3,4-³H]-Leucine was administered to fasted guinea pigs which were then killed at each timepoint indicated. Values given are the averages of either two (8 and 10 h) or four (13 and 18 h) experiments.

should not be metabolized to other amino acids. The proteins of double-labeled homogenates and cell fractions were hydrolyzed and the distribution of label among the various amino acids was determined by thin layer chromatography: over 92% of both ¹⁴C and ³H counts were recovered in the leucine spot (results not shown). (c) The rates of synthesis of the proteins of the membranes under study should be similar at both labeling timepoints. The nutritional status of the guinea pigs was maintained unchanged during the study since changes in diet and feeding schedule can result in changes of protein turnover.

It should also be pointed out that 13 h after isotope injection the specific activity of salt-extracted proteins was of the same order of magnitude as that of membrane proteins (Fig. 5). Therefore, the small contamination by secretory proteins is ex-

However, the time between the second injection and the sacrifice was sufficiently long to minimize errors. Furthermore, in the pancreas, due to their massive incorporation into secretory proteins, there is little chance of reutilization of amino acids in synthesis of nonsecretory proteins.

pected to have only a minor effect on the estimates of membrane proteins turnover.

The results of the *in vivo* experiments are reported in Tables II and III and Figs. 6-11. The relative turnover of all membranes involved in the intracellular transport is very slow (= low double-isotope ratio), not only in comparison with secretory proteins, but also with the homogenate and mitochondria. This is quite clear for RM and ZG membranes, while the turnover of SM membranes is slightly faster (Tables II and III, Figs. 6-8).

Doubly labeled membrane fractions obtained both in the 4 day and in the 6 day interval experiments were studied by SDS-polyacrylamide disk gel electrophoresis. In these experiments 10.5% gels, 9 mm in diameter and 20 cm in length, were used (except for ZG membranes which were separated in 10.5%-5 mm-12 cm gels).⁵ As opposed to the situation found in the *in vitro* experiments, the label was not concentrated into large peaks but appeared spread over the entire gel. Little coincidence was found between the distribution of protein, as revealed by the absorbancy profiles of the stained gels, and the distribution of radioactivity. This finding, which is probably dependent on the limitations of the technique used, i.e. on the insufficient resolution of the electrophoretic separation, prevented us from analyzing the turnover of individual membrane polypeptides. Nevertheless, the results obtained clearly indicate that the turnover of the proteins of the membranes under study is markedly heterogeneous. Thus, the double-isotope ratio was not the same in different gel slices but in general decreased from the origin to the front of the gel. Since in SDS gel electrophoresis the relative rate of migration of polypeptides depends primarily on their size (21), these results suggest the existence of a general correlation between the molecular weight of a polypeptide and its turnover: large polypeptides turn over faster

⁵ The experimental conditions under which polyacrylamide gel electrophoresis was carried out in the *in vitro* experiments are different from those of the *in vivo* experiments, and the absorbancy profiles are, therefore, necessarily different. However, in each case the patterns were reproducible. The latter observation confirms our previous results, obtained by a number of different experiments (17), indicating that, in our conditions of cell fraction isolation and processing, proteolytic zymogens are not activated and, therefore, extensive digestion of cell proteins does not occur.

TABLE II
 $^{14}\text{C}:$ ^3H Ratios of Guinea Pig Pancreas Cell Fractions (4 Day Interval between Injections)*

	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$		^{14}C	^3H	$^{14}\text{C}/^3\text{H}$
	(dpm/mg protein)				(dpm/mg protein)		
TH	10,220	8,291	1.23	RM-soluble protein	6,350	5,905	1.06
Mitochondria	7,235	9,911	0.73	RM membranes	7,750	14,623	0.53
TM	7,204	8,004	0.90	Ribosomes	4,115	7,620	0.54
RM	6,582	7,396	0.89	SM-soluble protein	6,720	6,109	1.10
SM	7,430	7,582	0.98	SM membranes	9,550	14,253	0.67
ZG	8,950	2,486	3.60	ZG-soluble protein	8,120	2,255	3.60
				ZG membranes	7,050	11,557	0.61

* Each of two guinea pigs was injected i.p. with 1 mCi of L-[4,5- ^3H]leucine. 4 days later each guinea pig received 300 μCi of L-[U- ^{14}C]leucine. 13 h after the last injection the animals were killed, pancreases were pooled, and the cell fractions isolated as described under Methods. TH, total homogenate.

TABLE III
 $^{14}\text{C}:$ ^3H Ratios of Guinea Pig Pancreas Cell Fractions (6 Day Interval between Injections)*

	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$		^{14}C	^3H	$^{14}\text{C}/^3\text{H}$
	(dpm/mg protein)				(dpm/mg protein)		
TH	12,300	5,700	2.16	RM-soluble protein	6,120	3,221	1.90
Mitochondria	7,814	6,915	1.13	RM membranes	8,790	11,416	0.77
TM	6,904	4,512	1.53	Ribosomes	4,902	6,248	0.78
RM	7,290	5,027	1.45	SM-soluble protein	6,300	3,224	1.93
SM	6,870	4,215	1.63	SM membranes	9,623	8,828	1.09
ZG	8,366	2,140	3.91	ZG-soluble protein	8,300	2,000	4.15
				ZG membranes	6,247	7,349	0.85

* Experimental details as in Table II except the interval between first and second dose of isotope was 6 days. TH, total homogenate.

than small polypeptides. The same phenomenon had been observed previously in liver microsomes and plasma membranes by Schimke and his associates (25-28). The correlation is quite clear in RM and ZG membranes. In the experiment with the 6 day interval between injections, the ratios ranged between 0.85 and 0.38 (RM) and between 1.25 and 0.45 (ZG) (Figs. 6 and 8). In SM membranes the correlation is still evident (ratios between 1.47 and 0.76). However, three groups of proteins with anomalously high turnover are present. Their mol wt are around 50,000, 24,000, and 19,000 daltons (Fig. 7).

It is worth mentioning that the general correlation between size and turnover of polypeptides appears valid only within each membrane, whereas proteins of the same size present in different membranes can have different turnover. This is particularly striking in RM and SM membranes: their electrophoretograms are quite similar (compare Figs. 6 and 7, continuous line), while the

turnover of SM membrane proteins appeared always faster than that of the RM membrane proteins of the same size.

In order to determine whether our estimates of membrane protein turnover were influenced by contaminating secretory proteins, soluble proteins released from RM, SM, and ZG were also studied by SDS-polyacrylamide disk gel electrophoresis. We found that in both microsomal-soluble fractions only a limited proportion of the radioactivity was accounted for by secretory proteins, as shown by the relatively low $^{14}\text{C}:$ ^3H ratios. The rest was contributed by long-lived proteins, probably adsorbed from the cytoplasm or stripped from membranes (Figs. 9 and 10).

A rather different situation was found with the ZG which still retained an appreciable amount of radioactive secretory proteins, as reflected by the higher $^{14}\text{C}:$ ^3H ratios (Fig. 11). However, due to their low specific activity, contaminating secretory proteins did not have an appreciable effect on the

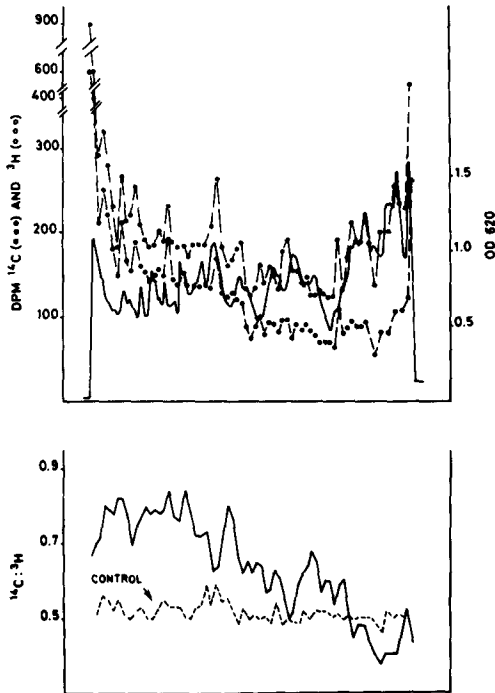


FIGURE 6 Relative turnover of pancreas RM membrane proteins. Each of two guinea pigs received 0.3 mCi of L-[U- ^{14}C]leucine 6 days after the administration of 1 mCi of L-[4,5- ^3H]leucine. Animals were killed 13 h thereafter. Protein ($\sim 700 \mu\text{g}$) was applied to 10.5% high resolution SDS-polyacrylamide gels which were 20 cm long and 0.9 cm in diameter. The upper panel includes the optical density tracings of stained gels and the distribution of ^3H and ^{14}C counts in ~ 70 2.8-mm thick gel slices. In the lower panel, the continuous line refers to the $^{14}\text{C}:^3\text{H}$ ratios of the slices. These data are representative of two gels run in parallel. The dotted line in the lower panel shows the results of a control experiment in which comparable animals were administered 125 μCi of L-[U- ^{14}C]leucine and 240 μCi of L-[4,5- ^3H]leucine simultaneously and sacrificed 13 h later. Electrophoretic migration from left to right. Specimen: record ratio = 1:1.

estimates of ZG membrane protein turnover (compare the distribution of the label in Figs. 8 and 11).

So far, the turnover of membranes and membrane components has been defined only in relative terms as double-isotope ratios. However, it has been shown that these values can be adequately converted to give half-life values (26, 29) which are in good agreement with those obtained by following with time the loss of nonreutilizable amino acids from proteins (26).

The half-lives of RM membrane proteins thus estimated are on the average ~ 5 days, with a mini-

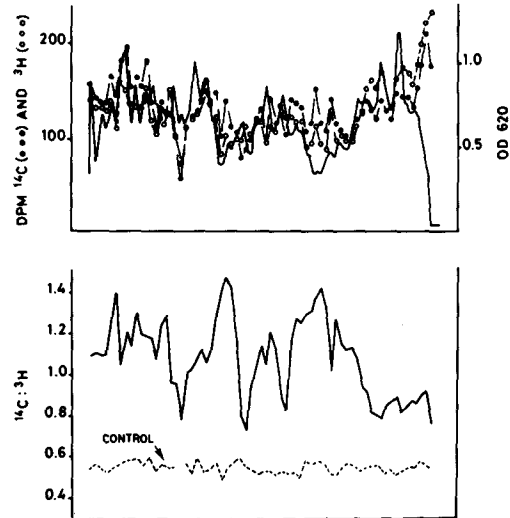


FIGURE 7 Relative turnover of pancreas SM membrane proteins. Experimental conditions as in Fig. 6.

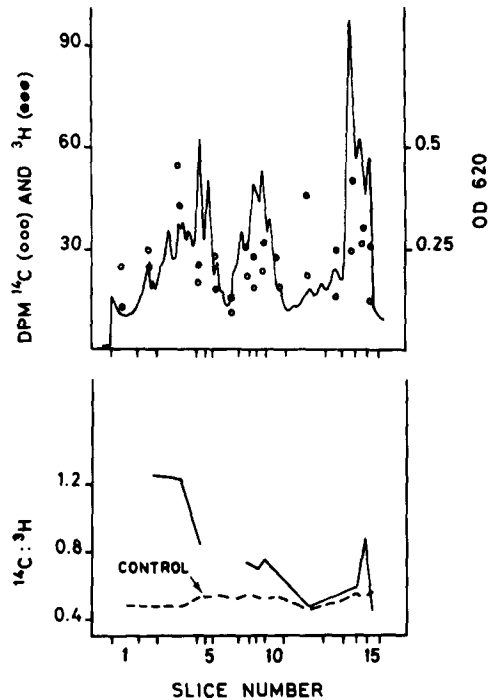


FIGURE 8 Relative turnover of pancreas ZG membrane proteins. Experimental conditions as in Fig. 6, with the following exceptions: (a) the load was $\sim 70 \mu\text{g}$; (b) the gel was 12 cm long and 0.5 cm in diameter and was cut by following the distribution of stained bands into 15 unequal slices as indicated on the abscissa; (c) only one gel was run; samples were counted three times for 100 min, and the data given are the averages of the results obtained, which were consistent within 8%.

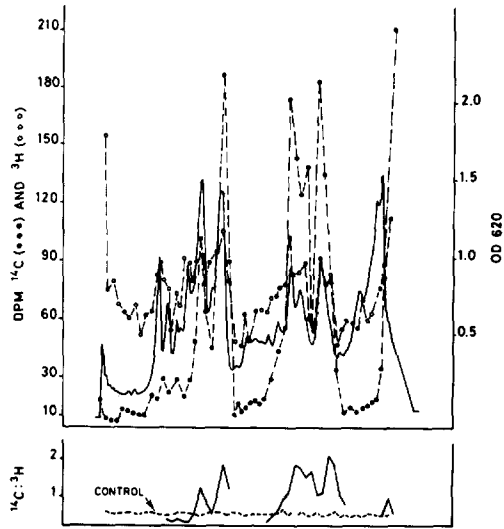


FIGURE 9 Relative turnover of pancreas RM-soluble proteins. Experimental conditions as in Fig. 6.

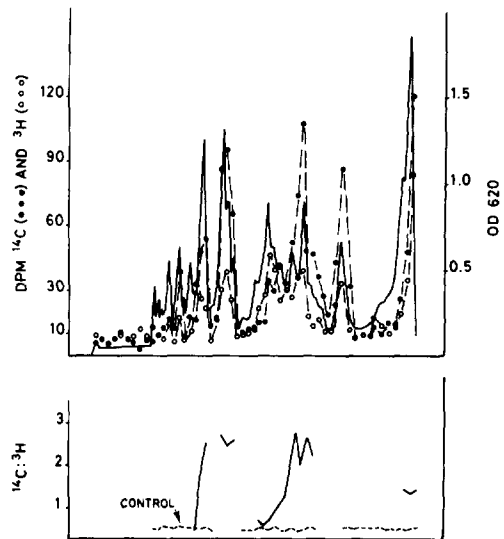


FIGURE 10 Relative turnover of pancreas SM-soluble proteins. Experimental conditions as in Fig. 6.

imum of ~ 4.5 days for high molecular weight and a maximum of ~ 28 days for small proteins. The corresponding values for SM membranes are ~ 3.5 , ~ 2.75 , and ~ 5 days; for ZG membranes, ~ 4.5 , ~ 3 , and ~ 13 days.

The half-life of secretory proteins cannot be estimated from our data because of the long time intervals between the two isotope injections. Thus, at the time the animals were killed virtually all the ^3H -labeled secretory proteins had already been

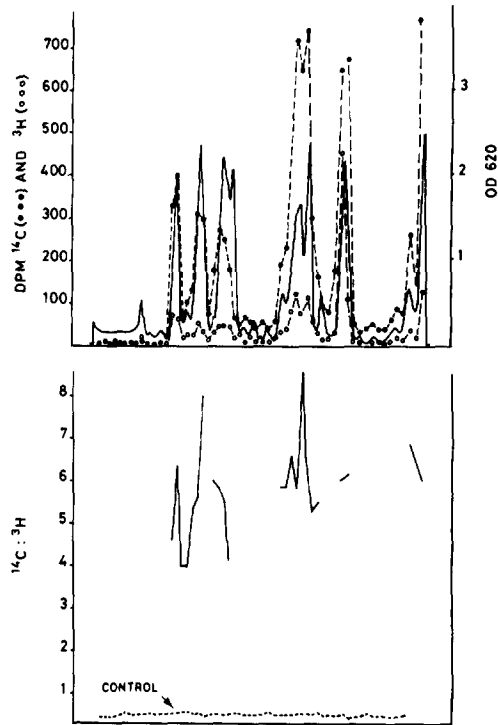


FIGURE 11 Relative turnover of pancreas ZG-soluble proteins. Experimental conditions as in Fig. 6, except that the load was 1 mg of protein.

released from the cells, as shown by the analogous double-isotope ratios found in ZG and ZG-soluble fractions with both 4 and 6 day intervals between injections (Tables II and III) and by the distribution of ^3H counts in the ZG-soluble gel. However, all our data indicate that the turnover of secretory proteins is very fast. In the rat pancreas the half-life of five purified secretory hydrolases was found to vary between ~ 10 and ~ 20 h, depending on the diet (30).

DISCUSSION

In this work the synthesis and turnover of the cytoplasmic membranes of the guinea pig pancreas acinar cells were investigated by two different types of experiment: (a) *in vitro* pulse-chase experiments, which were carried out to determine the rate of synthesis and the possible biogenetic relationships of the membranes under study, and (b) *in vivo* double-label studies, which were devised to investigate the average turnover of the protein moiety of each of these membranes, as well as the turnover of the various membrane polypeptides. Both these

approaches suffer from technical limitations. Thus, due to the large difference in labeling of secretory and membrane proteins, a large proportion of the radioactivity recovered in the in vitro-labeled membrane fractions was accounted for by small amounts of contaminants. Furthermore, due to the extreme complexity of the membrane protein composition (17), the separation method that we used was inadequate to yield detailed information on individual membrane proteins, and the results therefore refer only to groups of polypeptides separated according to their molecular weight. However, even with these limitations, our results provide clear evidence indicating that in pancreatic acinar cells, a cell type highly specialized toward protein secretion, replacement of cytoplasmic membranes takes place by mechanisms analogous with those already identified in cells of different origin and function (hepatocytes (25-29, 31, 32), neurons (33), various cultured cell lines (34), etc.; for a review see reference 35). As in these other cell types, pancreatic membranes appear to be long-lived structures, whose molecular components are, however, involved in the dynamic turnover of the cell.

The evidence that we obtained is incompatible with the idea that the membranes participating in the intracellular transport are synthesized concomitantly and transported *pari passu* together with secretion products to be destroyed at the end of each secretory cycle (11-13). Thus, all membrane proteins were very little labeled in comparison with secretory proteins after a short pulse in vitro with radioactive leucine, and their specific activity did not change appreciably during chase incubation. In agreement with these data the in vivo studies revealed that the average turnover of RM, GC, and ZG membranes is very slow relative to that of the secretion products. Furthermore, the turnover of the different proteins of the membranes under study is greatly heterogeneous, i.e., individual membrane proteins appear to be replaced at their own rate, independently from one another. This finding is incompatible with the idea that transfer of pieces of complete membranes is a major mechanism of membrane replacement, since this would imply synchronous loss and replacement of all membrane components.

While our observations are compatible with the idea that the different cytoplasmic membranes participating in the intracellular transport of secretory proteins could be synthesized independently from one another, they do not exclude the existence

of any kind of biogenetic relationships among them. Even if the site of synthesis of membrane proteins (whether the bound or the free ribosomes or both) is still debated (36, 37), the possibility that proteins could be originally integrated into a membrane and later transported to a membrane of different type has been repeatedly envisaged (38-40). The route(s) of this transport are completely unknown. However, we cannot exclude that, at least in some cases, such a transport would involve the physical transfer of membrane patches. This type of membrane flow would be perfectly compatible with our results provided that (a) it is much slower than the flow of the secretion products and (b) it is coupled with modifications of the transported membrane in the new location: changes in membrane molecules (for instance, glycosylation of glycoproteins), removal of old and insertion of new membrane components. So far, the existence of this type of flow is only hypothetical. Much better methods for the resolution of membrane components than those used in this work and a characterization of the proteins of the different membranes are needed for solving these problems. In any event, it should be emphasized that this type of flow would be only indirectly related to the intracellular transport of secretory proteins. Rather, it would be a part of the basic phenomena of membrane biogenesis and turnover.

Since the intracellular transport of secretory proteins and the biogenesis of the membranes involved in that process appear regulated according to greatly different time scales,⁶ it follows that pancreatic acinar cells necessarily reutilize extensively their intracellular membranes or components thereof.

As discussed in detail by Jamieson and Palade (14), sites of membrane circulation or reutilization should be located between the RER and the GC and between this region and the plasmalemma.

⁶ The faster turnover found in SM membrane proteins relative to RM and ZG membranes does not seem to depend on contamination by secretory proteins (compare Fig. 7 and Fig. 10). It could rather depend on the heterogeneity of the fraction, which is primarily accounted for by Golgi elements but also includes minor components such as pinocytic vesicles, apical vacuoles, and small fragments of PM (2, 18) which could have high turnover. However, in agreement with previous observations in other cell types (12, 41), it could also be that in the acinar cells of the pancreas GC membranes are replaced faster than RM and ZG membranes.

Here the continuity of the secretory pathway could be established by the specific interaction (fusion-fission) of the participating membranes. Whether or not such reutilization requires the disassembly of the membranes to the corresponding macromolecules, followed by their specific reassembly at a different cell site, is still unknown, even though morphological evidence favoring the preservation of membrane structure during the cycle has been reported (14, 42).

On the other hand, as indicated by our previous studies, intracellular transport must be carried out by mechanisms which do not lead to membrane intermixing, since the membranes involved have distinct differences in their molecular and enzymic composition (15-17). Hence, their interactions must be nonrandom, in the sense that the identity of fusing-fissioning membranes is preserved during the process. This conclusion implies that subtle mechanisms, which are still completely unknown, can regulate not only the specific fusion of different membranes, but also the specific retrieval of previously incorporated membrane patches from membranes of different nature.

Finally, the question might be asked as to whether in other cell systems secretion can be carried out by cellular mechanisms analogous to those identified in pancreatic acinar cells. This is likely to be the case in liver cells, since marked heterogeneity in the turnover of microsomal and plasma membrane proteins has long been established (25-29, 31, 32). Moreover, the average half-lives of these membranes are approximately two orders of magnitude longer than the time needed for secretion products (such as albumin and lipoproteins) to be cleared from the cells (6, 25, 28, 29, 31, 32). However, in the liver the turnover of the Golgi and secretory granule membranes has not been estimated yet.

In other cell systems, such as the chromaffin adrenal cells (7, 43-47), acinar cells of the parotid gland (42, 48), cells of the adenohypophysis (46), adrenergic nerve (49) and neurohypophysis terminals (46), cholinergic neuromuscular junctions (50, 51), attention has been given to the sequence of events occurring after incorporation of the membrane of secretory granules into the plasmalemma. While most of the authors agree that excess membrane is removed from the plasmalemma by endocytosis (11, 42-51), there have been various conjectures as to the final fate of endocytosed membrane: based on morphological, cytochemical, and biochemical evidence, both extensive reutilization

(7, 46, 50, 51) and prevailing or even complete degradation (11, 43, 44, 48, 49) have been suggested.

We feel that the evidence suggesting degradation should be reconsidered for the following reasons: (a) since secretory proteins have a great tendency to adsorb onto membranes (19, 52) it is difficult to obtain an uncontaminated membrane fraction. Due to the high turnover and the large excess of protein of the granule content relative to the membrane, an unsuspected contamination, even of moderate degree, could greatly affect the results. (b) In the case of catecholamine-storing granules, calculations on membrane turnover are based primarily on the determination of the activity of dopamine- β -hydroxylase, an enzyme which is partially bound to adrenergic vesicle membrane and partially localized in the granule content (43, 49). (c) In some cases, the fate of granule membranes was looked at in unphysiologically stimulated secretory cells, and the possibility that exhaustive stimulation could impair the mechanisms of membrane reutilization was not investigated (43, 44).

It would be highly desirable to study other cell systems in a carefully controlled manner, in order to establish the generality of our findings on pancreatic acinar cells.

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