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THE USE OF RADIOACTIVE CHOLINE AS A LABEL FOR MICROSOMAL MEMBRANES

II. STUDIES ON MEMBRANE–RIBOSOME INTERACTION, SUBFRACTIONATION AND FUNCTION

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SUMMARY

(1) The specificity of radioactive, methyl-labelled choline for membrane lecithin in rat liver microsomes enabled its use as a marker to follow membranes in a number of microsomal subfractionations: (a) Solubilization of membranes by means of the detergents, sodium deoxycholate and Triton X-100. (b) Separation of free ribosomes from microsomal membranes on 5–20% sucrose gradients. (c) Fractionation of microsomal membranes according to their approximate isopycnic densities on 30–60% sucrose gradients. (d) Location of membranous contaminants on 10–30% sucrose gradients during the sedimentation analysis of free polysome preparations and investigation of the interactions between membranous contaminants and polysomes.

(2) Tritiated choline served as a membrane marker in double labelling experiments with [^{14}C]glucosamine, in which the latter was used to measure the ribosomal initiation of carbohydrate prosthetic group attachment onto glycoproteins during their biosynthesis. These confirmed previous results in showing that glycoprotein prosthetic group initiation was virtually restricted to endoplasmic reticulum bound ribosomes.

(3) Using choline-labelled lecithin as a radioactive substrate for phospholipase C it was easy to follow the hydrolytic breakdown of phospholipids in intact membranes under very gentle conditions at 0°. This facilitated a study of the role of phospholipids in the attachment and stabilization of bound ribosomes on the endoplasmic reticulum membranes.

INTRODUCTION

It was shown in a previous paper[1] that radioactive choline, injected along with non-radioactive amino acids was incorporated *in vivo* by rat liver microsomes exclusively into lecithin. Lecithin is a major component of microsomal membranes and the virtual absence of incorporation under our conditions into other microsomal components including non-membrane lipoproteins[1], permits the use of radioactive choline as a membrane label. Labelled lecithin can be counted directly in acid insoluble

precipitates without isolation and purification and this allows multiple samples to be monitored rapidly and conveniently on automated counters.

This report describes the application of choline labelling in four different types of microsomal subfractionation and to investigate the interaction between free polysomes and membranous components which contaminate them during isolation. Double labelling experiments using [^{3}H]choline and [^{14}C]glucosamine to investigate the specificity of free and bound ribosomes in glycoprotein biosynthesis are also discussed. Procedures are outlined for breaking down the bulk of the phospholipids in intact microsomal membranes, under very gentle conditions, using phospholipase C at 0°. Phospholipid hydrolysis was readily followed by the release of radioactive phosphorylcholine from labelled membranes. This technique facilitated a study of the role of phospholipids in the attachment and stabilization of bound ribosomes on the endoplasmic reticulum membranes.

MATERIALS AND METHODS

Preparation of labelled fractions

Rats were injected with [*Me*-^{3}H]- or [*Me*-^{14}C]choline and 10 mg of an amino acid mixture, 30–35 min before killing, as described previously[1]. Liver microsomes were prepared as before[1] but various ions were added to the sucrose media. (Medium A: Tris (pH 7.6), 50 mM; KCl, 25 mM; Medium B: Tris (pH 7.6), 50 mM; KCl, 25 mM; $MgCl_2$, 5 mM.) Free polysomes were prepared by the discontinuous 1.5 M/2.0 M sucrose gradient method described by BLOEMENDAL *et al.*[2].

Use of detergents to prepare ribosomes

To a post mitochondrial supernatant prepared from a 20 % liver homogenate in 0.35 M sucrose–Medium B, was added 1/3 vol. of the appropriate concentration detergent solution (Triton X-100 or deoxycholate) in 0.35 M sucrose–Medium A. The suspension was clarified by careful inversion 6 times and centrifuged in the Spinco 50 rotor at 150000 × *g* for 60 min. The clear yellow supernatant was floated off by gently introducing 1.0 M sucrose just above the tightly packed ribosome pellet. In no case was any "fluffy layer" observed, such as was reported with 0.26 % deoxycholate by ERNSTER, SIEKEVITZ AND PALADE[3]. The rinsed pellet was finally resuspended in water before analysis.

Use of deoxycholate to prepare membranes

One third of a volume of 2 % deoxycholate was added to a suspension of the microsomal subfraction containing 10 mg protein per ml in 0.35 M sucrose–Medium B. The clarified suspension was centrifuged in the Spinco SW 50 rotor at 150000 × *g* for 90 min to sediment ribosomes and the clear yellow supernatant taken for analysis.

Sucrose gradients

Linear sucrose gradients were prepared using a mixing device as described by BRITTEN AND ROBERTS[4]. The linearity of the gradients was established by the use of the dye methyl green. After layering microsomes, purified endoplasmic reticulum membrane fragments or free polysomes (1–2 ml) on top of the gradients these were centrifuged in the SW 25 rotor of the Spinco at 0° and stopped without braking. The

tubes were punctured with a needle and equal sized fractions 1.5–2 ml in volume were collected from the bottom of the tube.

Analysis of free polysomes on sucrose gradients

20 ml linear 10–30 % sucrose gradients were prepared above a cushion of 7 ml of 50 % sucrose in Spinco tubes: the sucrose contained Medium B. The free polysome suspension (in 1 ml volume approximately equivalent to 0.5 mg RNA) was layered on top of the gradient and centrifuged in the SW 25 rotor at 64000 × *g* for 2 h at 0° and fractions collected as above. The absorbance of each fraction was measured at 260 mμ and radioactivity was determined on 0.1 ml samples from each fraction as described below.

Assay of ribosome detachment from endoplasmic reticulum membranes in vitro

To measure ribosome detachment accurately by the method of SABATINI, TASHIRO AND PALADE[5] the endoplasmic reticulum membranes must be first carefully purified from free ribosomes and polysomes. This preliminary purification was performed by a technique described previously, which utilizes the large differences in density between ribosomes and membranes[6]. Post mitochondrial supernatant (5 ml) was layered atop a discontinuous sucrose gradient consisting of 4 ml of 0.5 M sucrose and 3 ml of 2.0 M sucrose–Medium B. The system was centrifuged at 105000 × *g* in the Spinco 40 rotor for 4–16 h, so that the high density ribosomes and polysomes sedimented into the 2.0 M sucrose layer while the endoplasmic reticulum membranes remained at the 0.5 M/2.0 M sucrose interface whence they were collected by pipetting. After dilution to a concentration of 1 g liver equivalent per ml the endoplasmic reticulum membranes were treated with phospholipase and ribonuclease and the effect of these treatments upon ribosome binding was assayed by the SABATINI method[5]. This assay consisted of sedimentation of the membranes through 28 ml of a continuous 5–20 % sucrose gradient for 2 h at 64000 × *g* in the SW 25 rotor. The membrane bound ribosomes pelleted to the bottom of the tube while any released ribosomes remained in the supernatant and were decanted. Ribosome release was measured by the difference in RNA content between the experimentally treated membranes and controls treated with water, both being run on the Sabatini gradient.

Treatment with phospholipase and ribonuclease

Samples of purified endoplasmic reticulum membranes or free polysomes were incubated with various amounts of phospholipase C at 0° or 37° in the presence of 1–5 mM $CaCl_2$ and 10–75 mM maleate buffer (pH 7.1). When phospholipase was followed by ribonuclease treatment, an exactly calculated amount of EGTA (pH 7.8) was added to remove Ca^{2+} before ribonuclease was added and the samples were stood a further 30 min at 0°.

Analysis of fractions

The radioactivity of acid insoluble components was determined on glass fibre discs as described previously[1]. Protein was estimated by the method of LOWRY *et al.*[7]. RNA was determined by the Schmidt–Thannhauser procedure as modified by FLECK AND MUNRO[8], omitting lipid solvent treatment[9]. The RNA content of the reacidified alkali digests was calculated using an $E^{1\%}_{1\,cm}$ of 312 (ref. 10).

RESULTS

The isolation of ribosomes using detergents

Since the early work of LITTLEFIELD *et al.*[11], various concentrations of deoxycholate have been used to prepare ribosomes freed of membranes. The concentrations generally lie in the range 0.2 to 1.3%. Recently the non-ionic detergent Triton X-100 at a concentration of 1% has also been used to prepare liver ribosomes[12,13]. The degree of membranous contamination of ribosomes prepared with different concentrations of detergents can be seen from Table I. Satisfactory removal of choline labelled material (*i.e.* membranes) only occurs when deoxycholate is used at concentrations of 1% or above. However Triton X-100 at concentrations of 1% leaves a substantial proportion of membranous contaminants in the ribosome fraction and concentrations of 3-4% Triton are needed to give adequate membrane solubilization. This difference in efficiency between deoxycholate and Triton X-100 was observed in 7 separate experiments in which detergent treatment was carried out either directly on the post mitochondrial supernatant or else on isolated microsomes. Triton also showed much more variable efficiency for membrane removal, particularly at concentrations of 1 and 2%.

TABLE I

THE RECOVERY OF RNA AND MEMBRANOUS MATERIAL IN RIBOSOME PELLETS PREPARED USING DETERGENTS

The ribosomes were prepared as described in MATERIALS AND METHODS from a rat injected with 25 μC [^{3}H]choline containing amino acids, 30 min before killing. The ribosomal pellets were counted and analysed for RNA The recoveries are expressed as the percentages of RNA content and radioactivity of water treated controls.

Detergent	*Final concn.* (%)	*RNA recovery* (%)	*Radioactivity* (%)
Deoxycholate*	0.26	84	17
	0.5	99	5.1
	1 0	90	1.0
Triton X-100	1.0	85	9.9
	2.0	95	2 6
	3.0	86	1.9
	4.0	91	1.4

* Obtained from E. Merck AG, Darmstadt, Germany.

Separation of free from bound ribosomes on 5-20% sucrose gradients

CAMPBELL, SERCK-HANSSEN AND LOWE[14] have shown differences in the metabolic activities of free and membrane bound ribosomes from rat liver by separation of these two classes on continuous 5-20% sucrose gradients. Centrifugation on these relatively low density gradients for a short time exploits differences in the sedimentation rate of components, with the free, monomer ribosomes and smaller free polysomes trailing the membrane bound ribosomes, which themselves sediment to the cushion of 50% sucrose at the bottom of the tube. The position of the smooth or agranular membranes in this system has not hitherto been considered, but application

of the choline labelling method to follow membrane distribution clearly shows (Fig. 1) that the smooth membranes sediment together with the rough membranes, only one labelled peak being obtained. Identical results were obtained in two experiments. This continuous gradient procedure would appear to offer a method for preparing free ribosomes and polysomes much less contaminated than those obtained by the discontinuous gradient methods, which yield fractions seriously contaminated by membranes[15,16].

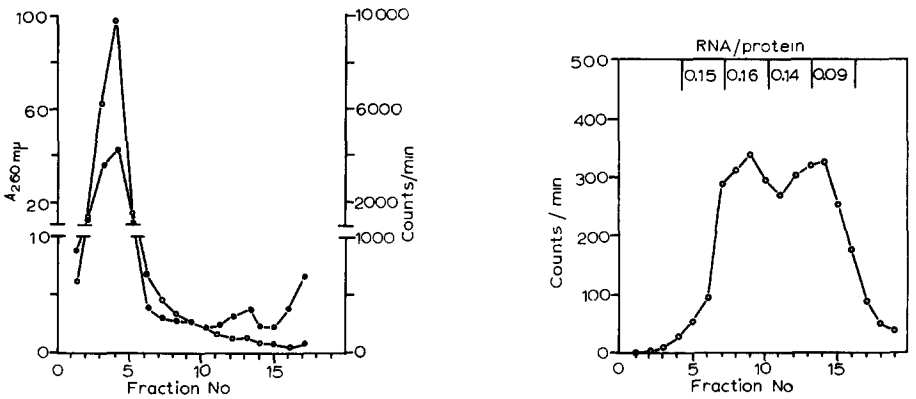


Fig. 1. Rat liver microsomes prepared and fractionated on a 5–20% sucrose gradient (20 ml) above a 50% sucrose cushion (5 ml) containing ions as described by CAMPBELL, SERCK-HANSSEN AND LOWE[14]. The rat was injected with 50 μC [^{3}H]choline containing amino acids, 30 min before killing. The gradient was centrifuged at 22000 rev./min for 135 min in the Spinco SW 25 rotor. ●—●, $A_{260\,m\mu}$; ○—○, counts/min.

Fig 2. Isopycnic density gradient centrifugation of microsomes on a 30–60% sucrose gradient (25 ml) containing Tris (pH 7.6), 50 mM, KCl, 25 mM; CsCl, 15 mM atop an 80% sucrose cushion (5 ml), centrifuged for 16 h at 25000 rev./min in the SW 25 rotor. The microsomes were prepared in the presence of Medium A and resuspended in 0.25 M sucrose containing Tris, KCl and CsCl as above. The rat was injected with 25 μC [^{3}H]choline containing amino acids, 35 min before killing.

Resolution of microsomal membranes on dense sucrose gradients

Separation of smooth from rough microsomal membranes is technically difficult and cannot be achieved simply by differential centrifugation[17]. Use of discontinuous gradients[17–19] allows a separation commensurate with the graduations in density used. However, a continuous gradient centrifuged near to equilibrium will separate the complete range of microsomal membranes on the basis of their density[20].

Prior to using choline to follow microsomal membranes when they are fractionated on gradients, the uptake of radioactive choline into rough and smooth surfaced membrane fractions of microsomes, freed of ribosomes by centrifugation after deoxycholate treatment, was investigated. The results (Table II) show that the radioactive choline content of both classes of membranes generally reflects their protein content quite closely, under our conditions of labelling, though the smooth membranes showed slightly more incorporation than the rough in one experiment.

In Fig. 2 choline is applied as a membrane marker in the fractionation of microsomes on a 30–60% sucrose gradient containing caesium. The membranes are separated into two peaks, which we suppose on the basis of their RNA/protein ratios and density to represent impure granular and agranular membrane fractions. A similar

TABLE II

UPTAKE OF LABEL INTO THE ROUGH AND SMOOTH SURFACED MEMBRANE FRACTIONS OF MICROSOMES

The fractions were prepared as described by the authors mentioned in the table. They were treated with deoxycholate as described in MATERIALS AND METHODS and the solubilized membranes were analysed for protein and radioactivity after removal of the ribosomes by centrifugation at 150000 × *g* for 90 min The rats were injected with labelled choline containing amino acids 35 min before killing.

Method of preparation	*Isotope*	*Counts/min per mg protein*	
		Rough membranes	*Smooth membranes*
Ref. 25	^{3}H	7 440	7 420
Ref 26	^{3}H	11 600	11 600
Ref 18	^{14}C	850	710
Ref. 25	^{3}H	3 840	4 570

separation was obtained in two other experiments. Caesium was used in order to increase the density of granular membranes, while reportedly not affecting the agranular membranes[18]. The presence of magnesium with no caesium led to a poorer separation of the peaks, as DALLNER's findings would predict[18]. The 16 h centrifugation appears to have brought the membranes near to equilibrium, and the free ribosomes (density 1.5–1.6 g/l) appear to have sedimented right through the sucrose cushion (density 1.3 g/l) and form a pellet at the bottom of the tube with an RNA/protein ratio of 0.8.

In addition to the two major peaks there is some less well defined material which sediments between them. The RNA/protein ratio of this material (0.15–0.08) suggested that it might be intermediate in nature between fully granular and agranular endoplasmic reticulum and this has now been confirmed by further investigations in this laboratory, which show that membranes of an intermediate character exist over the complete density range between fully granular and agranular reticulum[20,*].

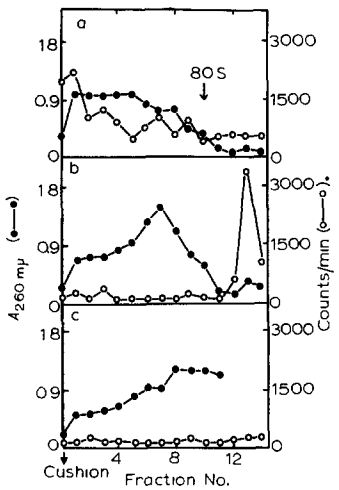
Stabilization of free polysomes by membranes

TSUKADA AND LIEBERMAN[21] observed that free polysomes were broken down to monomer ribosomes by lipases or phospholipases and suggested that the polysomes may be stabilized in some fashion by the phospholipids of membranes cosedimenting with them during their isolation. An investigation of this phenomenon was undertaken using analysis on 10–30 % sucrose gradients to check polysome integrity while membranes were located by their radioactivity after choline labelling. Fig. 3a shows a typical sedimentation pattern for control free polysomes. They are almost exclusively in polysomal aggregates with very few contaminating monomers or dimers, however, membranes are distributed throughout the gradient with a noticeable concentration towards the bottom. Fig. 3b demonstrates the effect of 0.5 % deoxycholate. This effectively solubilizes most of the membranes, converting them to material which remains atop the gradient. However it does not produce any significant increase in monomers or dimers and therefore does not cause polysome breakdown though there

* T. HALLINAN AND RANA MUNNS, unpublished results

is a noticeable shift to a smaller apparent aggregate size for some of the polysome species.

A similar pattern was observed after treatment with phospholipase C in the presence of liver postmicrosomal supernatant, which provided ribonuclease inhibitor to inhibit any traces of ribonuclease which might be present in the phospholipase C preparation (Fig. 3c). Phospholipase C catalysed extensive hydrolysis of the phospholipids, but contrary to the findings of TSUKADA AND LIEBERMAN[21] caused little detectable breakdown of polysomes. It is likely therefore, as suggested previously by BLOEMENDAL *et al.*[2], that the breakdown of polysomes accompanying phospholipase action is due to contaminating ribonucleases. The absence of significant polysome breakdown when most of the contaminating membranes or their phospholipids have been demonstrably removed (*cf.* ref. 2), suggests that neither the membranes nor their phospholipids play an essential role in polysome stabilization.



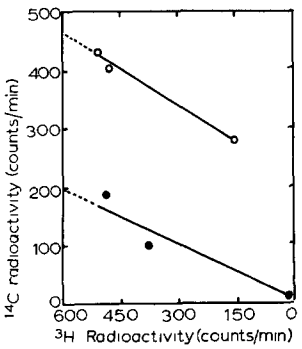


Fig. 3. Gradient analysis of free polysomes prepared according to BLOEMENDAL *et al.*[2]. After carefully removing any material adhering to the wall of the tube above the free polysome pellet, this was surface rinsed and resuspended in 0.25 M sucrose–Medium B. Samples a–c, treated as indicated, were layered atop a 20-ml linear 10–30% sucrose–Medium B gradient and centrifuged at 64000 × *g* for 2 h and fractions were collected as described in MATERIALS AND METHODS The rat was injected with 100 μC [^{3}H]choline containing amino acids 35 min before killing. a, Untreated control; b, made 0.5% to deoxycholate; c, treated with 50 μg of phospholipase C at 37° for 30 min in the presence of 1 mM $CaCl_2$, 75 mM maleate buffer (pH 7.1) and 12.5% post microsomal supernatant. ○—○ counts/min., ●—●, $A_{260\,m\mu}$.

Fig. 4. Plot of [^{14}C]glucosamine counts in glycoproteins *versus* [^{3}H]choline counts in membrane lecithin from samples of bound ribosomes and total free ribosomes (polysomes, dimers and monomers), prepared variably contaminated with membranes[15]. Variable membrane contamination was achieved by treating the crude fractions of purified bound ribosomes[15] and total free ribosomes with increasing concentrations of deoxycholate, 0.25, 0.5 and 1%. Those samples from both fractions which exhibit most [^{3}H]choline counts were treated with 0.25% while those with least choline counts were treated with 1% deoxycholate. Amounts of less than 200 μg of protein were plated onto glass fibre discs and prepared for counting in a Packard 3375 liquid scintillation spectrometer using settings chosen to give less than 10% spillover of carbon counts into the tritium channel and zero tritium spillover. The rats were injected with 25 μC of [^{3}H]choline–amino acids, 35 min before killing and 20 μC of [^{14}C]glucosamine, 15 min before killing ○—○, bound ribosomes; ●—●, free ribosomes

Investigation of the roles of free and endoplasmic reticulum bound ribosomes in glycoprotein biosynthesis: a double labelling study

Other investigators of glycoprotein biosynthesis have shown that the attachment of carbohydrate prosthetic groups onto liver glycoprotein apopolypeptides is initiated by the linkage of glucosamine residues onto the polypeptide chains while these are still bound nascent to their ribosomal sites of synthesis (reviewed in ref. 15). However assay of ribosomal prosthetic group initiation using radioactive glucosamine is greatly hindered by the presence of contaminating membranes which contain highly radioactive glycoproteins[15]. It was possible to overcome this difficulty by using [^{3}H]choline to estimate membranous contamination while [^{14}C]glucosamine incorporation was used to measure prosthetic group initiation. Fig. 4 shows the capacity of free and bound ribosomes with decreasing proportions of membranous contaminants to incorporate [^{14}C]glucosamine. It can be seen that removal of membranous contaminants from free ribosomes causes a simultaneous reduction in their capacity to incorporate glucosamine, which extrapolates almost to zero in the absence of membranes. However bound ribosomes continue to show substantial glucosamine incorporation even when their membranous component has been almost wholly removed. This double label experiment confirms earlier results which showed that ribosomal initiation of carbohydrate prosthetic group attachment onto glycoproteins is probably an exclusive function of bound ribosomes[15].

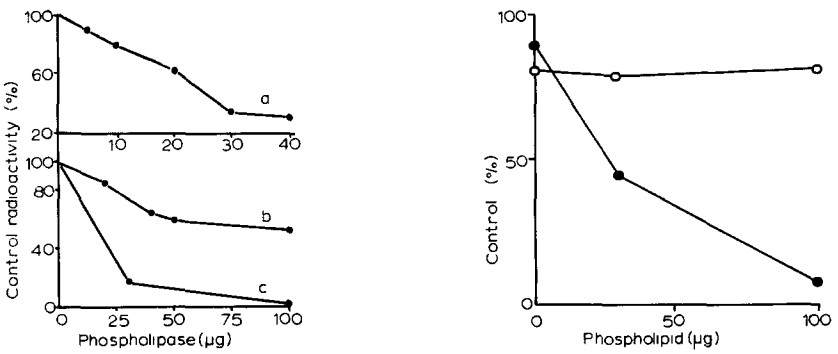


Fig. 5. Digestion of purified, [^{14}C]choline-labelled microsomal membranes with phospholipase C. Membrane samples equivalent to 0.5 g wet liver were treated with 5–100 μg of phospholipase C in the presence of 5 mM $CaCl_2$ and 75 mM maleate buffer (pH 7.0) as described in MATERIALS AND METHODS and samples were plated on glass fibre discs and counted. Recovery of counts was compared with controls maintained at 0° in the complete incubation mixture *minus* phospholipase C. a, incubated 15 min at 37°; b, treated 30 min at 0°; c, treated 15 min at 0° in the presence of 110 μg of ribonuclease

Fig. 6. The extent of attachment of bound ribosomes to endoplasmic reticulum membranes subjected to the stepwise breakdown of their lecithin by phospholipase C: effect upon the susceptibility of the bound ribosomes to ribonuclease. Purified microsomal membranes[6] equivalent to 0.5 g wet liver were treated for 30 min at 0° with 30 and 100 μg of phospholipase C in the presence of 1 mM $CaCl_2$ and 10 mM maleate buffer (pH 7.1) They were then made 1 mM to EGTA to remove calcium and treated a further 30 min at 0° with 50 μg/ml ribonuclease before being loaded on the SABATINI gradient[5] to assay for ribosome release. A further sample was treated identically except that phospholipase C was omitted from the first incubation *i.e.* it was only digested with ribonuclease. Comparison was made with duplicate controls incubated in the absence of both phospholipase and ribonuclease and then analysed directly without centrifugation on the Sabatini gradient. The rat was injected with 10 μC [^{14}C]choline–amino acids, 35 min before killing. ●—●, phospholipid; ○—○, RNA

Membrane phospholipids and the attachment and stabilization of endoplasmic reticulum bound ribosomes

As well as a convenient marker, radioactive choline incorporated into membrane lecithin can also serve as a substrate for phospholipases, and used thus, provides a simple radiochemical assay for their action upon intact membranes. It has been possible using phospholipase C to remove the lecithin from intact microsomal membranes under very gentle conditions at 0° as well as at 37° with the formation of two, non-lytic products, phosphorylcholine, which is acid soluble and diglyceride (Fig. 5). In the course of this study we adventitiously observed that ribonuclease often stimulates phospholipase C at 0° (Fig. 5c). This effect is yet to receive detailed attention, but it is possible that ribonuclease, which is a polycation, partly fills the absolute requirement of phospholipase C for cationic activators, though it cannot wholly replace calcium ions in this capacity.

Using granular endoplasmic reticulum fragments carefully purified from free ribosomal contaminants[6] a study was made of the role of membrane phospholipids in attaching ribosomes to the reticulum and in protecting the bound ribosomes from ribonuclease at 0°. This resistance of bound ribosomes to ribonuclease was recently demonstrated by BLOBEL AND POTTER[22]. Fig. 6 shows that the controlled hydrolysis of over 90 % of the lecithin of granular reticulum by phospholipase C caused no detectable release of bound ribosomes; this lack of effect of phospholipase C in detaching ribosomes was observed in four separate experiments. Fig. 6 also shows that lecithin removal does not alter the resistance of bound ribosomes to digestion by ribonuclease at 0°. The amount of ribonuclease used (50 μg/ml) is 10 times the minimum amount required to degrade free ribosomes under these conditions[22].

DISCUSSION

The choline labelling method offers great versatility and ease of application to many problems involving membranes, especially microsomal membranes. An injection of 50 μC of [^{3}H]choline into a 200-g rat gives liver microsomal phospholipids a specific activity of 60 000 disint./min per mg after 30 min. The counting efficiency was 33%, so with a background of 25 counts/min, less than 1 μg of phospholipid (or 0.04 μg of lipid phosphorus) can be accurately detected. This is about 50 times more sensitive than Fiske–SubbaRow type assays commonly used for lipid phosphorus[23]. Increasing the dose will give better sensitivity, the upper limit of which is determined solely on economic grounds: the risk of interference by radiation damage to animals during a 30-min period of labelling by ^{3}H or ^{14}C is considered negligible. Furthermore the response range of modern counters is such that it is rarely necessary to consider adjusting the concentration of samples so they fall within the range of the assay procedure. This factor, taken together with the short time in which the samples can be prepared for automatic counting (20 samples were routinely prepared in 3–4 h), makes the method particularly suited to gradient analysis. In addition, protein, RNA and glycoprotein can be also directly counted in acid insoluble precipitates, so that simultaneous monitoring of selected components using double labelling techniques can be readily obtained. Finally, the technique is by no means restricted to liver as spleen, small intestine and kidney all show sufficiently high incorporation for radioactive choline labelling to be usefully applied to them also.

Perhaps our most interesting application of the choline labelling technique to date is coupled with phospholipase C digestion at 0° to follow the controlled breakdown of phospholipids in intact membranes. This has facilitated what is probably the first study of the role of membrane phospholipids in attaching bound ribosomes to the endoplasmic reticulum and in protecting them from digestion by ribonuclease. It was found that lecithin, at least, and probably phosphatidylethanolamine and sphingomyelin which are also susceptible to phospholipase C (ref. 24), play no apparent role in either attaching or protecting bound ribosomes. This tends to make models of ribosome attachment to the reticulum, *via* salt linkages between RNA-phosphate and lecithin-choline, highly unlikely. However, phosphatidylserine and the phosphoinositides appear resistant to phospholipase C, so these could still conceivably function in ribosome attachment to the reticulum.

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