

CONCOMITANT SYNTHESIS OF MEMBRANE PROTEIN AND EXPORTABLE PROTEIN OF THE SECRETORY GRANULE IN RAT PAROTID GLAND

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ABSTRACT

After enzyme secretion the membrane of the secretory granule, which had been fused to the cell membrane, was resorbed into the cell. Experiments were therefore carried out to test whether formation of new secretory granules involves reutilization of the resorbed membrane or synthesis of a new membrane, *de novo*, from amino acids. Incorporation of amino acids-¹⁴C into proteins of various cell fractions was measured *in vivo*, 30, 120, and 300 min after labeling. At all times the specific radioactivity of the secretory granule membrane was about equal to that of the granule's exportable content. At 120 and 300 min the specific radioactivity of the granule membrane and of the granule content was much higher than that of any other subcellular fraction. It is therefore concluded that the protein of the membrane is synthesized *de novo* concomitantly with the exportable protein. The proteins of the granule membrane could be distinguished from those of the granule content by gel electrophoresis. All major bands were labeled proportionately to their staining intensity. The amino acid composition of the secretory granule membrane was markedly different from that of the granule's content and also from that of the mitochondrial membrane. The granule membrane showed a high proline content, 30 moles/100 moles amino acids. The analyses show that the radioactivity of the granule membrane is indeed inherent in its proteins and is not due to contamination by other fractions. The possibility is considered that the exportable protein leaves the endoplasmic reticulum already enveloped by the newly synthesized membrane.

INTRODUCTION

Quantitative studies recently established that enzyme secretion in the parotid gland occurs by fusion of the secretory granule membrane with the cell membrane at the acinar lumen (1). An opening is formed at the point of fusion of the two membranes through which the content of the granule flows directly into the lumen. Excess membrane which accumulates at the lumen through the fusion process appears to be subsequently resorbed in the form of small vesicles. These disappear later on,

concomitantly with the formation of new secretory granules. The observations noted above pose the problem of the fate of the granule membrane after secretion. The membrane might be reutilized in the formation of new secretory granules, or alternatively, it might be completely degraded so that the formation of new granules would require *de novo* synthesis of a new membrane. The question of membrane circulation in different cell systems (3, 4) and, in particular, in the exocrine pancreas

(5, 6) and in the adrenal medulla (7, 8) was raised earlier by a number of investigators. However, for lack of experimental evidence, no definite conclusions could be drawn. It should be pointed out that the various protein and lipid components of the membrane need not all have the same fate. Therefore, it was decided to study first the question whether the protein of the secretory granule membrane is reutilized or synthesized *de novo* when a new granule is formed. If the membrane protein is reutilized it will show a negligible incorporation of labeled amino acids relative to the newly synthesized secretory protein which is enveloped by the membrane. On the other hand, if the membrane protein must be synthesized each time a granule is formed it will show a specific radioactivity equal to that of the content which is destined for secretion. Obviously a partial reutilization of the preexisting membranes will result in an intermediate specific radioactivity. The outline of the work was therefore: brief labeling *in vivo* with radioactive amino acids, isolation of a purified secretory granule fraction, and comparison of the specific radioactivity of the protein of the membrane with that of the exportable content.

There are no known specific markers of the secretory granule membrane. Furthermore, the membrane protein comprises only a very small fraction, about 5%, of the total granule protein (9, 10). Because of this low relative amount, various precautions had to be adopted to ensure minimal contamination of the isolated membranes by granule content and by other cell fractions such as mitochondria and microsomes which are rich in membranes. It also seemed pertinent to compare the extent of incorporation into membrane protein of the secretory granule with the incorporation into membrane protein of a different subcellular structure which is not directly involved in the secretion process. The mitochondrial membranes were chosen for this purpose.

The findings presently reported lead to the conclusion that the membrane protein of the secretory granule is synthesized *de novo* concomitantly with the exportable protein.

METHODS

Labeling of Experimental Animals

Albino rats weighing 260 ± 20 g fed *ad libitum* were used. Light was kept "on" from 6 a.m. to 6 p.m. Under such conditions the eating and secretion

cycle of the animals are standardized (11). Three animals for each time point were each injected intravenously under light ether anaesthesia with 0.6 ml of saline containing 12 μ Ci of a mixture of uniformly labeled L-amino acids- 14 C at pH 7.4 (1.5 mCi/mg). 15 min later the animals received an intravenous injection of a concentrated mixture of unlabeled amino acids (6 mg per animal). At 30, 120, and 300 min after injection of labeled amino acids the animals were sacrificed under ether anaesthesia by heart incision. The parotid glands were immediately removed and placed in cold homogenizing medium. In order to increase the amount of tissue for cell fractionation, glands were also removed from five additional, nonlabeled animals which were kept under the same conditions.

To obtain a higher specific radioactivity of the proteins for analysis by gel electrophoresis, the above procedure was modified as follows. Four animals were each injected with 30 μ Ci of the amino acid mixture shown above, followed, 15 min later, by 8 mg of unlabeled amino acids. The glands taken from the labeled animals 2 hr after injection of the label were admixed with an equivalent amount of glands from a similar group of nonlabeled animals. All subsequent procedures were carried out at 0°-5°C.

Cell Fractionation

The glands were washed twice in the homogenizing medium containing 0.3 M sucrose adjusted to pH 7.5 with Na_2CO_3 to which 0.2 μ g/ml N,N'-diphenyl *p*-phenylenediamine (DPPD) was added to prevent lipid peroxidation (12). The tissue was cut into small pieces with scissors, homogenized in the sucrose medium, and fractionated as shown in Fig. 1.

The sediment of the secretory granules consisted of a tightly packed white pellet with a tan layer of mitochondria on top. The mitochondria were removed by swirling the tube with small volumes of added sucrose medium and were combined with the 1200 g supernatant. The secretory granule pellet was resuspended, resedimented, and the trace of remaining mitochondria was removed, as described above, and discarded. The sediment of mitochondria contained at the bottom of the pellet a small white layer of tightly packed secretory granules. The mitochondria were collected by swirling and decantation three times, adding a small amount of medium, and taking care to leave the secretory granule layer at the bottom undisturbed. The latter was discarded. The mitochondrial suspension was sedimented twice more, repeating the above procedure for removal of residual secretory granules. The sediment of microsomes was washed once. All fractions were finally suspended in 4 ml of sucrose medium.

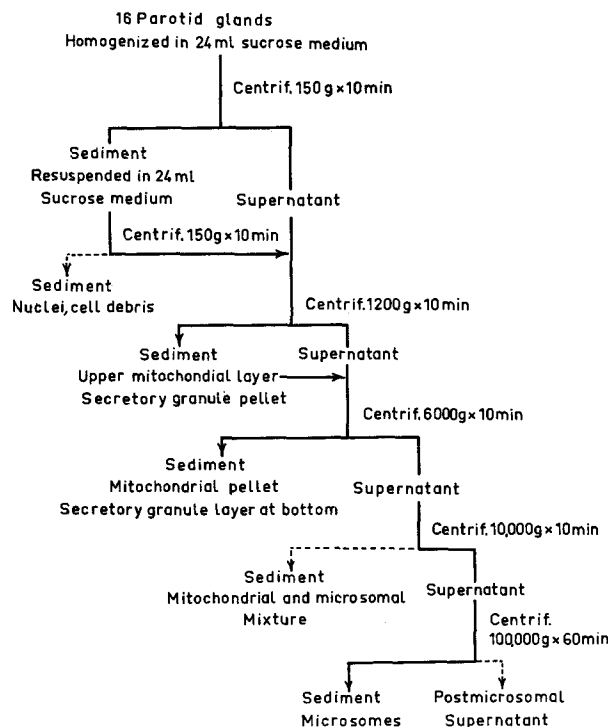


FIGURE 1 Scheme describing the fractionation procedure for rat parotid gland.

Isolation of Membranes

In order to obtain highly purified membrane preparations and to preserve their integrity as much as possible, samples were dialyzed overnight against a hypotonic medium at alkaline pH. Of each subcellular fraction, 2.7 ml were dialyzed for 12 hr against 300 volumes of 10 mM Tris buffer pH 8.5, which contained also 0.2 $\mu\text{g}/\text{ml}$ DPPD and 0.05 mM ethylenediaminetetraacetate (EDTA). Subsequently the inside of the dialysis bag was washed twice with 2 ml of freshly prepared buffer, as above, which was combined with the dialysate. Protein recovery after dialysis was 95–100%. Washing at very low ionic strength apparently facilitates further purification of the membrane material (13, 14). Therefore, after the first sedimentation the membrane pellet was washed twice in a medium containing 1 mM Tris pH 8.5, 0.05 mM EDTA, and 0.2 $\mu\text{g}/\text{ml}$ DPPD. Centrifugation conditions were as follows: secretory granule ghosts, $10^4 g \times 20$ min; mitochondrial membranes, $3 \times 10^4 g \times 30$ min; and microsomal membranes, $10^5 g \times 60$ min. The membrane material was gently resuspended each time with the aid of a polythene rod and finally suspended in 0.5–1.0 ml of H_2O . The supernatants of all the above fractions were optically empty as checked by phase-contrast microscopy. A parallel experiment in which all the membrane

preparations were centrifuged at $10^5 g \times 60$ min gave the same yield of membrane protein, indicating that the membranes were quantitatively sedimented also when a lower centrifugal field was applied as above. In order to obtain a more concentrated protein solution for gel electrophoresis the following changes were introduced in the procedure for isolation of secretory granule membrane and content. The pellet of granules was resuspended in only 1 ml of sucrose medium, and 0.9 ml of the suspension was dialyzed against 1 liter of Tris buffer. The dialysis bag was washed only once with 0.5 ml of the buffer. The final centrifugation was carried out at $30,000 g \times 30$ min. No inactivation of the exportable proteins amylase and DNase occurred during dialysis.

Chemical and Enzymatic Assays

Amylase and DNase served as markers of the secretory granule content (11, 15). Succinic dehydrogenase was chosen as a marker for the mitochondria (16). 5'-Nucleotidase was used as a marker for smooth microsomes since it was recently shown that the smooth microsomal fraction of the parotid gland is 15-fold enriched in this enzyme as compared to the rough microsomal fraction (17, see also reference 18). RNA (19) served as a marker for rough

microsomes. Protein was determined according to Lowry et al. (20).

Unless otherwise specified, the enzymatic analyses were performed on the total fractions rather than on the membranes after dialysis in order to avoid errors due to enzyme inactivation when calculating cross contamination. Amylase was determined by the procedure of Bernfeld (21), enzyme units and specific activity being defined as outlined in a previous report (22). The presently reported specific activities are somewhat lower than described previously (22) because of the different source of the soluble starch substrate. Deoxyribonuclease was determined with the diphenylamine reagent (23), the assay conditions and units of activity being as previously described (15).

Succinic dehydrogenase was measured by following indophenol reduction (16). A unit of activity was defined as the amount of enzyme that catalyzes a decrease of 0.001 OD/min at 600 nm at 38°C. 5'-Nucleotidase was assayed as described by Widnell and Unkeless (24). RNA was measured, after hydrolysis in hot trichloroacetic acid (25), by its pentose content according to Mejbaum (26). 1 μ mole equivalent of pentose was calculated to be equal to 0.7 mg RNA (27).

Gel Electrophoresis

Proteins of secretory granule membranes and of the granule content were run by electrophoresis on acrylamide gels by using a modification of the method of Takayama (28, 29). Samples of 100–300 μ g protein were placed in a mixture of phenol–acetic acid–water (4:2:1, w/v/v) containing 1% of the detergent Nonidet P40 and 0.5% β -mercaptoethanol (29). Acrylamide gels were prepared as described by Eytan and Ohad (29), except that ethylene diacrylate was replaced by 0.2% bisacrylamide.

Electrophoresis of samples was carried out without prior extraction of lipid since the lipid is not stained by amido black as used in this work. Nor did the lipid show any significant amount of radioactivity. Samples were first run at 0.5 ma per tube for 30 min and then at 3.0 ma per tube for 4.25 hr. Densitometer tracings of the stained gels were made with a Gilford-Beckman spectrophotometer Model 2000 equipped with a linear transport scanner and using a 0.05 mm slit at 600 nm. The radioactivity of the protein bands was measured in a toluene–Triton X-100 scintillation mixture after the zones of the gels corresponding to the main stained bands were cut and dissolved overnight in 30% H_2O_2 at 37°C.

Amino Acid Analysis

Samples were hydrolyzed in 6 N HCl in a sealed ampoule under vacuum at 110°C for 22 hr. Amino

acids of the hydrolysate were analyzed in a Beckman Model 120 B.

Radioactivity Measurements

A mixture of uniformly labeled amino acids- ^{14}C rather than a single labeled amino acid was used to compare incorporation into the different proteins. Protein in soluble fractions was precipitated by cold trichloroacetic acid (TCA) at a final concentration of 5%. TCA precipitates and the dialyzed washed membrane preparations described above were finally dissolved in 1% sodium dodecyl sulfate. Samples were counted in a toluene–Triton X-100 scintillation mixture. Counting rates were corrected for quenching which was in the range of 10%. Previous and present tests showed that additional extractions by organic solvents and hot TCA did not remove significant amounts of radioactivity from the precipitated fractions (30). Therefore, only negligible amounts of radioactivity could be present in the lipid components of the fractions.

Table I demonstrates that the total amount of radioactivity found in the gland after injection of the radioactive amino acids into the animal did not change drastically within the time period of 30–300 min. It is also shown that within the first 30 min after injection most of the label was already incorporated into TCA-precipitable protein. It is therefore not very likely that, after 30 min, marked changes in the specific radioactivities of the different fractions can be due to the further synthesis of labeled protein.

Calculations of the amount of cross contamination in the isolated cell fractions and computation of the true specific radioactivity of cell components are given in an appendix to this paper. In no case did the com-

TABLE I
Labeling of the Parotid Gland as a Function of Time after Injection of Amino Acids- ^{14}C

	Time after injection of amino acids- ^{14}C		
	30 min	120 min	300 min
Total cpm in homogenate	170,000	215,000	205,000
% of total cpm precipitable in TCA	73	81	92
% of total cpm soluble in TCA	26	17	10

15 min after injection of the amino acids- ^{14}C a chase of unlabeled amino acids was given as described in the section on labeling of experimental animals. The total radioactivity in the homogenate is defined as 100%.

puted values vary from the experimental values by more than 35%.

Electron Microscopy

All fractions were prepared as described in the section on cell fractionation. Suspension of the membrane preparations before the final centrifugation was done in phosphate buffer, pH 8.5, instead of Tris buffer. To obtain tightly packed pellets of the secretory granules, mitochondria and the membrane fractions therefrom, the centrifugation time was increased three- to fivefold over that specified in the section on cell fractionation.

The pellets of membrane fractions were fixed at 4°C in 4% glutaraldehyde adjusted to pH 7.0 by NaOH and containing also 10 mM phosphate buffer, pH 7.4. After 4 hr the pellets were washed free of fixative with cold 10 mM phosphate buffer, pH 7.4, and postfixed at 4°C for 8 hr in 2% OsO₄ in 30 mM phosphate buffer, pH 7.4. The same procedure was used also for fixation of intact secretory granules, mitochondria, and microsomes, but all media contained also 0.3 M sucrose. All pellets were subsequently dehydrated by transfer through increasing concentrations of ethanol and were embedded in Epon (31). Sections were cut through the entire depth of the pellet to permit scanning from top to bottom. An LKB Ultram III microtome was used. Sections were stained with uranyl acetate and lead citrate (32). Electron micrographs were obtained with the Philips EM 300 operated at 60 kv.

Materials

L-amino acids were purchased from Mann Research Labs Inc., New York. Amino acids-¹⁴C

(uniformly labeled) mixture was obtained from New England Nuclear Corp., Boston, Mass. (purified L-amino acids in the same relative proportions as found in a typical algal protein hydrolysate, approximately 40 mCi/m atom carbon). Soluble starch for α -amylase assay was the product of Mallinckrodt Chemical Works, St. Louis, Mo.

RESULTS

Purity of Subcellular Fractions of Secretory Granules, Mitochondria, and Microsomes

The protein yield, RNA content, and the specific activities of marker enzymes of the various fractions are shown in Table II. The data represent the average for three independent experiments at 30, 120, and 300 min after injection of labeled amino acids. As judged from the specific activity of succinic dehydrogenase in the secretory granule fraction as compared to the mitochondrial fraction, the former fraction was contaminated by about 2% mitochondrial protein. The isolated membranes of the secretory granule fraction had a very low 5'-nucleotidase activity (0.25 units/mg protein) as compared with the microsomal fraction. Because of this low activity of 5'-nucleotidase and the negligible RNA content, the secretory granule fraction was apparently almost free of smooth and rough microsomes. The mitochondrial fraction contained about 10% secretory protein, as calculated on the basis of the specific activity of amylase in the secretory granule fraction. If one assumes that all the 5'-nucleotidase and RNA in

TABLE II
Specific Activities of Marker Enzymes and Chemical Analyses of Subcellular Fractions of Parotid Gland

Fraction	Protein mg	Specific activities units/mg protein				RNA/protein weight ratio
		α -amylase	DNase	Succinic dehydrogenase	5'-Nucleotidase	
Homogenate	290.0 \pm 40	210 \pm 10	22 \pm 2	70 \pm 7	0.49 \pm 0.05	0.056 \pm 0.004
Secretory granules	22.0 \pm 3	450 \pm 30	71 \pm 6	23 \pm 8	\leq 0.04	\leq 0.020
Mitochondria	5.5 \pm 1	40 \pm 4	—	1100 \pm 100	0.39 \pm 0.06	0.057 \pm 0.01
Microsomes	30.0 \pm 1	5 \pm 1	—	22 \pm 7	2.00 \pm 0.1	0.25 \pm 0.02

Protein represents the yield of homogenate and purified fractions from 16 glands. Findings are presented as the mean of the three experiments in which animals were sacrificed 30, 120, and 300 min after injection of labeled amino acids. The range of the deviation from the mean for the different experiments is also shown.

the mitochondrial fraction represent smooth (17) and rough microsomes, contamination by these structures would amount to about 20% of the mitochondrial fraction on the basis of protein. It is also shown in Table II that the microsomal fraction was essentially free of mitochondria. The small amount of amylase in the microsomes is probably intrinsic to this fraction.

The relative amounts of soluble protein and insoluble membrane protein in the isolated subcellular structures are given in Table III. It should be noted that the purified membrane of the secretory granule represented about 4% or 1/25 of the total granule protein. The same percentage of membrane protein had been obtained previously when the secretory granules were lysed by dilution in hypotonic buffer (10). The membrane was contaminated by 0.1% of the original soluble content of the granule as measured by both amylase and DNase. It can therefore be calculated that only 2.5% of the protein of the secretory granule membrane fraction represent contamination by residual content. This calculation is based, of course, on the assumption that all other secretory proteins were removed to the same extent as the amylase and DNase which were measured.

Additional washing of the secretory granule membranes decreased the residual amylase and DNase to below detection. However, such a procedure was not routinely adopted in order to minimize the damage to the membranes. It should be further emphasized that the ratio of membrane protein to total protein was about 10 times higher in the mitochondria than in the secretory granules. Therefore, the contamination of 2% mitochondrial protein in the intact secretory granule fraction might increase to a relative value of 20% in the isolated membrane fraction of the granules. When the microsomal fraction was examined, the recovery of protein was rather low. This is probably due to gradual removal of soluble contents and detachment of ribosomes from the membrane during washing in the hypotonic EDTA medium after dialysis (compare Figs. 6 and 7).

Characterization of the Subcellular Fractions by Electron Microscopy

Representative micrographs of secretory granules, mitochondria, and microsomes as well as purified membranes from these fractions are shown in Figs. 2-7. The fraction of secretory granules con-

TABLE III
Fractionation of Secretory Granules, Mitochondria, and Microsomes into Soluble and Membrane Components

Fraction	% of total of each subcellular fraction		
	Protein	α -amylase	Deoxyribonuclease
Secretory granules			
Membranes	3.7 \pm 0.6	0.09 \pm 0.02	0.11 \pm 0.04
Soluble component	91 \pm 6	95 \pm 8	90 \pm 10
Mitochondria			
Membranes	44 \pm 7	—	—
Soluble component	44 \pm 3	—	—
Microsomes			
Membranes	45 \pm 3	—	—
Soluble component	21 \pm 6	—	—

Findings are represented as the mean of the three experiments in which animals were sacrificed 30, 120, and 300 min after injection of the labeled amino acids. Fractionation was carried out by dialysis against hypotonic buffer, followed by centrifugation and washing of the precipitated membranes. The procedure is described in detail under Methods. The amount of protein and enzyme activity of each fraction placed in the dialysis bag is defined as 100%. The relative amount of soluble fraction refers to the supernatant of the first centrifugation, while the relative amount of membranes refers to the washed sediment. The deviation from the mean in the three experiments reported in Table II is shown.

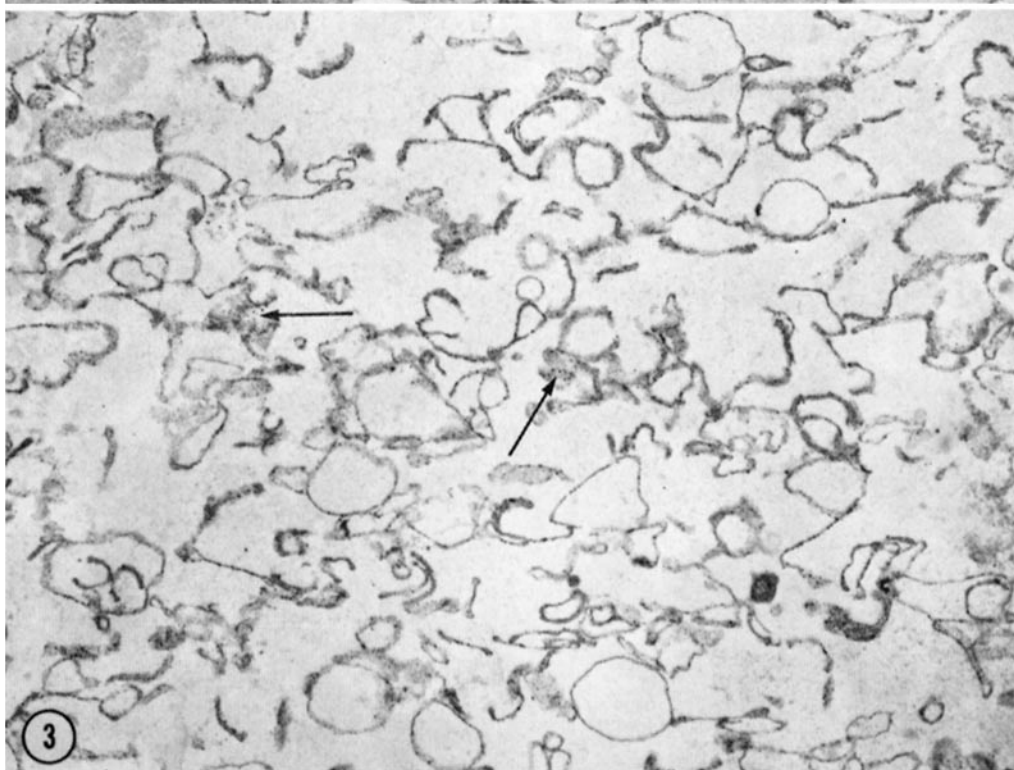
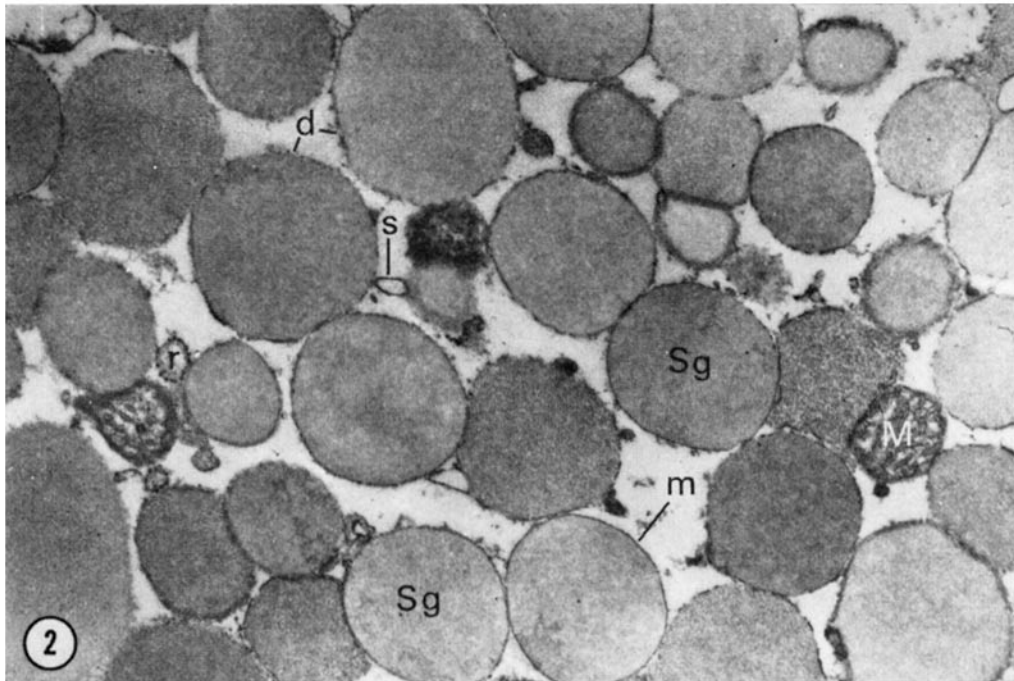


FIGURE 2 A section through the pellet of the secretory granule fraction. Secretory granules (*Sg*) constitute the main component. They are surrounded by a continuous unit membrane (*m*) which in some places seems to be disrupted (*d*). The fraction is only slightly contaminated by mitochondria (*M*) and smooth (*s*) and rough (*r*) microsomes. $\times 19,000$.

FIGURE 3 A section through the pellet of the purified membranes obtained from the secretory granule fraction. Only vesicles and fragments of smooth membranes are present. The fine granulation (arrow) is probably due to membrane-bound osmium precipitates. It is also seen in the mitochondrial membranes shown in Fig. 5. $\times 20,000$.

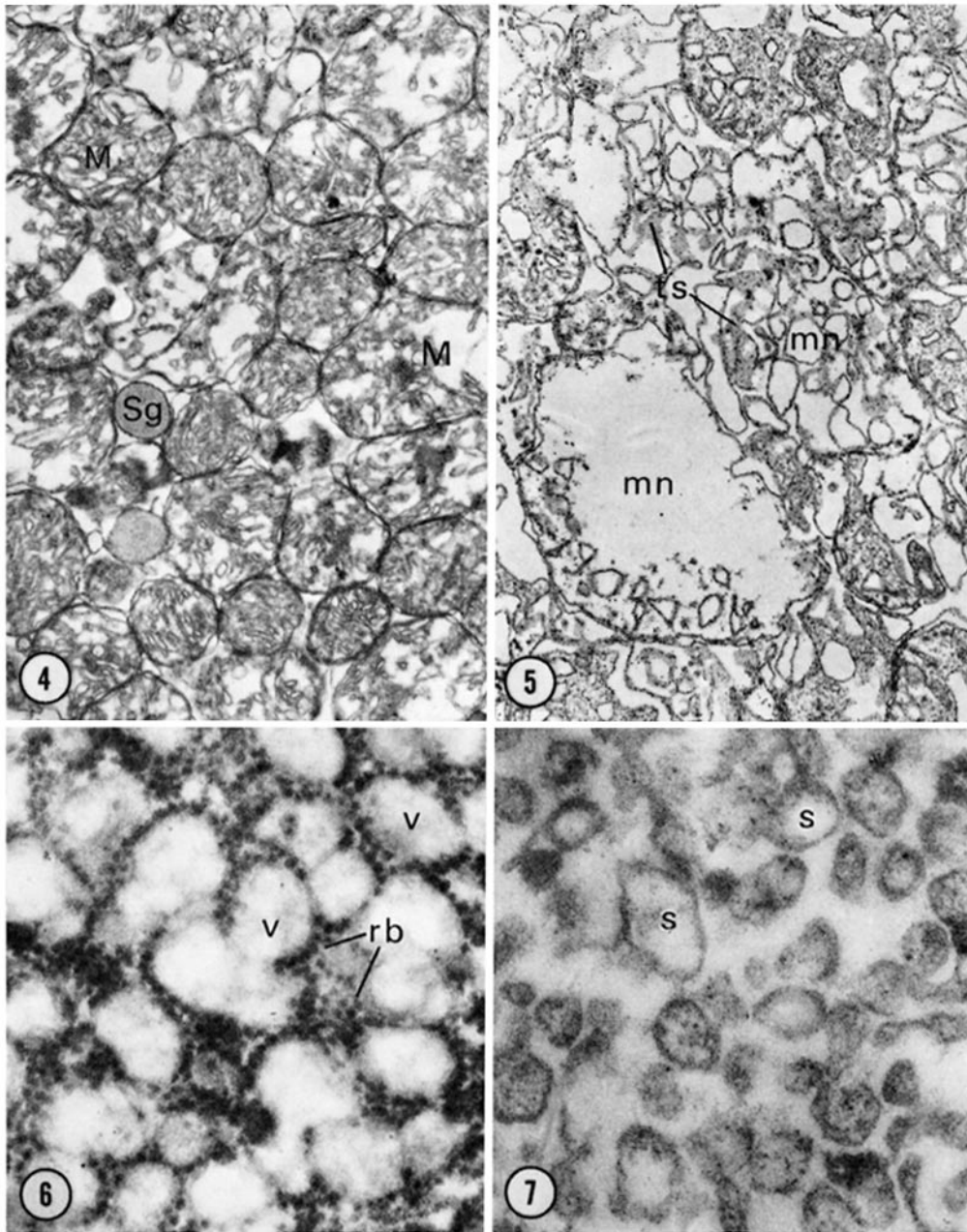


FIGURE 4 A section through the pellet of the mitochondrial fraction. It is slightly contaminated with small secretory granules (*Sg*) (compare with Fig. 2). *M*, mitochondria. $\times 19,000$.

FIGURE 5 A section through the pellet of the purified membranes obtained from the mitochondrial fraction. Swollen and disrupted mitochondria devoid of matrix still can be identified (*mn*); *ts*, tangential section of membranes. A fine granulation is noted on part of the membranes (see also Fig. 3). $\times 19,000$.

FIGURE 6 A section through the microsomal pellet. Microsomal vesicles (*v*) are lined with tightly packed ribosomes (*rb*) which mask the underlying membrane. $\times 61,000$.

FIGURE 7 A section through the pellet of purified membranes obtained from the microsomal fraction. Notice the complete absence of the ribosomes. The pellet is constituted solely of smooth-surfaced vesicles (*s*). The ribosomes had apparently been removed during the dialysis and subsequent washing in the medium containing EDTA. $\times 61,000$.

tains mainly intact, dense granules enveloped by a distinct single membrane (Fig. 2). The main foreign structures in this fraction are the mitochondria. Few smooth and rough microsomes are present. The secretory granule membrane preparation consists solely of smooth membranes in the form of fairly large, empty vesicles and membrane fragments (Fig. 3). Intact, unextracted, or partially extracted granules were not detected when all layers throughout the pellet were examined. The mitochondrial preparation was contaminated by a few microsomes and by secretory granules, the latter being mainly located in the lower region of the pellet. The mitochondrial membrane preparation consists of large membrane structures which enclose many internal vesicles (Figs. 4, 5). The microsomes show a large number of attached ribosomes which were completely lost after dialysis and subsequent washing in 1 mM Tris buffer, pH 8.5, containing also EDTA (compare Figs. 6 and 7).

Amino Acid Analysis

The relative amino acid composition of the secretory granule membrane, of the granule con-

TABLE IV
Amino Acid Composition of Secretory Granule Membranes and Soluble Content and of Mitochondrial Membranes

Amino acid	Secretory granule		Mitochondrial membranes
	Membrane	Content	
	<i>moles per 100 moles amino acid</i>		
Alanine	2.9	5.0	8.8
Arginine	5.9	3.7	5.1
Aspartic acid	4.4	12.2	8.5
Glutamic acid	17.5	10.4	11.3
Glycine	17.2	9.9	9.7
Histidine	1.3	1.9	2.3
Isoleucine	2.1	4.0	5.1
Leucine	3.7	9.1	9.3
Lysine	3.2	3.6	7.0
Methionine	0.7	1.0	0.0
Phenylalanine	1.9	3.5	4.5
Proline	29.8	11.4	4.8
Serine	2.9	8.0	6.1
Threonine	2.1	4.3	5.6
Tyrosine	1.4	3.0	3.6
Valine	2.8	6.5	7.0

Aspartic acid includes asparagine, and glutamic acid includes glutamine. Tryptophane, cysteine, and cystine were not determined.

TABLE V
Specific Radioactivities of Membranes and Soluble Content of Subcellular Structures in Rat Parotid Gland

Fraction	Time after injection of amino acids- ¹⁴ C		
	30 min	120 min	300 min
	<i>specific radioactivities cpm/mg protein</i>		
Secretory granules			
Membranes	390	1300	900
Soluble content	470	1300	1050
Mitochondria			
Membranes	230	240	180
Soluble content	600	450	310
Microsomes			
Membranes	370	300	210
Soluble content	1200	460	280

The fractions are defined as outlined in Table III.

tent, and of the mitochondrial membranes was analyzed. The results show significant differences in the composition of the different fractions (Table IV). The proline content of the secretory granule membrane amounts to 30% (relative mole per 100 moles) as compared with only 4.8% in the mitochondrial membranes and 11.5% in the secretory granule content.

Radioactivity of the Subcellular Components

The specific radioactivities of the membrane protein and the soluble protein content of the different subcellular structures are presented in Table V and Fig. 8. The most striking finding was that the specific radioactivity of the secretory granule membrane was about equal to that of the exportable content of the granule at all the time periods tested. At 30 min after injection of labeled amino acids the highest protein specific radioactivity is found in the soluble content of the microsomal fraction. The microsomal membrane showed a relatively low specific radioactivity. The soluble content of the mitochondria showed a rather high specific radioactivity which might be due to highly labeled exportable protein located in microsomes, small secretory granules, or condensing vacuoles which contaminate the mitochondrial fraction.

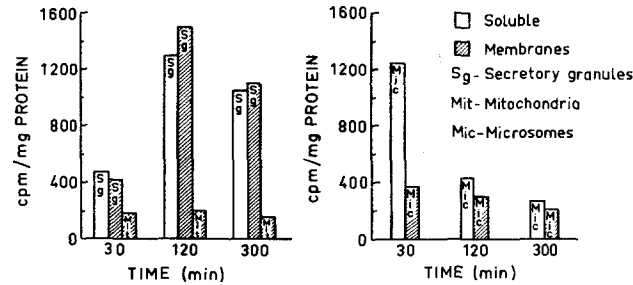


FIGURE 8 Specific radioactivities of subcellular fractions corrected for contamination by components from other cell fractions.

Earlier findings that strongly support this assumption have been reported (30, 33). The fact that the specific radioactivity of the soluble content of the mitochondrial fraction decreases considerably with time is also in line with this explanation. Since the amylase in the mitochondrial fraction is probably of different specific radioactivity than that of the secretory granules, the calculations for correction of the specific radioactivity described in the Appendix cannot be applied. Therefore, corrected values for the soluble fraction of the mitochondria are not given in Fig. 8. Since contamination of the microsomal fraction by mitochondria and secretory granules was very small, the correction introduced was minimal.

2 hr after labeling, the highest specific radioactivity is that of the secretory granule contents which is equal to that of the granule membrane. At the same time, the specific radioactivity of the microsomal content has markedly decreased. It should be further noted that the specific radioactivity of the secretory granule membrane is, at this time, about 5.5-fold higher than that of the mitochondrial membrane.

The specific radioactivities of each cell fraction were corrected for contamination by other cell components. The calculated values are given in Fig. 8. The specific radioactivity of the secretory granule membrane remained about equal to that of the granule contents at all times after labeling. At 2 and 5 hr after labeling, the specific radioactivity of the secretory granule membrane was seven times higher than that of the mitochondrial membrane. 5 hr after injection of label, the interrelationships of the specific radioactivities did not change significantly. As compared to 2 hr, a general decrease in the specific radioactivities is observed and is probably in part due to continued synthesis of unlabeled protein.

Gel Electrophoresis and Radioactivity of the Secretory Granule Proteins

The electrophoretic pattern of the proteins derived from the secretory granule membrane is different from that of the proteins of granule content (Figs. 9-10). Two intensely stained bands (m_2 , m_3) and up to five fainter bands were identified in the membrane preparation. The pattern of bands in the preparation from the granule content consisted of four major bands (c_2 - c_4 and c_6) and two minor bands (c_1 and c_5) (Figs. 9-10). The electrophoretic pattern of a mixture of granule membrane and granule content preparations is also shown in Fig. 9. It can be seen that bands c_2 and c_3 of the granule content appear as a slight contaminant in the granule membrane preparation. The densitometer tracing (Fig. 10) discloses also a slight contamination of the membrane preparation by the content protein band (c_6). It can therefore be concluded that the membrane protein bands m_1 , m_2 , and m_3 are unique to the membrane preparation. It should be noted that while all content protein migrated into the gel, part of the membrane protein preparation, estimated at about 10% of the total radioactivity, remained at the gel origin.

The electrophoretic patterns obtained with five different secretory granule preparations, from both starved and fed animals, showed no significant departure from the electrophoretic pattern shown in Figs. 9-10. The radioactivity of protein bands from both the granule content and membrane preparations was proportional to the staining intensity of each band (Fig. 10). The degree of contamination of the membrane proteins by content protein is estimated to be about 20%. This calculation of contamination is based on the radio-

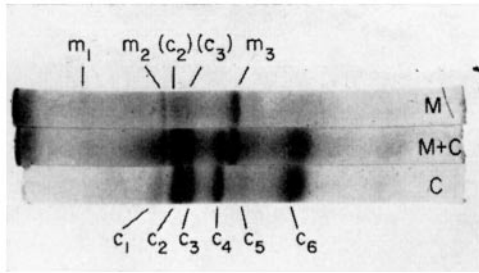


FIGURE 9 Electrophoretic patterns of secretory granule fractions. *M*, membrane preparation; *C*, granule content, and *M + C*, mixture of membrane and content preparations in a protein ratio of 1.5:1. Parentheses around symbols signify that the appropriate bands are presumed to be contaminants. For explanation, see Results.

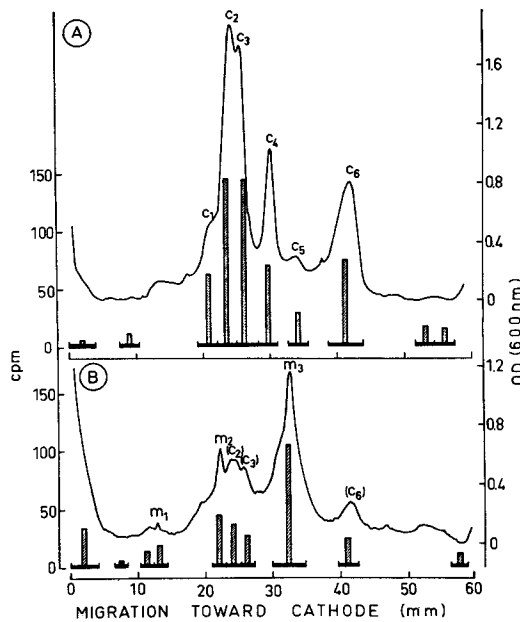


FIGURE 10 Densitometer tracing and radioactivity distribution in electrophoretograms of secretory granule membrane and content proteins. A, secretory granule content preparation. B, secretory granule membrane preparation. The letters used to identify the different peaks are the same as used in Fig. 9. The height of the dashed bars represents the amount of radioactivity in each band, while the width of the bars' bases indicates the length of the gel cut for counting. The results shown are the average of duplicate runs. The deviation in the duplicates from the mean radioactivity in the different peaks was less than 10%. Notice that the radioactivity in the peaks of both membrane- and content-specific proteins is proportional to the respective OD value. The radioactivity was negligible in those gel regions where staining intensity was very low.

activity found in the contaminating peaks (c_2 + c_3) + (c_3) + (c_6) relative to the total amount of radioactivity found in the gel (Fig. 10 B). The value of 20% contamination is much higher than that calculated on the basis of amylase and DNase activities in the purified membrane preparation. The higher contamination is probably due to the procedure of isolating the membranes for electrophoresis in a highly concentrated form so that removal of granule content is less efficient (see Methods).

It is estimated that the maximal radioactivity which could be contributed to the main membrane band m_3 (Fig. 10 B) by the adjacent content contaminants c_4 and c_5 (Fig. 10 A) cannot amount to more than 20% of the radioactivity of this band. This estimate is based on the fact that the radioactivity corresponding to the content proteins c_2 and c_3 (Fig. 10 A) is reduced to one-fifth in the membrane preparation (Fig. 10 B). It is assumed from the above calculation that a similar reduction would occur in the amount of the contaminants c_4 and c_5 .

DISCUSSION

The information available from previous work on the parotid and pancreas glands appeared to indicate that the secretory granule membrane might be reutilized, after secretion, for the formation of new secretory granules (1, 34). After its fusion with the cell membrane during secretion, the granule membrane appeared to be converted into small vesicles. It seemed likely that these vesicles return to the "pool of membranes" in the Golgi region. Since the new secretory granules always originate in the periphery of the Golgi complex, it could be supposed that the membrane of the new granule is contributed by the pool of preexisting membranes. The present work shows that the labeling of membrane-specific protein of the secretory granule is as high as that of the granule's exportable content. It is therefore quite clear that at least the protein part of the membrane is synthesized *de novo*. The ratio of the specific radioactivity of the membrane to that of the content remained constant at about 1/1 while the absolute values changed with time according to the known pattern of synthesis and transport of the content (35-37, 30, 33, 38). It therefore seems reasonable to assume that the membrane protein is synthesized and transported concomitantly with the content protein.

A most critical point in this study is the purity of secretory granule membrane preparation. The

enzymatic analyses indicated that the granule membrane preparation obtained at 30, 120, and 300 min after injection of label contained only small amounts of mitochondria, microsomes, and exportable protein. The electron micrographs of the secretory granule membranes confirmed the biochemical analysis as to the purity of the granule membrane fractions. The amino acid composition and the pattern of gel electrophoresis further substantiated the conclusion that the secretory granule membrane preparation consists mainly of proteins specific to this membrane. The gel electrophoresis also showed that all the protein bands contained radioactivity in proportion to their staining intensity. Taking all these criteria into consideration, there can be little doubt that the measured radioactivity of the membrane fraction is indeed inherent in the protein of the secretory granule membrane.

It has been shown by Morimoto et al. and by Jamieson and Palade that transport of exportable proteins from the site of synthesis on the ribosomes to the secretory granules does not require continuous protein synthesis (46, 34). These findings can be readily explained by the present work which shows that the protein of the membrane which would be required for the construction of the secretory granule has already been synthesized at the same time as the product to be transported. Furthermore, it seems reasonable to suggest that the transitional elements (33, 34, 39), through which the exportable proteins leave the endoplasmic reticulum, represent also the site of formation of the future secretory granule membrane. Thus, the exportable protein would leave the endoplasmic reticulum already enveloped by the new membrane. According to this concept, the newly synthesized membrane protein should have a high specific radioactivity while it is still part of the endoplasmic reticulum. However, it is not possible to isolate a pure fraction of transitional element membranes. In practice we obtained a microsomal fraction which probably contains the transitional elements mixed with a large amount of constitutive endoplasmic reticulum, plasma, and Golgi membranes. It is therefore not at all surprising that the microsomal membrane fraction showed a low specific radioactivity at all times measured. The concept that the transitional elements could be the site of formation of a membrane different from that of the rest of the reticulum implies heterogeneity of this membrane system. Findings for (40, 18), and

against (41), regional differentiation within the endoplasmic reticulum have been reported.

The membrane of the secretory granule had a very high content of proline and was also rich in glycine and glutamic acid. It is of interest to note that a soluble glycoprotein isolated from human parotid saliva shows a striking similarity in its amino acid composition (2) to the insoluble membrane protein of the secretory granule. Because of this similarity it is tempting to speculate that glycosylation, because of the hydrophilic properties of the sugar, might convert a membrane protein into a soluble protein and vice versa.

The findings of the present work imply that the protein of the granule membrane is degraded after secretion. It may be assumed that the processes which cause fusion of the granule membrane with the cell membrane and the subsequent conversion to small vesicles induce changes in the structure of the membrane which invite degradative processes. In this respect, it is of interest to note that structures apparently of lysosomal nature were prominent after secretion and before reaccumulation of secretory material (42). In contrast to the membranes and content of the secretory granules, the mitochondrial membranes which are not directly involved in the secretion process seem to have a slow turnover as reflected by their relatively low incorporation of labeled amino acids. This conclusion appears to hold also for most of the membranes represented in the microsomal fraction. It has been previously estimated that constitutive proteins in exocrine pancreas are synthesized at a rate about one-sixth of that of the exportable proteins (33). A similar ratio is obtained in the present work when peak incorporation into protein of the secretory granule is compared to incorporation into mitochondrial and microsomal membranes. It should be emphasized that the findings described in the present work were obtained *in vivo* without any period of starvation and without application of an external stimulator of secretion. It may therefore be concluded that the findings apply to the normal function of the gland. Experiments not reported in the present work showed that secretory granule membrane and content proteins were equally labeled also when the gland was induced to secrete *in vivo* by isoprenaline.

Studies to date have revealed the striking similarities in the structure and function of the various exocrine glands. Indeed, the workings of endocrine glands with respect to the processes of packaging and extrusion of exportable substances seem also

not to be very different (7, 8, 43-45). It is therefore likely that the *de novo* synthesis of membrane protein for the packaging of exportable material as found in the present study may apply to the other gland systems. Fusion of the secretory granule membrane with the cell membrane has often been called reverse pinocytosis. It remains to be seen whether in pinocytosis proper the membrane protein of the pinocytotic vesicle formed from the cell membrane will also turn out to be nonreutilizable.

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APPENDIX

Calculations to Correct the Specific Radioactivity of Cell Fractions Accounting for the Contaminating Components Present

The following procedure was used to calculate the true specific radioactivity of a cell structure by taking into account the contamination by other cell components in the fraction. To make such a calculation feasible, it had to be assumed that the radioactivity of a specific protein is the same in the contaminated and in the contaminating fractions. The relative amount of a contaminant in any fraction is calculated using the following symbols: A, fraction purified for component *a* identified by marker enzyme *a** contaminated by small amounts of component *b*. B, fraction purified for component *b* identified by marker enzyme *b** contaminated by small amounts of other components. The relative amount of component *b* in fraction A is defined as C.

(equation 1)

$C = \frac{\text{specific enzyme activity of } b^* \text{ in fraction A}}{\text{specific enzyme activity of } b^* \text{ in fraction B}}$

For the purpose of the calculation shown in equation 1, it is assumed as an approximation that the specific enzymatic activity of component *b* is equal to that of fraction B in which it is the predominant component.

Contamination was measured in the total frac-

tions rather than on the isolated membrane fractions, to avoid errors due to possible enzyme inactivation. The relative amount of contaminants in a purified membrane fraction was therefore derived as follows:

Let *Y* be the ratio of membrane protein to total protein in fraction A.

Let *X* be the ratio of membrane protein to total protein in fraction B. The relative amount of membranes of component *b* in the membrane preparation isolated from fraction A will then be $C \frac{X}{Y}$.

The true specific radioactivity of the membrane of component *a* can now be derived: The symbol SR represents specific radioactivity (cpm/mg protein) and *m* represents membranes. The measured specific radioactivity SR_{Am} consists of the specific radioactivity of the major component SR_{Am} plus the contribution of the relative amount of contaminant *bm*, $\left(C \frac{X}{Y}\right)$, multiplied by its specific radioactivity SR_{Bm}. Thus, one can calculate the true specific radioactivity of the major component SR_{Am} according to equation 2:

$$SR_{Am} = \left(SR_{Am} - SR_{Bm} \times C \frac{X}{Y} \right) / \left(1 - C \frac{X}{Y} \right)$$

By the same type of calculation the specific radioactivity of any soluble or membrane fraction can be corrected for contamination by any other soluble or membrane fraction.

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