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THE USE OF RADIOACTIVE CHOLINE AS A LABEL FOR MICROSOMAL MEMBRANES

I. SELECTIVITY OF LABEL FOR ENDOPLASMIC RETICULUM AND SPECIFICITY FOR LECITHIN

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SUMMARY

1. Radioactive choline serves as a highly efficient and specific label for the lecithin of rat liver microsomes, provided that it is administered with non-radioactive amino acids to inhibit incorporation of radioactivity into protein.

2. The radioactivity of lecithin can be directly measured by counting acid-precipitated fractions. The extraction of lipids before counting is thus avoided, allowing the rapid monitoring of multiple samples.

3. With a labelling time of 30–35 min choline incorporation by rat liver *in vivo* occurs selectively into the microsomal fraction. Evidence is presented that this incorporation does not occur by a simple exchange reaction, but probably *via* the pathway involving phosphorylcholine and CDP-choline.

INTRODUCTION

Microsomes can be separated into a number of subfractions containing different proportions of ribosomes, membranes and other components such as glycogen and ferritin[1–4]. Membranous components contain virtually all the phospholipid and therefore phospholipid provides a useful marker for following membrane distribution among subfractions[4,5]. However conventional procedures of phospholipid analysis require that this be extracted and purified before estimation, normally by phosphorus determination. This is both time-consuming and unsuited to the handling of multiple samples with widely differing lipid contents as obtains in gradient analysis.

It was considered that the use of a radiochemical marker might fill the need for a more versatile method of following the membrane content of fractions. Radioactive choline was chosen since it is known to be incorporated rapidly into the phospholipids of rodent liver and was thought to be fairly specific for lipids[6,7].

This paper demonstrates the absolute specificity of ^{14}C- and ^{3}H-labelled choline for lecithin in rat liver microsomes. This specificity is a necessary condition for the use of choline as a marker for microsomal membranes as described elsewhere[8].

MATERIALS AND METHODS

Preparation of labelled subcellular fractions

Adult albino rats (Wistar) kept on a normal stock diet were fasted overnight (16 h) before use, with water given *ad libitum*. To label phospholipids animals were injected intraperitoneally with 5–10 μC [*Me*-^{14}C]choline (New England Nuclear Corporation, Mass.) or 20–50 μC of [*Me*-^{3}H]choline (Radiochemical Centre, Amersham) contained in a volume of 0.5 ml. In later experiments, the choline was dissolved in water with Bacto casamino acids (Difco Laboratories, Michigan) (20 mg/ml), so that the proportion of counts in non-lipid material could be reduced (see RESULTS AND DISCUSSION).

Rats were killed 30–35 min after injection and the livers immediately removed and chilled. Nuclei, mitochondria, lysosome, microsomes and the supernatant fraction were prepared quantitatively from a 0.25 M sucrose homogenate as described by DE DUVE *et al*[9]. Microsomes were also prepared from a 0.35 M sucrose postmitochondrial supernatant by centrifugation for 60 min at 105 000 $\times$ *g*. These were resuspended in distilled water at a protein concentration of 10 mg per ml (ref. 10).

Direct counting of subcellular fractions

Duplicate 0.1-ml aliquots of subcellular fractions containing less than 10 mg protein/ml were plated onto glass-fibre discs[11] (Whatman GF/C 2.1 cm) and washed 6 times in 0.2 M $HClO_4$ in small beakers. This treatment removes all acid-soluble material, while leaving all acid-insoluble material on the disc. Discs were stood in ammonia vapour for 30 min to neutralize $HClO_4$ and prevent the subsequent formation of a yellow colour. After drying under an infrared lamp, the discs were placed in 5 ml nonpolar scintillant (0.1 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 4 g 2,5-diphenyloxazole, 1 l toluene) in small stoppered disposable vials (1.5 cm $\times$ 5 cm) which were inserted into the standard counting vials. The counting efficiency is only reduced 1–2 % by this procedure, and the more expensive counting vials can be reused if required. All counting was performed in a Packard Tri-Carb liquid scintillation spectrometer (314A or 3375). The radioactivity recorded bore a linear relation to the amount of material plated, up to 1 mg protein per disc, both for [*Me*-^{14}C]- and [*Me*-^{3}H]choline.

Isolation, separation and counting of phospholipids

Phospholipids were isolated quantitatively from subcellular fractions by 3 extractions with chloroform–methanol (2:1, v/v)[12]. The extracts were purified by saline washing to remove all labelled small precursor molecules[7]. After evaporation and digestion of the washed extracts, phosphorus was estimated by the method of ALLEN[13]. Phospholipid was calculated by multiplying the phosphorus content by 25 (ref. 3).

Separation of phospholipid components was carried out by thin-layer chromatography on Silica Gel G (E. Merck, Darmstadt, Germany) of 500 μ thickness. The plates were developed with chloroform–methanol–water (16:7:1, v/v/v)[14], and phospholipids located by staining with Rhodamine G. Phospholipids were eluted from the stained spots by 3 extractions with chloroform–methanol (2:1, v/v)–0.1M HCl.

The eluates were evaporated to a small volume at room temperature and exposed to ammonia vapour to neutralize acid before being taken to dryness.

The evaporated phospholipids were dissolved in 0.1 ml hyamine and 0.1 ml methanol and 15 ml of polar scintillant (5 g 2,5-diphenyloxazole, 90 g naphthalene, 360 ml dioxane, 216 ml ethanol, 360 ml toluene) added for counting. A uniform quench of about 20 % was observed in all samples and radioactivity was determined as disint./min by recounting every sample with internal standard added.

Isolation of intact RNA

RNA was extracted undegraded with phenol–sodium laurylsulphate–8-hydroxyquinoline, including the ribonuclease inhibitors naphthalene 1,5-disulphonate and bentonite, as described by HALLINAN AND MUNRO[15]. The purification of extracts was continued to the third ethanol precipitation. Further purification was carried out in two separate ways (i) the RNA was re-precipitated three more times with ethanol, or (ii) the RNA solution was dialysed exhaustively against the salt buffer at 0°. RNA was counted by plating on glass-fibre discs in non-polar scintillant.

RESULTS AND DISCUSSION

The specific activities of each subcellular fraction[9] from the liver of a rat killed 35 min after the injection of [^{14}C]choline are shown diagramatically in Fig. 1. Fractions were isolated quantitatively, the recovery of radioactivity being 93 % and of protein

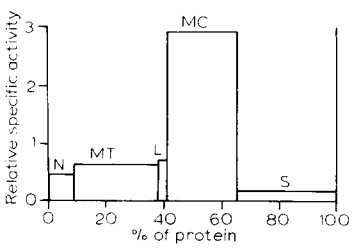

Fig. 1. Relative specific activities of choline-labelled rat liver subcellular fractions. The specific activity was measured as counts/min per mg protein, but is expressed here relative to that of the whole homogenate (3470 counts/min per mg protein) whose specific activity is taken as unity. N, nuclei; MT, mitochondria; L, lysosomes; MC, microsomes; S, soluble fraction.

92 %. It is seen that of all the fractions microsomes, which contain 69 % of the activity incorporated, have by far the greatest uptake of choline into acid-insoluble components, which is consistent with previous demonstrations of the localisation of lecithin biosynthesis in this fraction[16,17]. The contribution to microsomal radioactivity of labelled compounds from other subcellular fractions contaminating the microsomes can thus be regarded as very small when considering the later results of the studies on microsomal lipids. However, this assumption may only be justified for the comparatively short labelling times used in this study. A 30–35-min labelling time was used in all experiments because of a previous observation[7] that phospholipids from the granular and agranular microsomal membranes exhibit approximately the same specific activity about this time. Shorter periods of labelling often caused greater differences in specific activity between granular and agranular microsomal mem-

branes, while more prolonged labelling might lead to more activity in subcellular fractions other than microsomes.

The proportion of radioactivity in microsomal lipid components

Resuspended [^{14}C]choline-labelled microsomes were plated and acid washed. Some discs were further extracted with chloroform–methanol (2:1, v/v) 3 times.

TABLE I

REMOVAL OF ACID-INSOLUBLE RADIOACTIVITY FROM CHOLINE-LABELLED MICROSOMES BY LIPID AND NUCLEIC ACID EXTRACTION PROCEDURES

Microsomal protein (mg)	*Material*				
	Acid insoluble (counts/min)	*Non-lipid*		*Non-lipid, non-nucleic acid*	
		(counts/min)	*% of total**	*(counts/min)*	*% of total**
0.50	7 330	440	6.0	390	5.3
0.75	11 680	583	5.0	567	4.8
0.85	12 700	699	5.5	683	5.4
1.00	13 620	801	5.9	845	6.2

* Non-lipid radioactivity averaged 5.6 % and non-lipid, non-nucleic acid radioactivity 5.4 % of the total, respectively.

Others were additionally treated with 0.2 M $HClO_4$ at 85–90° for 30 min after lipid extraction, to remove nucleic acids also. All discs were then ammonia treated, dried and counted. The results (Table I) show that over 94 % of the radioactivity present is in lipid, and that scarcely any additional radioactivity is removed from the disc when nucleic acids are extracted. Virtually identical results were obtained with [^{3}H]-choline-labelled microsomes, and similar results with another sample of microsomes labelled with [^{14}C]choline, although here only 88 % of the radioactivity was present in lipid and 2.3 % was extracted along with the nucleic acids by hot $HClO_4$.

Radioactivity of RNA when choline is injected

The preceding experiment suggests that RNA contained little or no radioactivity. This was verified directly by comparison of the specific activities of RNA and phospholipids isolated from the same microsomal fraction, labelled with [^{14}C]choline.

The purity of the intact RNA isolated as described in MATERIALS AND METHODS was established by measuring the ultraviolet spectra of the two purified samples. In both cases the RNA content as calculated from phosphorus measured by ALLEN's method[13], and that calculated from absorbance at 260 mμ agreed to within 2 %.

The specific activities of the RNA samples were both less than 7 counts/min per mg, while the extracted phospholipids had 72 000 counts/min per mg. Even allowing for varying proportions of RNA and phospholipid in different microsomal subfractions, this very low activity of RNA shows that it could never contribute a significant proportion of counts.

Radioactivity in protein when choline is injected

It would appear that the remaining radioactivity not in phospholipid can be accounted for by protein. Glycogen is unlikely to be labelled, since in the fasted rats

used here there is a very reduced amount of liver glycogen, and most of this is acid soluble[18]. An experiment (not described in detail here) where the insoluble material after lipid extraction of labelled microsomes was subjected to a partial alkaline hydrolysis supported the idea that the radioactivity in this non-lipid soluble portion was in protein. The solubilisation of protein by hydrolysis exactly paralleled the loss in radioactivity.

TABLE II

THE EFFECT OF AMINO ACID INJECTION ON NON-LIPID RADIOACTIVITY

Casamino acids (10 mg) were injected prior to choline at the times indicated. Animals were killed after 30 min choline labelling and acid-insoluble radioactivity in non-lipid material was determined. The control received 0.5 ml of 0.9 % NaCl, 8 min before choline injection.

Time (min)	*% radioactivity in non-lipid material*
20	2.6
8	1.1
5	1.1
0	1.0
control	4.4

TABLE III

THE EFFECT OF AMINO ACIDS ON THE INCORPORATION OF RADIOACTIVITY INTO MICROSOMAL PHOSPHOLIPID

Two pairs of matched, 100-g rats were injected each with 5 μC [^{14}C]choline, 30 min before killing. One pair was also given 10 mg of casamino acids with the choline.

Treatment	*mg phospholipid per mg protein*	*Phospholipid (counts/min per mg)*
With casamino acids	0.40	13 900
Without casamino acids	0.39	13 200

The methyl label from choline may be incorporated into serine[19], methionine[6], and into some other amino acids less directly. It was thought that by flooding the amino acid pools of the rat, incorporation of label into protein would be reduced. Accordingly a solution of 10 mg of Bacto casamino acids was injected at various times before the injection of [^{14}C]choline. The animals were killed 30 min after the choline injection and the acid-insoluble radioactivity of liver microsomes determined. Some discs were also extracted with chloroform–methanol so that the proportion of counts in lipids could be determined (Table II). The proportion of counts in lipid can be seen to be increased to 99 % when amino acids are given with the choline. This distribution of radioactivity in the presence of casamino acids was checked in 6 different experiments and non-lipid radioactivity was found to average 0.95 $\pm$ 0.45 % (S.D.) over the entire series of 20 samples.

The isolated phospholipids of microsomes of two pairs of matched rats, injected with or without casamino acids, showed identical specific activities (Table III). The

extent of incorporation of radioactivity into the lipids is thus not affected by the administration of amino acids. The radioactivity in these extracted lipids was exactly the same as that of the acid precipitates from which they were isolated. This equality of radioactivity in isolated lipids and acid-insoluble precipitates has been demonstrated for both [^{3}H]- and [^{14}C]choline labelling in a whole range of microsomal subfractions containing up to 1 mg protein when counted. The method thus has the important advantage that the lipids can be counted at maximal efficiency without the need for the time-consuming extraction and purification procedures.

The nature of the labelled lipid components

Sonication, under conditions whereby most of the nascent, intracisternal proteins are released and rendered no longer sedimentable with microsomes[20], only releases 1 % of the radioactivity from choline-labelled microsomes. It was concluded therefore that the labelled choline enters membrane structural lipids and that no significant amount is incorporated into nascent proteins or lipoproteins associated transitorily with the microsomes.

With regard to the distribution of the label amongst individual lipid components, the relative radioactivity of separated liver microsomal phospholipids from animals injected with [^{14}C]choline is shown in Table IV. Phosphatidyl choline accounts for 98 % of the radioactivity recovered.

Radioautography of the thin-layer chromatogram revealed only one spot (Fig. 2), corresponding to the positions of lecithin. This shows that the incomplete recovery of 80 % (mentioned in Table IV) was due to inefficiency of elution, and not to the failure to detect and count some other radioactive compound.

TABLE IV

RADIOACTIVITY IN SEPARATED MICROSOMAL LIPIDS

The rat was injected with 10 μC [^{14}C]choline containing amino acids, 35 min before killing. Microsomal lipids were extracted, separated and counted as described in MATERIALS AND METHODS. Overall recovery of radioactivity was 80 %.

Spot number	*Component*	% *Radioactivity*
1	lysolecithin	0.1
2	sphingomyelin	1
3	lecithin	98
4	cephalins	0.3
5	unidentified*	0.0

* This spot contained no phosphorus

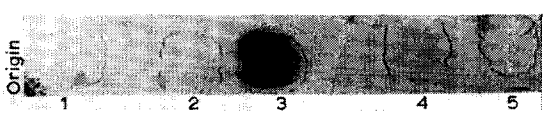

Fig. 2. Radioautogram of separated microsomal-lipid components from a rat injected with [^{14}C]-choline containing amino acids. The position of spots which stained with Rhodamine G are outlined. The numbering of spots corresponds to that used in Table IV; thus the only radioactive spot (No. 3) corresponds to lecithin. Identical results were obtained with 5 separate samples of radioactive phospholipid subjected to thin-layer chromatography and radioautography.

Further supporting evidence for the radioactivity being found only in lecithin was obtained from an experiment in which lipids from [^{14}C]choline-labelled microsomes were subjected to the selective hydrolysis procedure of DAWSON[21]. Almost all (98 %) of the radioactive material was hydrolysed by 0.03 M ethanolic NaOH, and rendered water soluble. This confirms the preceding data (Table IV) that less than 2 % of the radioactivity is found in sphingomyelin and plasmalogen, since these would not be hydrolysed under these conditions. Two-dimensional chromatography of the water-soluble hydrolysis products[21] revealed only one spot on radioautography. The position of this labelled spot corresponded precisely to that of a glycerylphosphoryl choline marker applied at the same time.

Mechanism of choline incorporation

There is strong evidence[7,16] that much of the choline incorporated into rat liver lecithin follows the Kennedy pathway via phosphoryl choline and CDP-choline.

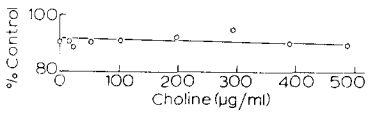

Fig. 3. Displacement of radioactivity from the lecithin of microsomes labelled *in vivo* with [^{14}C]-choline by incubating with unlabelled choline *in vitro*. Incubation was carried out in Tris buffer (pH 9) in the presence of 0.01 M $CaCl_2$ for 30 min, conditions previously reported to give exchange[22]. The radioactivity of incubated samples is expressed as a percentage of that in unincubated control microsomes.

However it has also been reported that free choline can be incorporated *in vitro via* a Ca^{2+}-activated pathway which by-passes phosphoryl choline and CDP-choline and possibly involves a phospholipase D-catalysed exchange between free choline and pre-formed lecithin[22]. Evidence for this pathway was sought by determining the ability of increasing amounts of non-radioactive choline *in vitro* to chase radioactivity from microsomes containing lecithin prelabelled with [^{14}C]choline *in vivo*. These labelled microsomes were incubated with 0–500 µg per ml of [^{12}C]choline under the reported optimum conditions for the exchange reaction[22]. Fig. 3 shows however that no evidence of choline-dependent exchange was obtained. Altering the calcium concentration over the range 0–0.01 M, the pH over the range 6–11 and substituting phosphoryl choline for the choline chaser all failed to elicit any exchange reaction though 7 separate microsome fractions were examined. No evidence for exchange was found either in nuclei, lysosomes, mitochondria or supernatant fractions incubated at pH 7.1 in the presence of 0.01 M $CaCl_2$. These results are consistent with the absence of phospholipase D from animal tissues[23] and thus the existence of the exchange reaction in rat liver must be regarded as doubtful.

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