LOSS OF LIPID DURING FIXATION FOR ELECTRON MICROSCOPY

THOMAS E. MORGAN and GARY L. HUBER. From the Departments of Medicine and Anesthesiology, the University of Washington, Seattle. Dr. Huber's present address is the Second and Fourth Medical Service, Boston City Hospital, Boston, Massachusetts

INTRODUCTION

For many years it has been assumed tacitly that lipids and proteins are retained during the process of fixation by various heavy metals. Experimental evidence documenting this assumption has been sparse but recent studies by Korn and Weisman (1) show that varying amounts of neutral lipids and phospholipids are lost from amebae during fixation for electron microscopy. Stein and Stein[1] also have reported marked lipid losses from hepatic tissue during preparation for electron microscopy. We too have become aware that significant amounts of radioisotopically labeled lipids were being lost from lung tissue during fixation for histologic examination. This first became evident during studies on the incorporation of tritiated choline into lung lipids by newborn guinea pigs. The specific activity of the lipid fractions was sufficient to enable us to study with precision the loss during various stages of fixation for electron microscopy.

[1] Stein, Y., and O. Stein 1966. Personal communication.

METHODS

Newborn guinea pigs within 48 hr of kindling were used in all experiments. All were weighed shortly after birth and again at 24 hr; only those

showing significant weight gain were used. Choline chloride tritium labeled in the methyl group (choline-methyl-^{3}H-chloride, 77.2 μc/μmole, obtained from New England Nuclear Corp., Boston, Mass.) was dried from its ethanol solution under a stream of nitrogen. 1 mc was then dissolved in 1.0 ml of 0.9% sodium chloride. Aliquots of this solution were taken for estimation of total radioactivity and other aliquots were injected intraperitoneally into the guinea pigs. In preliminary studies littermates were sacrificed at 0.5, 1, 2, 3, 12, and 30 hr. The animals were sacrificed after stunning by cross-clamping their necks with large Kelly forceps and decapitating them. A needle was inserted into the trachea and the chest was opened by sternal incision. The lung was inflated gently and the trachea was cross-clamped below the needle. The lung was removed en bloc and divided by sharp dissection for fixation or homogenization in an equal volume of isotonic saline using a glass tissue grinder.

Five volumes of methanol were added with stirring to each volume of homogenate. After standing for 15 min, 10 volumes of chloroform were added, mixed, and, after 30 min, two volumes of water were added with thorough mixing. A two-phase system resulted, the lower being removed. The upper phase was then reextracted twice with chloroform:methanol (2:1). The lower phases were combined and reduced under nitrogen at low pressure; aliquots were taken for measurement of phosphorus, cholesterol, total lipid, phospholipid distribution, and fatty acid analysis by methods previously described (2). Lipid fractions were separated by thin layer chromatography using a chloroform-methanol-water (95:35:4) ascending system and assayed for radioactivity. Lipid fractions were identified by brief exposure to iodine vapor. The fractions were separated by scraping them from the glass plate directly into a scintillation vial. The vial was filled with thixotropic gel and 15 ml of 4% BBOT in toluene. Thixotropic gel (Cab-O-Sil) and BBOT, 3,5 bis-[2-(5-*tert*-butylbenzoxazoly)]-thiophene, scintillation grade, were obtained from Packard Instrument Co., Inc., Downers Grove, Ill. The thin-layer scrapings were suspended by repeated inversion, cooled, and counted in a Packard Tri-Carb liquid scintillation spectrometer with automatic internal standardization. Aliquots of the water remaining after the lipid extraction and aliquots of the original saline solution of choline chloride were spotted on filter paper strips. These were placed in scintillation vials containing 15 ml of 4% BBOT in toluene and were counted. Counting was continued until an amount at least five times that of the background levels was reached. Efficiency of this system was 23.4%. Quenching was determined on individual samples by use of the automatic internal standard, but in no case exceeded 12%. A tabulation of the incorporation results is given in Table I. All results have been corrected for background and quenching.

Since the highest phosphatidyl choline specific activity was obtained at 3 hr after injection, a second experiment was conducted on a single day-old guinea pig. This animal was injected with 1 mc of choline-methyl-^{3}H-chloride in Tris-buffered isotonic saline, pH 7.4, and sacrificed at 3 hr. After inflation the right lung was removed, divided, and weighed quickly. Lipid determinations were made on one aliquot as before; the remaining aliquots were cut into 1 mm cubes and fixed in 10 volumes of fixative at room temperature according to the following scheme:

(*a*) fixation in *s*-collidine buffered 1.3% osmium tetroxide (OsO_4) for 2 hr (method of Bennett and Luft, (3); (*b*) fixation in 4% phosphate-buffered formalin, pH 7.4, for 3 hr followed by *s*-collidine buffered OsO_4 for 2 hr (based on a method suggested by Boatman);[2] (*c*) fixation in 2% glutaraldehyde buffered with cacodylate for 24 hr followed by *s*-collidine-buffered OsO_4 for 2 hr (method of Sabatini, Bensch and Barrnett, (4). (*d*) tricomplex fixation in palladium chloride-osmium tetroxide-sodium phosphotungstate for 2 hr according to the method of Elbers et al. (5).

TABLE I

*Incorporation of Radioactivity into Lipid Fractions**

Time after injection	Water soluble cpm × 10^4/g lung	Lipid soluble cpm × 10^4/g lung	% of Radioactivity in: Neutral lipid	Phosphatidyl choline	Other phospholipid	Specific activity,‡ phosphatidyl choline
hr						
0.5	69	38	—	33.2	0.3	0.58
1.0	79	74	—	46.0	1.5	1.23
2.0	104	171	—	59.7	1.1	2.17
3.0	317	624	0.1	63.8	2.9	3.67
12.0	195	304	0.2	57.5	5.3	1.38
30.0	86	227	2.1	54.9	10.1	1.33

* Newborn guinea pigs were injected intraperitoneally with 8.7×10^7 cpm choline-methyl ^{3}H-chloride and sacrificed in the manner described in the text. Lung samples removed at the times shown were extracted with lipid solvents and chromatographed to separate lipids. The radioactivity in each lipid and water sample was estimated by liquid scintillation (see text).

‡ μC/μmole lipid phosphorus.

[2] Boatman, E. S. Personal communication.

TABLE II

*Loss of Radioactivity from Guinea Pig Lung during Fixation and Dehydration**

Primary fixative	1.3% OsO_4		4% formalin		2% glutaraldehyde		Tricomplex	
Secondary fixative	None		1.3% OsO_4		1.3% OsO_4		None	
	cpm × 10^3	% of total	cpm × 10^3	%	cpm × 10^3	%	cpm × 10^3	%
Loss in								
Primary fixative	396	21.4	285	15.4	429	23.2	375	20.2
Secondary fixative	—	—	153	8.3	21	1.1	—	—
Alcohol	842	45.5	422	22.8	287	15.5	570	30.8
Propylene oxide	nil	—	nil	—	3	0.2	1	0.1
Total loss	1238	66.9	850	46.5	730	39.0	946	51.3
Radioactivity remaining in tissue	445	24.0	960	51.8	1050	56.7	919	49.3
Total radioactivity	1683	90.2	1810	98.1	1780	95.9	1863	100.6

Mean total radioactivity was 1851 × 10^3 cpm (range: 1829–1873 × 10^3 cpm).

* Samples were prepared as described in text. All values are averages of duplicate counts corrected for background and quenching; percentages are calculated on the basis of triplicate aliquots homogenized and extracted without fixative or dehydration.

Blocks were removed from fixative and dehydrated by successive 10-min immersions in 35, 50, 70, 90, and 100% ethanols. The final dehydration in absolute ethanol was repeated and followed by two immersions in propylene oxide. The tissue then was embedded in Epon 812 and sectioned (Luft, 6). Aliquots of the fixative, alcohol, and propylene oxide solvents were taken for direct counting after spotting on filter paper strips. In addition, the lung samples remaining after sectioning were subjected to wet oxidation (nitric-perchloric acid) by the method of Belcher, neutralized with 58% ammonium hydroxide, and counted in the PPO—POPOP:toluene—ethanol system recommended by Belcher (7). Appropriate blanks also were counted. Efficiency of this latter system was 9.28%. Quenching was estimated as described above. Two samples were subjected to fixation by each method. The results are given in Table II.

Other unfixed tissue blocks were dehydrated in ethanol according to the same scheme. The alcohol extracts were pooled, dried, and partitioned in a chloroform-methanol-water (8:4:3, v/v/v) system. Aliquots of aqueous and chloroform phases then were counted to determine the proportion of "water-soluble" and "lipid-soluble" radioactivity extracted.

Ultrathin sections embedded in Epon were examined using an RCA EMU-2A electron microscope.

RESULTS AND COMMENT

The incorporation of choline into phosphatidyl choline occurs rapidly in the newborn guinea pig (Table I). At the end of 3 hr about 12% of the injected radioactivity appeared in the lungs and 7.7% had been incorporated into lipid. At this time 96.2% of the lipid radioactivity was in the phosphatidyl choline fraction which had a specific activity of 3.67 $\mu c/\mu mol$. Presumably the labeling of this fraction conforms to normal metabolic patterns since a tracer amount (13 μmoles) was injected. Fatty acid analysis of the phosphatidyl choline isolated by thin-layer chromatography showed palmitate 55%, stearate 6%, oleate 6%, linoleate 15%, and linolenate 12%. In our experience this pattern is representative of guinea pig whole lung lecithin fatty acids.

In the second experiment water-soluble radioactivity in the lung was 29.1% of the total at three hours. The data presented in Table II indicate that, even if all of this water-soluble radioactivity were removed from the tissue by fixation and dehydration, there must be an additional loss of 37.8, 17.4, 9.9, or 22.2% of total radioactivity during preparation by OsO_4 alone, with formalin and OsO_4, with glutaraldehyde and OsO_4, and by tricomplex fixation, respectively. Since these additional losses of radioactivity cannot be explained as losses of water-soluble components, we may conclude that from 9.9 to 37.8% of tissue radioactivity is lipid which is lost during preparation for electron microscopy.

Confirmation of this estimate of lipid loss was obtained by measurement of the water- and lipid-soluble radioactivity extracted by ethanol from unfixed lung blocks. When no fixative was used, ethanol dehydration extracted 73.6% of lipid-soluble radioactivity and 61.2% of lipid phosphorus.

The lipid loss does not appear to be related entirely, as suggested by Riemersma (8), to the degree of saturation of fatty acids present in the lipid fraction, since fixation of lipids of constant composition by various heavy metals was quite variable. Glutaraldehyde-OsO_4 seemed to be most effective in preventing lipid loss although losses were considerable even with these fixatives. Stein and Stein have reported up to 18% loss of choline-labeled phospholipids from rat liver[1] during glutaraldehyde fixation, and Korn and Weisman (1) reported losses of up to 25% of phospholipid from amebae with various fixatives and dehydration schemes. The higher lipid loss from lung may be due to the much higher content of saturated fatty acids in lung although tricomplex fixation (which does not depend on unsaturation) might have been expected to show less loss.

We can make no definitive statement regarding the retention or loss of lipid in tissues other than lung, but we do raise the question whether, in view of the present results, electron-opaque masses seen on electron microscopy of the lung accurately represent lipids at the subcellular level. We also question whether it is reasonable to expect that surfactant dipalmitoyl lecithin in the alveoli will be retained for visualization by electron microscopy when sections are prepared by methods similar to those used here.

SUMMARY

Tritiated choline was injected intraperitoneally into newborn guinea pigs and the rate of its incorporation into lung phospholipid was determined. Samples of lung containing labeled phospholipid were prepared for electron microscopy by fixation with one of four fixative schemes, followed by alcohol-propylene oxide dehydration and Epon 812 embedding. Losses of radioactivity were 66.9% of total radioactivity when fixed in *s*-collidine-buffered osmium tetroxide, 46.5% when fixed in 4% buffered formalin followed by osmium tetroxide, 39.0% when fixed in 2% glutaraldehyde followed by osmium tetroxide, and 51.3% when tricomplex fixation was employed. Although some of the radioactivity lost was water soluble, 9.9–37.8% of lipid-soluble radioactivity was lost during preparation for electron microscopy.

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