DISTRIBUTION OF NEWLY SYNTHESIZED AMYLASE IN MICROSOMAL SUBFRACTIONS OF GUINEA PIG PANCREAS

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ABSTRACT

Amylase distribution was studied in guinea pig pancreas microsomes fractionated by centrifuging, for 2 hr at 57,000 *g* in a linear 10 to 30% sucrose gradient, a resuspended high speed pellet obtained after treating microsomes with 0.04% deoxycholate (DOC).[1] Amylase appeared in the following positions in the gradient: (*a*) a light region which contained ~35% of total enzymic activity and which coincided with a monomeric ribosome peak; (*b*) a heavy region which contained ~10% of enzymic activity in a sharp peak but which had very little accompanying OD_{260} absorption; (*c*) a pellet at the bottom of the centrifuge tube which contained ~20% of the enzymic activity. After 5 to 20 min' in vivo labeling with leucine-1-C^{14}, radioactive amylase was solubilized from these three fractions by a combined DOC-spermine treatment and purified by precipitation with glycogen, according to Loyter and Schramm. In all cases, the amylase found in the pellet had five to ten times the specific activity (CPM/enzymic activity) of the amylase found in the light or heavy regions of the gradient. The specific radioactivity (CPM/mg protein) of the proteins or peptides not extracted by DOC-spermine was similar for all three fractions. Hypotonic treatment of the fractions solubilized ~80% of the total amylase in the fraction from the heavy region of the gradient, but only ~20% of the amylase in the monomer or pellet fraction. Electron microscope observation indicates that the monomer region of the gradient contained only ribosomes, that the heavy region of the gradient contained small vesicles with relatively few attached ribosomes, and that the pellet was composed mostly of intact or ruptured microsomes with ribosomes still attached to their membranes. It is concluded from the above, and from other evidence, that most of the amylase activity in the monomer region is due to old, adsorbed enzyme; in the heavy region mostly to enzyme already inside microsomal vesicles; and in the pellet to a mixture of newly synthesized and old amylase still attached to ribosomes. Furthermore, the ribosomes with nascent, finished protein still bound to them are more firmly attached to the membranes than are ribosomes devoid of nascent protein.

INTRODUCTION

Recently, the involvement of various cell membranes in protein synthesis has received renewed attention as a result of reports that in both pancreas (1) and liver (2–4) the ribosomes attached to the membrane of the endoplasmic reticulum (ER)

[1] The abbreviations used in this paper: are DOC, Na deoxycholate; DEAE-cellulose, diethylaminoethyl cellulose; RNA, ribonucleic acid; RNase, ribonuclease; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl) aminomethane.

are more active in protein synthesis than ribosomes free in the cytoplasmic matrix. In the case of the guinea pig pancreas, for instance, the secretory zymogen, α-chymotrypsinogen, appears to be synthesized by attached ribosomes (1), although the latter represent only one-third to one-half of the total ribosomal population of the cytoplasm (5).

Various reasons have been given for this apparent membrane involvement: (*a*) in secretory cells of multicellular organisms, the membrane presumably provides a directing semipermeable barrier across which secretory proteins must be transferred before their exit from the cell (1, 2, 4, 6); (*b*) in hepatic (3) and bacterial (7) cells, the membrane could have a role in stabilizing the ribosome-messenger RNA complex; (*c*) finally, the phospholipids of the membrane have been assumed to play an intimate role in the reactions involved in protein synthesis (8, 9), perhaps by acting as carriers of amino acids (9).

The experimental results presented here bear on both the relationship of ribosomes with the ER membrane, and the methodology involved in obtaining ribosomes active in protein synthesis ("polysomes").

METHODS

Pancreatic glands were removed from 24-hr–starved adult guinea pigs, weighing 300 to 400 g. One g wet weight tissue was homogenized in 10 ml of cold 30% (0.88 M) sucrose, and the homogenate was centrifuged at 4°C at 20,000 *g* (average; No. 40 Spinco rotor) for 30 min to remove all particles larger than microsomes. The supernate was centrifuged for 60 min at 105,000 *g* (average; No. 40 Spinco rotor) to obtain a pellet consisting almost solely of microsomes bearing attached ribosomes, the free ribosomes remaining in the supernate (5). The microsomal pellet (10 to 15 mg of protein) from 1 g wet weight of tissue was resuspended with a Teflon homogenizer in 5 ml of 5% sucrose solution, and the suspension treated with sodium deoxycholate (DOC) in two different concentrations. In "low-DOC" experiments, 0.04 ml of 5% DOC, pH 7.4–7.6, was added to give a final DOC concentration of 0.04%; in "high-DOC" experiments, the amount added was 0.2 ml and the final concentration was 0.2%. The latter concentration of DOC is sufficient to detach ~90% of the ribosomes from the membranes of the microsomal fraction (5), whereas the former concentration only detaches about 50% (as determined by RNA measurements). In both cases, the partially clarified suspensions were centrifuged at 20,000 *g* (average; No. 40.3 Spinco rotor) for 10 min and the resulting supernates recentrifuged at 115,000 *g* (average; No. 40.3 Spinco rotor) for 90 min. The pellets obtained as a result of the last centrifugation were rinsed several times with 5% sucrose and then resuspended by homogenization in 1 ml of the same solution. The suspensions were then placed on 30 ml of linear density gradients, extending from 10 to 30% sucrose (in some experiments, various salts were added to the density gradients, as described in the Results section), and the tubes were centrifuged at 53,500 *g* (average; No. SW 25.1 Spinco rotor) for 120 min. At the end of the centrifugation, approximately 1 ml aliquots were collected at ~4°C, by means of a drop counter fraction collector. The small pellet, which always sedimented to the bottom of the centrifuge tube, was resuspended in 5.0 ml of 5% sucrose and assayed chemically and enzymatically as described below.

Aliquots of the collected fractions were diluted tenfold and their absorbancy at 260 mμ was determined as a measure of ribosome concentration in the gradient. Other aliquots of the same fractions (0.01 to 0.04 ml, always run in duplicate) were assayed for amylase activity by a method modified (10) after Bernfield (11). In some experiments, amylase activity was also determined in: (*a*) the original microsomal fraction; (*b*) the original DOC supernate and DOC pellet; and (*c*) the pellet obtained in the density-gradient centrifugation. Protein N was determined by nesslerization of Kjeldahl digests and RNA by the orcinol reaction on the "90°-20 min-5% TCA extracts" of TCA-precipitated proteins (cf. reference 5).

The labeling experiments and the isolation of radioactive amylase from various fractions were performed as follows. Two hundred μc of DL-leucine-C^{14} were injected intravenously into adult guinea pigs under ether anesthesia. The pancreatic glands were removed 5 to 30 min after injection and fractionated as indicated above. DOC-treated microsomal fractions were subjected to zone centrifugation in sucrose density gradients as described. Amylase-containing fractions, present in two regions of each gradient (see Results), were separately pooled, and to each pooled sample (4 to 6 ml) were added 0.2 ml of 5% DOC (0.15 to 0.20% final DOC concentration) and then 0.5 ml of a 0.05 M spermine–0.01 M $MgCl_2$ solution. The pellet at the bottom of the density gradient was resuspended in 5 ml of 5% sucrose and treated in the same way. The high DOC treatment was needed to break up membranous material (see below) still remaining in two of the fractions. The spermine-Mg^{2+} treatment, which has been shown to release over 90% of the secretory enzymes bound to ribosomes (12), was necessary, since it was found that the 40% alcohol step recommended by Loyter and Schramm (13) for amylase extraction from whole tissue released less than one-half of the amylase activity in the density gradient fractions. Usually, the DOC-spermine-Mg^{2+} mixtures were kept at 4°C overnight and then sedi-

mented at 105,000 *g* for 60 min. In all cases so treated, 70 to 100% of the amylase activity was solubilized and remained in the supernate. The solubilized amylase was then isolated as a glycogen complex, as described by Loyter and Schramm (13), except that four times as much glycogen as originally recommended was needed, supposedly because of the much smaller amounts of amylase present in our fractions. In our hands, the recovery of amylase activity by glycogen precipitation ranged from 40 to 100% for all fractions, but the average recoveries of amylase from each of the density gradient fractions were similar, being 55% from the heavy peak, 74% from the light peak (monomer region of gradient), and 66% from the pellet fraction. The amylase-glycogen precipitates were washed once or twice with cold 40% alcohol containing 0.01 M phosphate, pH 8.0, and then resuspended in 0.02 M phosphate buffer, pH 6.9. An aliquot was taken for amylase determination and the remainder dried down on planchets for radioactivity determinations using a thin-window, gas-flow Geiger counter. The pellets obtained by centrifuging the DOC-spermine-Mg^{2+}-treated gradient fractions were treated with 12% (final concentration) trichloroacetic acid and the precipitated residual proteins were washed, extracted, and counted as described earlier (1)

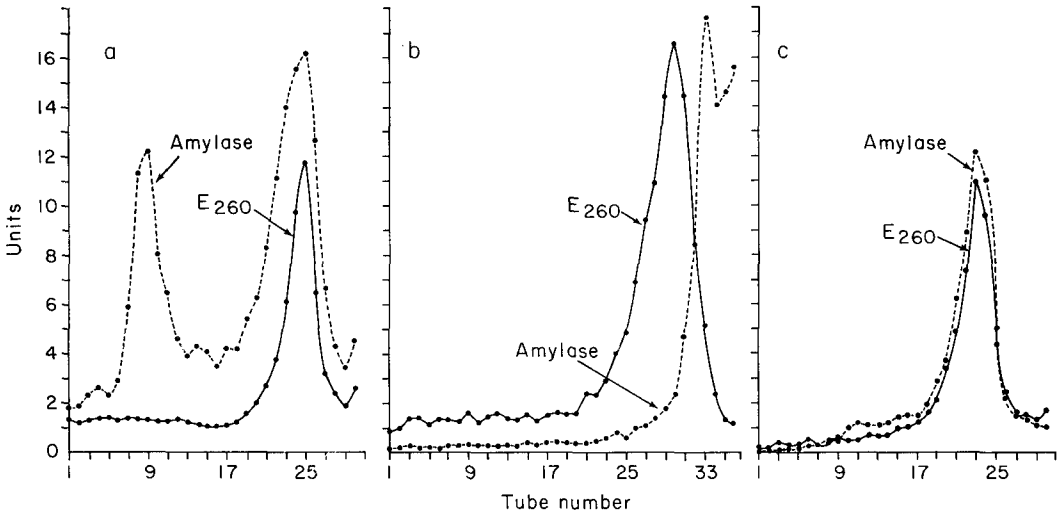

FIGURE 1 Sucrose density gradient profiles of DOC-treated pancreatic microsomes. The methods are given in the Experimental section. The linear density gradients went from 10 to 30% sucrose.

Fig. 1 *a* Microsomes treated with low-DOC; Fig. 1 *b* Microsomes treated with low-DOC but with 10 mM KCl, 1 mM Mg^{2+}, and 5 mM Tris, pH 7.4, in the sucrose density gradient; Fig. 1 *c* Microsomes treated with high-DOC. Procedures used for determining absorbancy at 260 mμ and amylase activity in each tube are given in the text. The units of measurement on the ordinate are arbitrary units both for OD_{260} and for amylase activity.

Electron Microscopy

The various density gradient fractions examined were pelleted without dilution at 105,000 *g* for 120 min. The resulting pellets were fixed by layering over them a 1% OsO_4 solution in 15% sucrose. Fixation was continued for ~15 hr at ~0°C and was followed by dehydration in graded ethanol and embedding in Epon. At the end of fixation or during dehydration, the pellet was cut into orientable strips to facilitate systematic top-to-bottom examination. In some cases, strips of fixed pellets were "postfixed" for 2 to 3 hr at 25°C in 0.5% uranyl acetate in acetate-Veronal buffer, pH 5.0, before dehydration. All sections were doubly stained (uranyl acetate followed by alkaline lead) and finally examined in a Siemens Elmiskop I operated at 80 kv, with a double condenser and 50 μ objective apertures.

RESULTS

Fig. 1 shows typical results obtained with three different types of microsomal preparations after centrifugation in 10 to 30% sucrose density gradients; the profile in Fig. 1 *a* was obtained after "low-DOC" treatment; that in Fig. 1 *c* after "high-DOC" treatment; and that in Fig. 1 *b* after low-

DOC treatment, but with 10 mM KCl, 5 mM Tris, pH 7.4, and 1 mM Mg^{2+} present in the density gradient. In the high-DOC experiment (Fig. 1 *c*), ~90% of the original microsomal RNA appeared in the monomer region (peak at tube 23) of the density gradient; in the low-DOC experiment (Fig. 1 *a*) only ~50% of the RNA appeared in the monomer region (peak at tube 25), the rest sedimenting as a pellet to the bottom of the tube.

Of particular interest is the distribution of amylase activity in these density gradients. In both low-DOC and high-DOC preparations amylase activity always appeared in a peak associated with monomeric ribosomes (henceforth called "light peak"). However, in low-DOC preparations, another sharp peak of amylase activity was found, about one-third of the way from the bottom (at peak tube 9 in Fig. 1 *a*). This amylase peak (henceforth called "heavy peak") always appeared in the same general region of the gradient; its position could be quantitated by dividing its peak tube number by the total number of tubes. Thus, in eleven experiments run the same way, this ratio ranged from 0.15 to 0.49 with an average of 0.32. In the same runs, the ratio of the peak tube number containing the monomeric ribosomes to total tubes ranged from 0.75 to 0.85 with an average of 0.79. Thus, the positions of the two peaks of amylase activity were reproducible; but the amount of amylase activity in the heavier region of the gradient was quite variable, ranging in various experiments from one-fourth that in the monomer region to almost equal amounts of amylase activity in both peaks. In all cases, amylase activity was also found in the pellet at the bottom of the tube. The amount of amylase in the microsomes from 1 g wet weight of tissue ranged from 150 to 400 μg in the various experiments. Values for the percentage of original microsomal amylase activity appearing in the microsomal subfractions were as follows: ~35% of the amylase activity was solubilized by the DOC treatment, and hence was not put on the density gradient; of the total amylase activity put on the gradient, 40 to 60% (average of 6 values = 54%) appeared in the light region; 10 to 30% (average of 6 values = 16%) in the heavy region of the gradient; and 25 to 45% (average of 6 values = 30%) in the pellet. Of the amylase activity placed on the density gradient, the percentage of total recovery (pellet and gradient) was 107, 87, 79, 111, 105 in the five experiments in which it was determined.

The structural correlates of the amylase activity in the sucrose density gradient, particularly that of the activity in the heavy peak, were further examined. The results show that the amylase activity of this peak has little to do with "polysomes," that is, with ribosomes actively engaged in amylase synthesis and presumably held together as polysomes (see references 14–18) by means of amylase messenger RNA. Thus, for one, very few ribosomes were found in this region, as noticed by the very low OD_{260} values. Again, when a higher DOC concentration was initially used to "solubilize" the microsomes, all the amylase activity was found in the light peak, with no activity appearing in the heavy region of the density gradient (Fig. 1 *c*). When KCl, tris buffer and Mg^{2+} were present in the density gradient, all the amylase activity was solubilized and appeared at the top of the gradient (Fig. 1 *b*). Finally, when tubes from the heavy amylase peak were pooled, diluted with water to a sucrose concentration of ~5%, and centrifuged at 105,000 *g* for 60 min, only 20 and 28% (two separate experiments) sedimented to the bottom while the balance, apparently solubilized, remained in the supernate. This was a "double osmotic shock" treatment, since the original microsome suspension was taken up in 5% sucrose before layering on the 10 to 30% sucrose density gradient. Evidently, it was the second osmotic treatment and subsequent centrifugation which resulted in the release of amylase. When the peak of amylase activity in the monomer region was subjected to the same procedure, 85 and 95% of the original peak activity (two separate experiments) remained sedimentable.

All the experiments point to the conclusion that much of the amylase in the heavy peak behaved as if enclosed within vesicles, which could be broken by high DOC concentrations or damaged (rendered leaky) by repeated osmotic shock. This assumption was confirmed when the microsomal material present in these regions was examined in the electron microscope. Fig. 2 shows that the light amylase peak contains typical ribosomes which, interestingly enough, do not appear as isolated particles but as chains of particles (rarely as clusters), although in the gradient they definitely sedimented only to the light peak, monomer region. The aggregation is probably the result of pelleting or fixation or both. Fig. 3 shows that the heavy amylase peak, located in the heavy region in the density gradient, contains small vesicles (diameter ≃400 A) with a dense content and a few ribosomes attached to the outer surface of

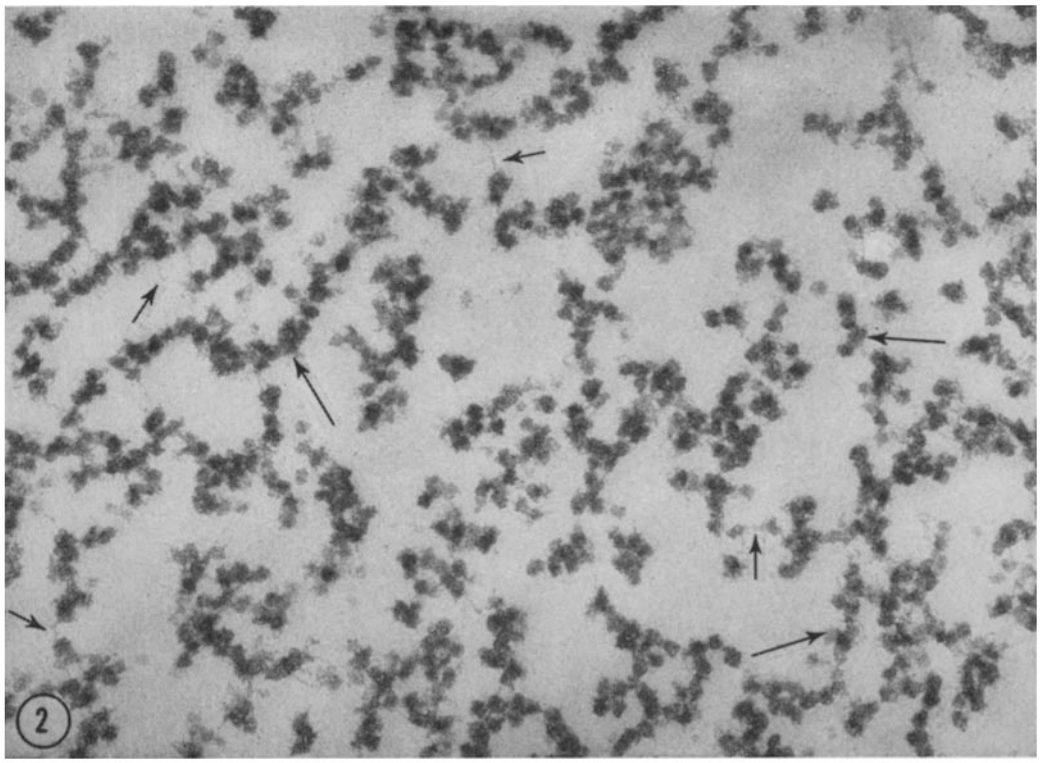

FIGURE 2 Section through the pelleted material of the light amylase peak (monomer region in the gradient). The peak is morphologically homogeneous and consists of ribosomes most of which are disposed in chains (long arrows). Fine connecting strands within or in between such chains are visible in a number of places (short arrows). Pellet fixed in 1% OsO_4 in 15% sucrose, and embedded in Epon. Sections stained with uranyl and lead. × 110,000.

their limiting membrane. When the material in this heavy amylase peak was incubated for 30 min at 4°C in KCl-tris buffer-Mg^{2+} (which released the amylase), then sedimented and fixed, the resulting pellet was found to consist of vesicles of about the same size, which have retained their attached ribosomes, but have lost all or part of their dense content (Fig. 4).[2] More significantly, when the tubes from this peak were diluted with water to a final sucrose concentration of ~5%, and centrifuged as above (conditions which resulted in the release of ~70 to 80% of the amylase activity), the vesicular content was found to be decreased in density or completely lost. The results were similar, but less pronounced than those obtained with KCl-tris buffer-Mg^{2+} extraction. Taken together, the biochemical and morphological findings strongly suggest that most of the amylase of the heavy peak is present in the contents of the vesicles of this fraction, rather than bound to ribosomes. It is clear that in the low-DOC experiments small fragments of the endoplasmic reticulum appear in the heavy region of the gradient; they still bear attached ribosomes and presumably contain amylase in their cavities. Indeed, other regions in the density gradient, immediately below and above the heavy amylase peak, were also found to contain small vesicles with a dense con-

[2] Although this treatment also released the amylase from the monomeric ribosomes of the light peak, we assume that the enzyme released from the heavy peak was originally present within the vesicles, rather than on the ribosomes of the latter fraction. The reasons for this assumption are: (*a*) the amount of ribosomes in the heavy peak is very much smaller than that in the monomer peak, as measured by the absorption at 260 mμ, whereas (*b*) the amount of amylase in this fraction is from one-third to two-thirds as much as in the monomer region.

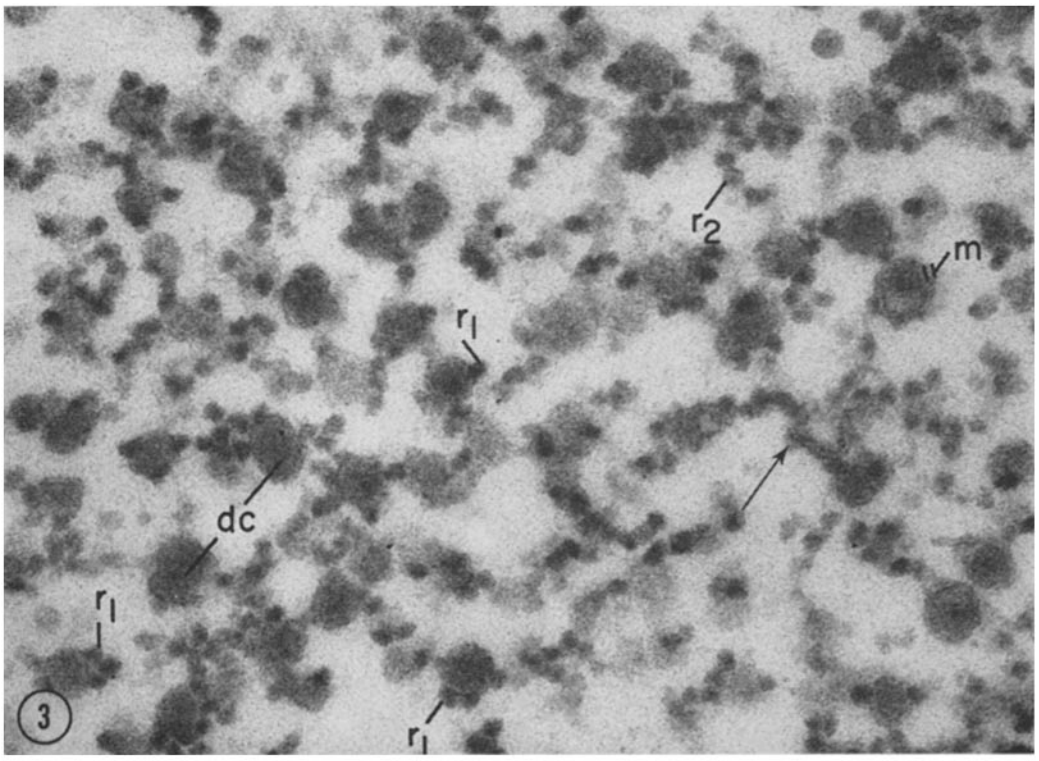

FIGURE 3 Section through the pelleted material of the heavy amylase peak. The pellet consists of small microsomal vesicles limited by a unit membrane (*m*) and filled with a dense homogeneous material (*dc*). Many of these vesicles still bear ribosomes (r_1) attached to the outer surface of their membrane. Ruptured membranes (arrow) and chains and clusters of ribosomes (r_2), most of which are still attached to or anchored on microsomal membranes, can be recognized among the microsomal vesicles. × 100,000.

tent and ribosomes still attached to their limiting membranes (see Discussion).

Further insight into this situation was provided by the results of the in vivo labeling experiments with DL-leucine-1-C^{14}. The amylase was isolated, as described, from the three fractions of the density gradient, i.e., the light region, the heavy region, and the pellet; and its specific radioactivities (CPM/enzymatic activity) were determined.[3] Table I gives the results. It is quite evident that the amylase in the heavy peak has no higher specific radioactivity than the amylase in the light peak, even at the earliest time point, thus once again demonstrating that the heavy amylase peak is not associated with typical polyribosomes (14–18). However, it was surprising to find that the pellet at the bottom of the density gradient contained amylase with a specific radioactivity much higher than that in any other fraction, particularly at the early time points. In the first two experiments in Table I, ~50% of the RNA and ~40% of the amylase activity placed on the gradient were recovered in the pellet, but the corresponding figures for amylase radioactivity were considerably higher: 79% in Experiment 1 and 72% in Experiment 2. There were no great differences in the specific radioactivity of the residual proteins among the three fractions, even at 30 min after injection (Table I).

The amylase of the pellet retained a high specific radioactivity even when microsomes were treated with high-DOC (Table I, Experiment 5). The pellet in this case contained only ~10% of the

[3] The amylase activity was arbitrarily converted to amylase amounts by using the conversion factor obtained for rat pancreas amylase (19). Thus, any comparison between the specific radioactivities of amylase and of residual protein (Table I) is not strictly valid.

microsomal RNA and ~5% of the microsomal amylase, i.e., ~15% of the amylase activity placed on the gradient, for ~75% of the microsomal amylase was lost to the DOC-supernate. When a similar pellet, obtained in a low-DOC experiment, was resuspended, treated with high-DOC, the suspension recentrifuged and the ensuing pellet again resuspended and run on a similar density gradient, the final pellet formed at the bottom of the gradient still had amylase with a higher specific radioactivity than that of the monomer peak (Table I, line 4). In this case, treatment with high-DOC resulted in a pellet which retained ~5% of the total ribosomes (by RNA measurements), ~10% of the total amylase activity, but ~50% of the total amylase radioactivity.

Since the specific radioactivities in Table I were determined by enzymatic assays of isolated amylase-glycogen complexes, we must consider the possibility that some of the enzyme of the pellet was inactivated. If so, the assays could not record the entire amount of enzyme protein in this fraction, thus leading to spuriously high values for amylase specific radioactivity. We do not think this is the case since, as mentioned above, recoveries of amylase on the gradient (as measured enzymatically) were satisfactory and recoveries of amylase as amylase-glycogen complexes from each of the three fractions were similar, though generally low (48%, 55%, and 66%, respectively). It should also be mentioned that the amylase-glycogen complex is reasonably pure with regard to protein contaminants (13); indeed, Redman et al. (19) have shown that labeled amylase from pigeon pancreas microsomes, isolated as the amylase-glycogen complex and then assayed for impurities by means of DEAE-cellulose chromatography, is free of contaminating protein and contaminating radioactivity as far as can be determined.

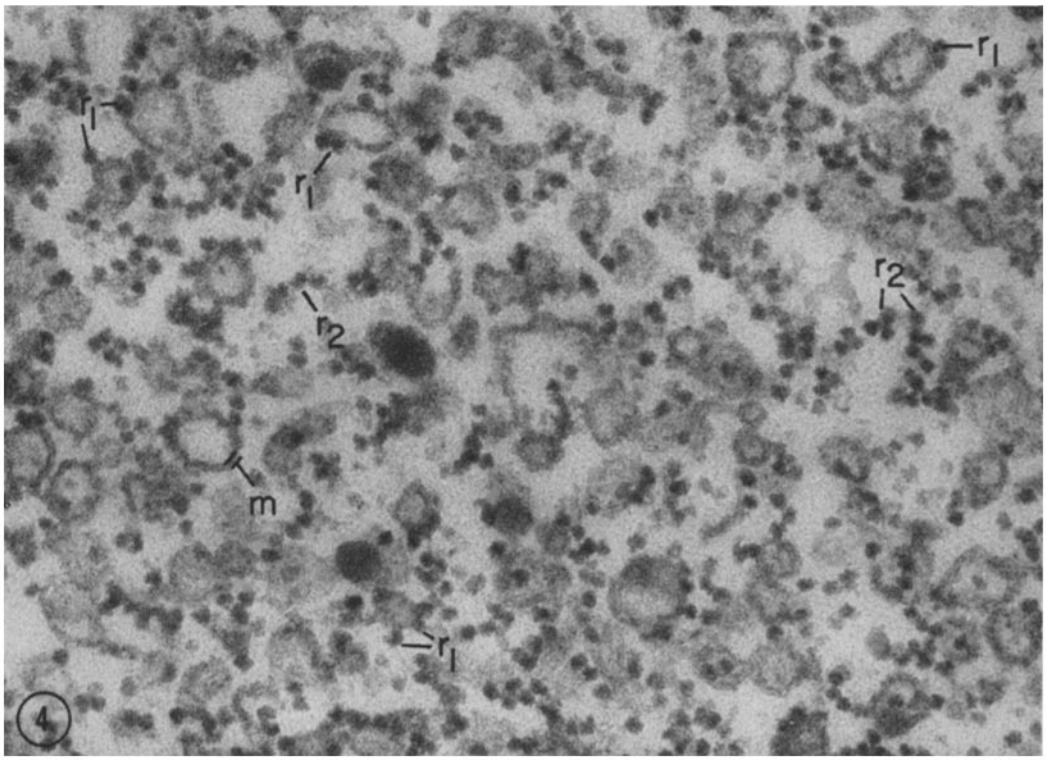

FIGURE 4 Section through the material of the heavy amylase peak pelleted after KCl-tris buffer-Mg^{2+}-treatment. The pellet is comprised of microsomal vesicles limited by a unit membrane (*m*). Most of these vesicles have lost their dense content, but have retained their attached ribosomes (r_1). Chains and clusters of ribosomes (r_2), some of which are probably still attached to microsomal vesicles, can be recognized in the rest of the field. × 100,000.

TABLE I

In Vivo Incorporation of Radioactive Leucine into the Amylase and Residual Protein of Various Pancreatic Microsomal Subfractions Separated by Density Gradient Centrifugation

Exp.	Time after injection	CPM/mg Amylase or Protein*					
		Heavy peak		Light peak		Pellet	
		Amylase	Protein‡	Amylase	Protein‡	Amylase	Protein‡
	min						
1	5	4,940	4,650	11,700	4,600	56,600	3,640
2	15	3,710	4,000	4,300	2,200	15,500	5,510
3	30	27,100	7,820	35,450	2,810	84,400	5,970
4	5	—	—	9,130§	2,310§	81,600‖	4,250‖
5	5	—	—	9,410¶	4,450¶	47,550¶	5,060¶

* Protein values were obtained from N determinations, while amylase values were obtained from enzymatic assays. The variations in specific activities from one experiment to another could be due to individual variations among animals or to differences in the actual amount of radioactivity reaching the pancreas in each case.

‡ Residual protein after secretory enzymes were extracted.

§ After low-DOC treatment; see Methods.

‖ After low-DOC treatment; pellet obtained then treated with high-DOC, and resedimented to obtain final pellet; see Results.

¶ After high-DOC treatment; see Methods.

By electron microscopy the pellet was found to consist of small vesicles similar in size to those of the heavy amylase peak. Some of them had a dense content, but many appeared broken (open) and empty. Attached ribosomes were present on their limiting membranes (Fig. 5). However, when such a pellet was resuspended in 5% sucrose and resedimented at 105,000 *g* for 60 min, a condition which is known to cause extraction of microsomal contents, 86% of its amylase was recovered in the sediment, a fact which indicates that most of the amylase in this fraction was bound to ribosomes. Electron microscope observations of similarly treated pellets showed a loss of the dense contents, but no appreciable diminution of ribosomes attached to membranes. Thus, the amylase seems to be bound to ribosomes in this fraction, and in this respect the enzyme is similar to that of the light amylase peak but quite unlike that of the heavy amylase peak, in which it appears to be enclosed within vesicular spaces.

DISCUSSION

The most probable explanation for the above results is that the ribosomes actively engaged in amylase synthesis (or synthesis of other proteins) are more firmly bound to the membranes of the ER than "resting" or "inactive" ribosomes. Our evidence for this statement may be summarized (Table II) as follows:

1. When pancreatic microsomes are incompletely disorganized by the use of low concentrations of DOC, various fractions can be obtained by sucrose density gradient centrifugation. These include a ribosomal fraction (a light peak in the gradient) which contains only about one-half of the ribosomes formerly attached to the microsomal membranes. This fraction also contains amylase bound to it (the enzyme cannot be removed by osmotic shock) but from the radioactivity data it appears that this bound amylase is a mixture of truly nascent amylase with a larger proportion of adsorbed enzyme.
2. The other two fractions resolved by the gradient are morphologically similar, i.e., they consist of what appear to be small microsomes or microsomal fragments. One of these fractions sediments in the gradient to a heavy region and contains but little RNA, while the other sediments to a pellet and contains about half the ribosomes placed on the gradient. These ribosomes appear to be bound to membranes. The two fractions can be resolved in that, while

both contain amylase, the one in the heavy region of the gradient releases its enzyme upon repeated osmotic shock, and hence presumably carries it within vesicles; while the pellet retains its amylase upon osmotic shock and hence carries the enzyme still bound to ribosomes attached to membranes.

3. These two fractions can be resolved biochemically in that the pellet contains most of the newly synthesized radioactive amylase of the microsomes, with a much higher specific radioactivity than the enzyme in the heavy gradient fraction, and indeed, than that in the ribosomal fraction.
4. When the microsomes were treated with higher concentrations of DOC, from 90 to 95% of the ribosomes were released from the membranes, ~90% appearing as monomers in the sucrose-density gradient, and ~10% in the pellet. At the same time, ~75% of the microsomal amylase is released into the DOC-supernate and hence is not placed on the gradient; this released enzyme probably comes from all three fractions mentioned above, and certainly from the heavy region, for this region virtually disappears upon treatment with high DOC.
5. In the above situation a comparison of the amylase in the pellet with that of the monomer region indicates that the same differences in specific radioactivity apply as in the case of the low-DOC experiment (Table I). In addition, this pellet, while containing only one-tenth of the RNA and about one seventh of the amylase activity of the ribosome fraction, still contains an amount of radioactive amylase equal to that in the latter fraction (Table II).
6. The simplest explanation we can offer for the above findings is that, when microsomes are gradually disrupted by detergent treat-

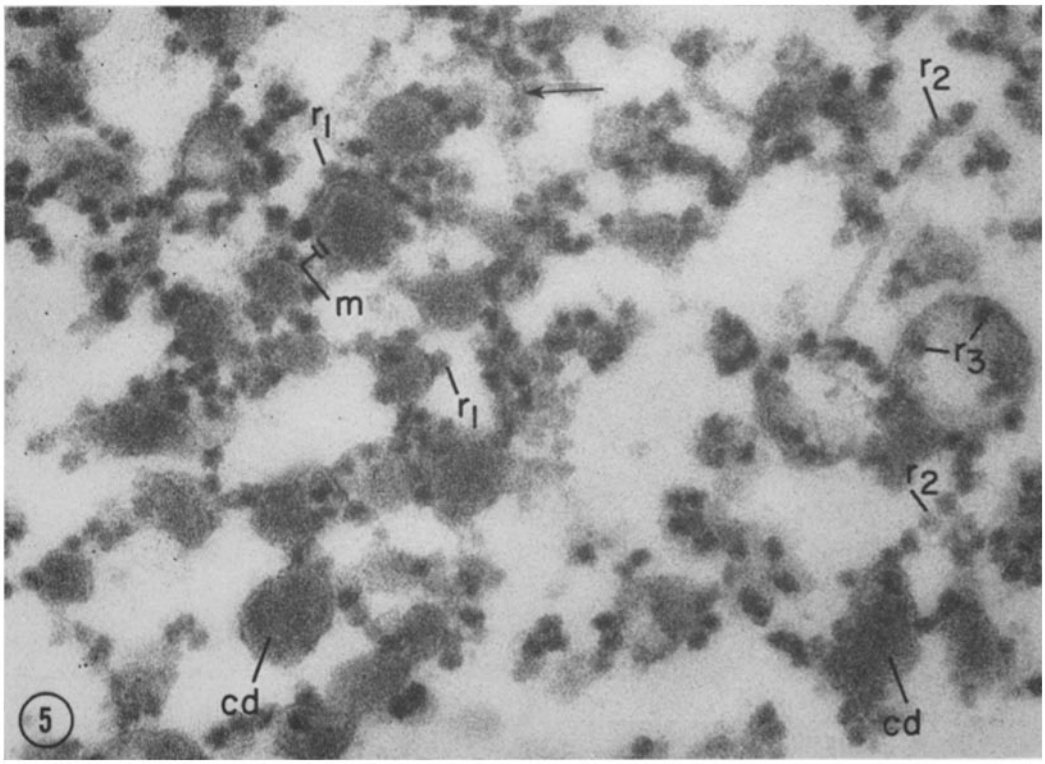

FIGURE 5 Section through the pellet obtained at the bottom of the sucrose gradient. The preparation consists of relatively large microsomes, clusters and chains of ribosomes (r_2) and ruptured membranes (arrow). The microsomes are limited by a unit membrane (m), still bear attached ribosomes (r_1), and have a content of varied, mostly high density material (cd). Some ribosomes have penetrated into ruptured microsomal vesicles (r_3). × 110,000.

ment, ribosomes begin to be detached from the membranes, and those detached most easily are those having low specific radioactive amylase (i.e., a mixture of nascent amylase with enzyme adsorbed from the soluble supernate). Even when disruption of the microsomes is stepped up by high-DOC treatment, there remains on microsomal membranes a small percentage (~10%) of ribosomes which are resistant to detachment by detergent, and contain an amylase of much higher specific radioactivity than that of the monomer-bound enzyme.

TABLE II

Correlations of RNA, Amylase Activity, and Amylase Radioactivity in Various Fractions of the Density Gradient

The figures give the percentages of the total RNA, amylase activity, and amylase radioactivity placed on the density gradient which were recovered in the three regions of the gradient. In the case of the low DOC experiments, the load on the gradient represented all the microsomal RNA and ~65% of the microsomal amylase; in the case of the high DOC experiments, it represented all the microsomal RNA and ~25% of the microsomal amylase. The low DOC figures are averages of Experiments 1 and 2, Table I, and the high DOC figures are averages of Experiments 3 and 4, Table I.

Condition		Heavy peak	Light peak	Pellet
		per cent		
Low DOC	RNA	—*	50	50
	Amylase activity	16	44	40
	Amylase radioactivity	4	20	76
High DOC	RNA	—*	92	8
	Amylase activity	0	87	13
	Amylase radioactivity	0	48	52

* Since no OD_{260} appeared in this region, no orcinol reactions were performed on the corresponding gradient fractions; thus it is assumed that the ribosomes in this region (see Fig. 3) represent 1% or less of the total ribosomes and that the pellet RNA and monomer RNA account for 100% of the microsomal RNA put on the gradient.

Since there is no difference in specific radioactivity among the residual proteins[4] of the three fractions investigated (Table I), it is clear that the presence of newly synthesized, completed protein (which can be assayed as enzyme activity) on the ribosomes coincides with a firmer attachment of the ribosomes to the membrane. One of the factors responsible for this situation might be that part of the amylase molecule is still firmly bound to the ribosome, while the rest of it is already anchored to the membrane of the ER. The membrane fragments containing the ribosomes having nascent enzymes might be rendered more resistant to the detergent action of DOC. An alternative explanation is that the attached ribosomes that contain the nascent protein also carry the postulated messenger RNA, and that it is this latter molecule which aids in the binding of the ribosome to the membrane.

A situation similar to that encountered in the pancreas has already been found by Sabatini et al. (20) in rat liver. After in vivo labeling of ribosomal protein, they solubilized by EDTA treatment ~70% of the ribosomal material (in the form of ribosomal subunits) of a microsomal preparation, but only 20 to 30% of the radioactive ribosomal protein, and concluded that the small proportion of ribosomes which remain attached to microsomal membranes contains the greatest proportion of newly synthesized protein.

We assume that the strong attachment to the subjacent membrane of ribosomes carrying nascent amylase is connected with the role of the endoplasmic reticulum as a passageway for secretory proteins. Indeed, in in vitro experiments (19) with pigeon pancreas microsomes capable of synthesizing amylase, it has been found that most of the synthesized enzyme is released from the ribosomes, not into the medium, but into the cavities of the microsomes (ER fragments) on which the ribosomes are bound. There is apparently a vectorial factor in the release of secretory enzymes from ribosomes, which leads to their transfer across the membranes of the ER.

The differential strength of the binding of certain ribosomes to the membranes of the ER is a

[4] The nature of this residual trichloroacetic acid–insoluble material is unclear. The method we have used extracts not only over 90% of the amylase but also over 90% of the other secretory proteins attached to the ribosomes. We look upon this residual material as unfinished polypeptide chains possible mixed with some nonsecretory proteins.

further complicating factor in experiments dealing with polyribosomes, particularly in those cells where ribosomes exist mostly attached to membranes. Thus, it has already been found that the treatment of microsomes with detergents gives different sucrose-density profiles in the case of hepatoma as compared to normal liver (21). Even in normal liver, the concentration of DOC has been found to be critical in giving consistent results (22). The use of DOC has been found to be important in differentiating the polysome profiles obtained from HeLa cells and from virus-infected HeLa cells (23–25). Finally, the work of Howell et al. (26) shows that liver microsomes can be separated into fractions whose ribosomes behave differently with regard to detachment by DOC and amino acid incorporation. All these results indicate that the metabolic state of the ribosomes must be considered in studies dealing with the isolation and protein-synthetic capacities of these particles. Even in bacteria it has been reported that the ribosomes present in a low-speed fraction, which also contains fragments of cell wall and plasma membrane, are more active in protein synthesis than the free ribosomes isolated from the same cells (8, 27–32), and it has been suggested that the nascent protein stabilizes the attachment of the ribosomes to the membrane (33). However, it is still questionable whether the relationship between the ribosomes of this low-speed bacterial fraction and the cell membrane is the same as that between pancreatic (1) or hepatic (4) ribosomes and the membrane of the ER: some of the bacterial ribosomes appear to be in the immediate vicinity of, rather than firmly attached to, the membranes involved, as shown in *Diplococcus pneumoniae* (34) and in *E. coli*[5] (35).

Finally, some comments are required concerning the presence of the peak of amylase activity found in the heavy region of the sucrose gradients in our experiments. It is not clear why the amylase-containing vesicles of this heavy peak should sediment as a sharp boundary instead of spreading throughout the gradient. A possible interpretation is that we are dealing with a mixture of polysomes and smooth surfaced vesicles of the type seen at the periphery of the Golgi complex in intact cells and that these vesicles are more resistant to low DOC treatment than the usual rough microsomes. In this case the rough microsome appearance of the elements in the heavy peak would be an artifact developed during fractionation or separation, a possibility which is not completely excluded by our morphological findings. However, a smooth microsomal fraction has recently been isolated from guinea pig pancreatic slices labeled in vitro (36) with leucine-C^{14}. In addition to its distinctive morphology, this fraction is characterized by a high specific radioactivity of its contained (exportable) proteins at 20 min after the beginning of incorporation. In our experiments (Table I) it is clear that there is no increase in specific radioactivity of the amylase in the heavy peak over that of the enzyme in the pellet at 15 and 30 min.

It should be noted that other regions of the density gradient, immediately lighter and heavier than the heavy amylase peak, were also found to contain small vesicles with a dense content and some ribosomes still attached to their limiting membranes. A good deal of chymotrypsinogen was found in the gradient, with two peaks of activity appearing, reproducibly, one in the middle of the density gradient (lighter than the amylase region), and the other in the monomer region. RNase activity was also found in the density gradient, with a sharp peak either coinciding with the ribosome peak or appearing in a region just heavier to it. Thus, it was found that there were vesicle-containing regions in the density gradient where one could detect amylase and chymotrypsinogen activities but not RNase activity or where chymotrypsinogen activity alone was detectable. It is also interesting to note that the higher the molecular weight of the enzyme (amylase > chymotrypsinogen > RNase), the closer to the bottom was its heavy peak of enzyme specific activity. Both chymotrypsinogen and RNase activities behaved like amylase activity in response to higher DOC treatment and to the presence of KCl-tris buffer-Mg^{2+} in the sucrose density gradient. A satisfactory explanation of the appearance of these distinctive profiles in density gradients is evidently not available, but the finding that partial separation of enzyme activities can be attained invites the speculation that there might be specialization and compartmentation within the rough endoplasmic reticulum of the exocrine cell for the production of specific secretory proteins; in other words, that some cisternae of the rough ER and their attached ribosomes are exclusively or predom-

[5] Unpublished observations on *E. coli* by Dr. J. D. Jamieson.

inantly engaged in the production of amylase, while others synthesize and segregate other digestive enzymes or zymogens.

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