METHODS

A Mild, Rapid, and Efficient Method of Lipid Extraction for Use in Determining Vitamin E/Lipid Ratios

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ABSTRACT

A new, general method for lipid extraction and measurement of vitamin E/total lipid ratios in tissue and cell samples has been developed. The new extraction procedure uses a combination of sodium dodecylsulfate, ethanol and *n*-heptane, and is mild, clean, convenient, efficient and rapid (≤5 min). The efficiency of the new method has been confirmed for human plasma, red blood cells and rat liver homogenate by the comparison of the yields of vitamin E, O-acyl lipid and cholesterol with the yields obtained following conventional extraction procedures. Extraction efficiency also has been confirmed for multilamellar vesicles composed of known quantities of vitamin E, egg lecithin and cholesterol.

Lipids 20:29-39, 1985.

INTRODUCTION

There is increasing interest in the involvement of in vivo lipid peroxidation in the general aging process and in the onset and development of associated diseases, such as heart disease and cancer, with special attention being paid to the effect of certain dietary compounds in retarding these degenerative processes (1,2). Lipid peroxidation, which involves the autoxidation of polyunsaturated fatty acids by a free-radical chain process, can be inhibited dramatically by very small quantities of lipid-soluble chain-breaking antioxidants (3-5). Vitamin E (1) and β-carotene (1,6,7) have received much recent attention with regard to their possible preventive role in these degenerative disease processes. These two compounds are believed to function in vivo as antioxidants, and it has been demonstrated clearly in vitro that each compound is an inhibitor of autoxidation (8,9).

In assessing the susceptibilities of different tissues, cells and organelles to peroxidative damage and the relevance of the findings to degenerative disease processes, it is now evident that lipid-soluble antioxidant levels (e.g., vitamin E, β-carotene) must be measured relative to the peroxidizable lipid (i.e., the polyunsaturated fatty acid residues) found associated with the antioxidants (10). Thus, for example, vitamin E has been reported to vary widely from tissue to tissue (11,12), but only rarely has its concentration been reported relative to total lipid (12,13) or the quantity of polyunsaturated fat (14).

In this paper we report the development of a convenient, new method for extracting lipid from cell and tissue samples which, because of its speed and mildness, greatly facilitates the measurement of small quantities of labile lipid components, such as vitamin E (determined by HPLC), and the measurement of polyunsaturated fatty acid (determined as part of the O-acyl lipid by GC after base-catalyzed transesterification of the lipid extract). The new procedure uses SDS to make membrane lipids amenable to extraction by a combination of ethanol and *n*-heptane. The efficiency of the method has been tested and confirmed by determining the quantities of vitamin E, cholesterol and O-acyl lipid in lipid recovered from aqueous MLV of known composition and also by determining and comparing these same lipid parameters in lipid extracted from red blood cells and rat liver homogenate by both the new procedure and by traditional methods (15-17). Blood plasma also has been used to determine the effect of varying SDS concentrations upon lipid yields by comparing results for lipid recovered by extraction with ethanol/*n*-heptane alone and by extraction with ethanol/*n*-heptane after the addition of SDS.

MATERIALS AND METHODS

Materials

Solvents were HPLC-grade (Fisher) except *n*-heptane (Fisher Spectranalyzed®) and abso-

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Abbreviations: SDS: sodium dodecyl sulfate; MLV: multilamellar vesicles; RBC: red blood cells; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; SP: sphingomyelin; 2-BHA: 2-*t*-butyl-4-hydroxyanisole; PMHC: 2,2,5,7,8-pentamethyl-6-hydroxychroman; CMC: critical micelle concentration.

lute ethanol (reagent grade). *t*-Butyl methyl ether (HPLC-grade) was obtained from Burdick and Jackson. The SDS detergent was obtained from Bio-Rad (electrophoresis grade) and from BDH (specially purified for biochemical work). Egg lecithin was bought from Avanti Polar Lipids (Birmingham, Alabama), and reference samples of PC, PE, PS, SP and cardiolipin were supplied by Mrs. A. Martin (NRC). Other chemicals required for reference and/or identification purposes were cholesterol and cholestane (Sigma); various fatty acid methyl esters and triheptadecanoin (Nu-Chek-Prep Inc., Elysian, Minnesota); 2*R*, 4′*R*, 8′*R*-α- and γ-tocopheral (Eastman); 2-BHA (Eastman, now discontinued); and PMHC (synthesized earlier [8] by a published procedure [18]). TLC was performed on silica gel 60 precoated on glass plates (Merck). Sephadex G-25 (coarse) was obtained from Pharmacia Fine Chemicals.

Fresh whole blood was obtained by venipuncture from a human volunteer (A.W.) and was mixed immediately with the anticoagulant, disodium ethylenediaminetetraacetate. Blood also was obtained from the Red Cross Blood Bank.

Rat liver was obtained from a young, male, adult Sprague-Dawley rat (ca. 200 g) and was homogenized with ca. 3 volumes of water in a Tissue Mizer homogenizer (medium setting; 4 x 15 sec).

All aqueous solutions were prepared using doubly-distilled water.

General Procedure for SDS Extraction of Lipid

A given volume of an SDS solution of known concentration (in the range 0.01-0.80 M) was added and mixed with the aqueous sample of plasma, RBC membrane or liver homogenate. A volume of absolute ethanol, usually equal to the combined aqueous volume, was then added and mixed by shaking or brief vortex-stirring. This causes the protein to precipitate immediately. Next, a known volume of *n*-heptane equal to, or in some cases less than, the volume of added ethanol was added and vigorously mixed by vortex-stirring for 30-60 sec. The aqueous and organic layers were conveniently and rapidly separated by brief centrifugation in a clinical, bench-top centrifuge (1-2 min). A known volume of the organic layer was carefully drawn off with a Pasteur pipette and transferred to a screw cap (foil-lined) vial and stored at −20 C. It should be noted that other solvents may be used instead of *n*-heptane. We have obtained identical results with, for example, *n*-hexane and *n*-octane. However, we do not recommend the use of *n*-hexane because of its toxicity (19).

Extraction of Lipid from Multilamellar Phospholipid Vesicles (MLV)

The efficiency of the SDS extraction method was tested by measuring the lipid extracted from MLV of known composition.

Known amounts of egg lecithin, cholesterol and α-tocopherol were dissolved in dichloromethane. The solvent from a 10 ml sample of this solution was removed under a stream of nitrogen and finally pumped off under vacuum. The MLV were formed by adding 10 ml water to the residue and vortex-stirring. Lipid was extracted by the SDS method from 1 ml aliquots of the MLV using 1 ml of SDS solution, 2 ml of ethanol and 2 ml of *n*-heptane. A reference sample of lipid was prepared by removing the solvent from a 1-ml aliquot of the dichloromethane stock solution and redissolving the residue in 2 ml of *n*-heptane. This reference sample was stored at −20 C in a tightly stoppered vial, and was later used to obtain reference values for the fatty acids, cholesterol and α-tocopherol.

Extraction of Lipid from Plasma

The conventional method for extracting plasma (20) is very similar to the SDS method. The recoveries of lipid obtained by the two methods were compared so that the efficiency of the SDS procedure could be evaluated.

Lipid was extracted in the conventional manner by mixing water (1 ml), ethanol (2 ml) and plasma (1 ml) in a glass tube. *n*-Heptane (1 ml) was then added and the mixture was vortex-stirred for 30-60 sec. The aqueous and organic layers were separated by brief centrifugation.

The SDS method was applied in exactly the same way using the same quantities of material but replacing the water with SDS solutions (1 ml) of different concentrations.

Extraction of Lipid from RBC

RBC, freed of plasma and the buffy coat after washing 3 times in 5 mM phosphate-buffered saline (pH 8.0) in the usual manner (21), were resuspended in the same buffer (hematocrit ca. 50%). Then 5 ml-samples of this suspension were lysed by dropwise addition to ca. 30 ml of 5 mM phosphate buffer (pH 8.0) contained in centrifuge tubes (13,21,22). The hemolysate was spun at 20,000 rpm for 10 min in a Sorvall RC2-B centrifuge equipped with a fixed angle SS-34 rotor (4.25 in. radius). Most of the supernatant was removed from each tube. The red-colored, hemoglobin-contaminated RBC ghost pellet that remained in each tube (ca. 2 ml) was transferred to a separate

16 ml test tube and 2 ml of 5 mM phosphate buffer/5 mM ascorbate (pH 7.0) was added, this being followed by the addition of 1 ml of an SDS solution of known concentration. Under these conditions with this quantity of RBC ghosts, the suspension became transparent when the concentration of the added SDS solution was 0.08 M or greater. Next, 5 ml of ethanol and 2 ml of *n*-heptane were added and mixed with the SDS/RBC ghost mixture in the same way as already described for the general SDS extraction procedure. The *n*-heptane layer that was obtained after centrifugation was colorless.

For comparative purposes, lipid also was extracted from the RBC ghosts by the Folch method that we have used previously (13) and that Nelson has reported extracts lipid very efficiently from plasma and whole RBC (23, 24). However, it is important to use hemoglobin-free ghosts in order to avoid the co-extraction of iron-containing pigments which can cause an almost complete loss of vitamin E (13). A sample of the same (red) ghost suspension used for the SDS extraction experiment was therefore washed twice following the improved procedure developed previously in our laboratory (22). The suspension of (now) white ghosts (ca. 2 ml) was added dropwise to stirred, ice-cold methanol (33 ml) and two portions of cold chloroform (33.5 ml each) were then added. The mixture was magnetically stirred for several minutes and then filtered onto ca. 2 g of Sephadex G-25 (coarse) in a round-bottomed flask. The ghost residue was rinsed with chloroform and the combined filtrate was concentrated by rotary evaporation under reduced pressure. In order to ensure the complete removal of water from the extract by the Sephadex, a further 10-ml portion of chloroform was added to the Sephadex residue and then removed again under reduced pressure. This was repeated twice more. The lipid was finally recovered from the Sephadex by extracting the latter with two 50-ml portions of chloroform and filtering. The combined filtrate was concentrated by evaporation under reduced pressure at a temperature below 30 C and finally to dryness with a stream of nitrogen. The lipid residue was redissolved in 2 ml of *n*-heptane.

The lipid extracts obtained by the new SDS and by the Folch methods were analyzed by TLC on silica gel in order to compare the recovery of the different types of phospholipid. The plates were developed in chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v) (25) and the spots were visualized by exposing the plates to iodine vapor.

Extraction of Lipid from Rat Liver

Rat liver homogenate, containing 0.25 g ml^{-1} of liver (wet weight), was extracted by the general SDS method and the Bligh and Dyer method (17,26).

Typically, the SDS procedure used 1 ml of rat liver homogenate, 1 ml of SDS solution, 2 ml of ethanol and 2 ml of *n*-heptane. A partial clarification of the homogenate occurred when the SDS solution was added.

In a typical Bligh and Dyer extraction, 2.5 ml of methanol and 1.25 ml of chloroform were vortex stirred for 2 min with 1 ml of rat liver homogenate. The mixture was centrifuged for 3 min in a clinical centrifuge, and the supernatant was removed with a Pasteur pipette. Then water (1.25 ml) and chloroform (1.25 ml) were added to the supernatant and mixed; this was followed by centrifugation to facilitate separation of the aqueous and organic layers. The aqueous layer was removed by aspiration and the organic layer was then dried over anhydrous sodium sulfate, filtered and evaporated under a stream of nitrogen. The residue was redissolved in 2 ml of *n*-heptane.

Measurement of the Tocopherols

PMHC and 2-BHA were used as non-interfering standards for measuring tocopherol concentrations. Aliquots (50 μl) of *n*-octane solutions of PMHC (8.30 nmol) and 2-BHA (9.23 nmol) were added by micropipette to 500 μl samples of the *n*-heptane lipid extracts. Samples of this solution (100 μl) were injected into a Varian 5000 HPLC instrument equipped with a 250 mm × 4 mm Hibar RT LiChrosorb Si 60 column (Merck) and were eluted with *n*-hexane/*t*-butyl methyl ether (3.0%)/2-propanol (0.05%) at 2 ml min^{-1}. Peaks were detected with a Varian fluorescence detector, equipped with a deuterium lamp, which was connected in series with a Varian Varichrome variable wavelength uv detector set at 295 nm. The fluorescence detector was equipped with a 220 nm interference excitation filter and the emission filter was a 2 mm thick Schott UG-1 glass band filter which gave 89% transmittance at λ_{max} ca. 358 nm and had a 279-419 nm "window" (10% transmittance limits). This emission filter replaced the 5 mm thick Corning 7-60 filter used earlier (13) and gave a more than 3-fold enhancement of the signal. The HPLC and associated detectors were interfaced with a Varian Vista CDS 401 control station and data-handling system.

Measurement of O-acyl-derived Fatty Acids and of Cholesterol

The quantity and composition of the fatty

acids in the O-acyl fraction of the lipid extract and the quantity of cholesterol each were determined by GC analysis after duplicate transesterification of the lipid extract (27).

Fatty Acid Methyl Esters

Transesterification was carried out by heating a mixture of 250-500 µl of the *n*-heptane lipid extract, 1 ml of benzene containing ca. 130 nmol of triheptadecanoin (as internal standard), and 2 ml of anhydrous 0.5 M sodium methoxide in methanol (Supelco) at 80 C for 20 min in a tightly-stoppered glass vial. The mixture was then allowed to cool and 0.1 ml of glacial acetic acid was added, followed by 5 ml of water. After extraction with three 5-ml portions of *n*-hexane, the combined hexane extracts were dried over anhydrous sodium sulfate containing 10% solid potassium bicarbonate. Following filtration, the filtrate was concentrated by evaporation under a stream of nitrogen.

The methyl esters were analyzed on a Varian 3700 GC instrument equipped with a flame-ionization detector using a 1.8 m × 3.2 mm (o.d.) stainless-steel column packed with 10% Silar-9CP on 100-120 mesh Chromosorb W-HP at a helium flow rate of 30 ml min^{-1}. The oven temperature was maintained at 150 C for 5 min and was then increased to 225 C at 3 C min^{-1}. The data were analyzed with a Varian Vista CDS 401 system.

The quantity of each fatty acid methyl ester was measured by comparison with the known quantity of methyl heptadecanoate formed from the triheptadecanoin. It was assumed that each fatty acid methyl ester gave the same peak area per unit weight, an assumption that was fully supported by results obtained with standards containing known amounts of the major fatty acid esters. Total fatty acid values were calculated for the acid form of the ester and included all the minor peaks (e.g., 16:1, 18:3, 20:3, 22:5). The percentage fatty acid composition was, however, based on a calculation restricted to the 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 set of fatty acids which together constituted well over 80% of the total fatty acid in most samples. Incidentally, for all the O-acyl lipid extracts examined in this work total fatty acid values, which are given in µmol ml^{-1}, can be converted to mg ml^{-1} by dividing by either 3.41 ± 0.03 for plasma, RBC and rat liver or by 3.62 ± 0.03 for MLV.

Cholesterol

Approximately 300 nmol of the internal standard, cholestane, were added to the *n*-hexane extract obtained after transesterification. The cholesterol was measured by GC using a 0.45 m × 3.2 mm (o.d.) stainless-steel column packed with 5% OV 101 on 80-100 mesh Chromosorb W-HP at 230 C with a helium flow rate of 30 ml min^{-1}.

Tests for Presence of SDS in *n*-Heptane Lipid Extracts

These tests were carried out by spotting the lipid extract onto a silica gel TLC plate and developing with dichloromethane/methanol 8:1 (v/v) (28). The TLC plate of an SDS lipid extract from RBC was developed with iodine vapor, and the plate of an SDS lipid extract from rat liver was developed by spraying with 5% sulfuric acid and heating at 80 C on a hot plate.

RESULTS

SDS Extraction of Lipid from MLV, Plasma and RBC Ghosts

The effect of SDS concentration upon the extracted amounts of α- and γ-tocopherol, as measured directly in the *n*-heptane extract, and cholesterol and O-acyl total fatty acid, as measured after the base-catalyzed transesterification, are shown for MLV, plasma and RBC ghosts in Figures 1, 2 and 3, respectively. The

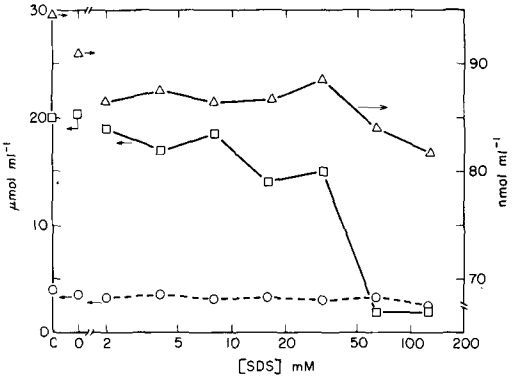

FIG. 1. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (Δ——Δ), total O-acyl fatty acid (□——□) and cholesterol (○ – – ○) from an aqueous MLV suspension by the SDS method (see Materials and Methods). Concentrations of extracted lipids are given per ml of aqueous MLV suspension (the α-tocopherol in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in µmol ml^{-1}). The data points at zero SDS concentration refer to extraction of the MLV suspension without SDS, while those on ordinate C refer to the control analysis of the dichloromethane stock solution used in preparing the MLV. The total O-acyl fatty acid can be converted from µmol ml^{-1} to mg ml^{-1} by dividing by 3.62 ± 0.03. Arrows indicate appropriate ordinate.

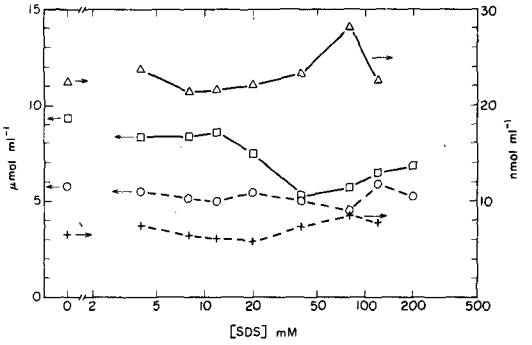

FIG. 2. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (△——△), γ-tocopherol (+---+), total O-acyl fatty acid (□——□) and cholesterol (○——○) from plasma (0.25 ml per ml of aqueous ethanol) by the SDS method (see Materials and Methods). Concentrations of extracted lipids are given per ml plasma (the tocopherols in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in μmol ml^{-1}). The 4 data points corresponding to zero concentration of SDS are reference values obtained by the conventional extraction procedure. The total O-acyl fatty acid can be converted from μmol ml^{-1} to mg ml^{-1} by dividing by 3.41 ± 0.03. Arrows indicate appropriate ordinate.

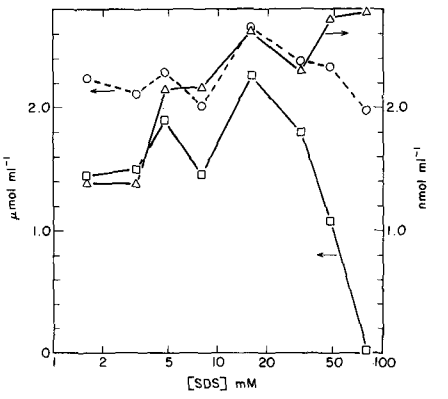

FIG. 3. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (△——△), total O-acyl fatty acid (□——□) and cholesterol (○— —○) from crude (hemoglobin contaminated) RBC ghosts (at a concentration corresponding to 0.25 ml packed RBC per ml of aqueous ethanol) by the SDS method (see Materials and Methods). The ghosts (ca. 2 ml) were derived from 2.5 ml of packed RBC (ex Red Cross) and were extracted with 2 ml of 5 mM phosphate buffer – 5 mM ascorbate (pH 7)/1 ml of SDS solution/5 ml ethanol/ and 2 ml *n*-heptane. Concentrations of extracted lipids are given per ml of packed RBC (the α-tocopherol in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in μmol ml^{-1}). There are no reference points at zero SDS concentration. All adjacent points have been connected, but it seems probable that some of the hills and valleys are experimental artifacts. The total O-acyl fatty acid can be converted from μmol ml^{-1} to mg ml^{-1} by dividing by 3.41 ± 0.03. Arrows indicate appropriate ordinate.

effects of SDS concentration on the corresponding fatty acid compositions are shown in Table 1.

MLV

As can be seen in Figure 1, α-tocopherol and cholesterol are very efficiently extracted by the SDS method. The reference points at C are based on analysis of the dichloromethane stock solution, and those at 0 SDS concentration on the results of an extraction without SDS. At SDS concentrations above 32 mM, the recovery of the fatty acids dropped dramatically, this drop coinciding with a clearing of the suspension after the ethanol was added.

Plasma

The data points in Figure 2 corresponding to zero concentration of SDS are the reference values that were obtained for lipid extracted in the conventional manner (i.e., with ethanol and *n*-heptane only).[2] It is against these points that the values obtained with SDS must be compared. It is apparent that the α- and γ-tocopherol, and cholesterol values are practically independent of the SDS concentration and are in satisfactory agreement with the reference values. (The somewhat greater scatter of the cholesterol values may reflect the fact that cholesterol is measured after chemical manipulation and that the internal standard is added after the transesterification.) The total fatty acid also agrees well with the reference value, provided the final SDS concentration in aqueous ethanol does not exceed ca. 12 mM. However, at higher SDS concentrations there is a fairly abrupt decline in total fatty acid to a rather lower value. This effect was observed consistently for plasma samples obtained from a number of different sources. Examination of Table 1 shows that this change in total O-ocyl fatty acid values is accompanied by significant changes in the composition of the fatty acids.

RBC Ghosts

In Figure 3 are shown the results obtained at various SDS concentrations using RBC obtained from a Red Cross blood sample. Once again, the

[2] We have shown by a comparison of vitamin E, O-acyl fatty acid, cholesterol and phosphorus values that extraction of plasma with ethanol/*n*-octane is at least as efficient as the Folch method. The latter method has been reported to provide near-quantitative yields of lipid (23,24).

TABLE 1

Effect of SDS Concentration on the Fatty Acid Composition of O-acyl Lipid Extracted from MLV, Plasma and RBC Ghosts

	SDS[a] (mM)	O-Acyl fatty acid[b] (μmol ml^{-1})	Percentage composition[b] (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
MLV[c]	Control[d]	20.0	32	11	35	17	5	1
	0[f]	20.3	31	10	38	17	3	1
	2[f]	19.0	30	10	38	17	4	1
	4[f]	17.1	31	11	37	17	3	<1
	8	18.4	30	11	39	17	3	1
	16	13.9	30	11	38	17	4	1
	32[g]	15.1	34	10	37	16	3	<1
	64	1.8[h]	–	–	–	–	–	–
	128	1.9[h]	–	–	–	–	–	–
Plasma[c]	0.0	9.38	19	8	23	43	8	t
	4.0	8.30	18	7	25	41	8	t
	8.0	8.42	19	7	25	41	8	1
	12.0	8.58	19	6	25	42	7	t
	20.0	7.44	19	7	24	42	8	t
	40.0	5.60	17	3	27	48	5	t
	80.0	5.90	16	2	28	48	6	t
	120.0	6.52	16	3	28	48	6	t
	200.0	6.81	16	2	29	47	6	t
		Change[e]:	−3	−6	+6	+4	−2	
RBC ghosts[c]	1.6	1.45	20	22	23	13	19	3
	3.2	1.50	20	22	22	14	19	3
	4.8	1.90	20	19	23	14	20	4
	8.0	1.46	21	19	23	14	20	3
	16.0	2.26	20	19	22	14	20	4
	32.0	1.80	19	20	23	13	21	4
	48.0	1.08	16	19	27	10	23	5
		Change[e]:	−4	−3	+4	−3	+4	+2

[a] Final concentration after addition of ethanol.

[b] Sum of all fatty acids from all O-acyl lipid. See Materials and Methods for details on measurement and calculation (t = trace).

[c] Results correspond to data points in Figure 1 (MLV), Figure 2 (plasma) and Figure 3 (RBC).

[d] Results obtained directly from an aliquot of dichloromethane stock solution (see Fig. 1 legend).

[e] Difference in composition of lipid fatty acid for highest and lowest SDS concentrations.

[f] Layer separation for these samples was difficult. The first fraction of the *n*-heptane extract, obtained in the usual way, was combined with a second fraction obtained after standing overnight at 4 C followed by centrifugation. The difficulty is caused by an emulsion and apparent precipitate at the interface.

[g] A clear solution was obtained after the addition of ethanol at this concentration of SDS and higher.

[h] Percentage composition values are not given because not all components were present in detectable amounts.

cholesterol value appears to be essentially independent of the SDS concentration, though no reference data (i.e., an extraction without SDS) were obtained with this particular blood sample. In contrast, the yield of extracted α-tocopherol is low at low SDS concentrations and does not reach its maximum until the final aqueous ethanolic SDS concentration is ca. 16 mM, thereafter remaining more-or-less steady. The total fatty acid extracted also increases slightly to ca. 16 mM SDS, but then declines rapidly to zero at higher concentrations of SDS. It can be seen from Table 1 that the rapid decline in the total fatty acid value is accompanied again by an appreciable change in its composition.

The SDS and Folch extraction procedures are compared in Table 2 for RBC ghosts prepared from a sample of fresh blood. The SDS method is clearly superior for the extraction of α-tocopherol. (Note that if the RBC ghosts are contaminated with hemoglobin, i.e., are red in color, then phosphate-buffered ascorbate should be added prior to the SDS to avoid a potentially substantial loss of α-tocopherol.) The cholesterol values, which again vary little with SDS concentration, show that recoveries by the SDS method are at least as good as by

TABLE 2

Comparison of Lipid Extracted from RBC Ghosts by the Folch and SDS Methods[a]

Method	α-Tocopherol[b,c] (nmol ml^{-1})	Cholesterol[b,d] (μmol ml^{-1})	O-Acyl lipid: Total fatty acid[b,d] (μmol ml^{-1})	Percentage composition[d] (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
Folch	1.84 ± 0.09	2.67 ± 0.13	2.57 ± 0.14	22	19	22	13	19	4
SDS (mM)[e]: 13	3.09 ± 0.43	3.06 ± 0.13	2.80 ± 0.03	22	20	22	13	20	3
27	3.15 ± 0.50	3.29 ± 0.10	2.80 ± 0.03	21	21	21	11	21	6
40	3.04 ± 0.67	2.62 ± 0.08	2.46 ± 0.03	20	21	21	12	21	5
80	2.56 ± 0.51	3.03 ± 0.10	0.44 ± 0.03	16	25	21	8	25	4

[a]Fresh blood sample from AW. SDS extractions were carried out on crude ghosts (without added phosphate-buffered ascorbate), and the Folch extraction was carried out on washed ghosts, as described in Materials and Methods.

[b]Lipid values refer to concentrations in 1 ml of packed RBC.

[c]Determined by HPLC. Each value is the mean and difference from the mean of the 2 results obtained consecutively with the fluorescence and UV detectors, respectively.

[d]Determined by GC after base-catalyzed transesterification (see Materials and Methods). Each cholesterol and total fatty acid value is the mean and difference from the mean of 2 results.

[e]Final concentration of SDS after the addition of ethanol. The concentration of ghosts corresponds to 0.25 ml of packed RBC per ml of aqueous ethanol, i.e., a 4-fold dilution of the original packed RBC.

the Folch method and perhaps slightly better. With final SDS concentrations in the 13-40 mM range, the total fatty acid extracted and its composition agree well with the results obtained by the Folch method. The similarity in composition suggested that the same types of phospholipid were being extracted in the same proportion by both the SDS and Folch methods. This was confirmed by TLC for PC, PE, PS and SP, which were found to be present in qualitatively similar proportions in lipid extracted by the two methods. At high SDS concentrations there is, once again, a drastic decrease in the total fatty acid and a pronounced change in its composition.

SDS was not found by TLC in the *n*-heptane extract of lipids from RBC ghosts.

SDS Extraction of Lipid from Rat Liver Homogenate

The results presented in Table 3 show that rat liver homogenate can be extracted successfully by the SDS method, and that this method is clearly superior not only in time and labor but also in terms of its recovery of α-tocopherol, cholesterol and probably total fatty acid, compared to the Bligh and Dyer method. Note that recoveries are insensitive to a remarkably wide range of SDS concentrations (ca. 12.5-ca. 50 mM in the aqueous ethanol). With this type of tissue, as with the others we have examined, too high a concentration of SDS (>50 mM) adversely affects the total fatty acid recovered and, once again, causes a dramatic change in the fatty acid composition (Table 3).

SDS was not found by TLC in an *n*-heptane lipid extract.

Other Observations Pertinent to the SDS Extraction Method

Lipid extracted from RBC ghosts and rat liver by the SDS method generally gave significantly "cleaner" HPLC traces when analyzed for vitamin E than did the corresponding Folch or Bligh and Dyer lipid extracts. (Also, the problem with over-long induction periods that we have occasionally experienced in the inhibited-autoxidation method (13) has not been encountered with lipid extracted from hemoglobin-free ghosts, though it may occur in lipid extracted from hemoglobin-contaminated, ascorbate-protected ghosts (13).) Extractions performed with SDS obtained from Bio-Rad showed a fluorescent impurity in the HPLC chromatograms which did not, however, interfere with the α-tocopherol or internal standard peaks. This impurity was not present in the SDS from BDH.

The effects of omitting SDS or ethanol or both upon lipid recovered from MLV are presented in Table 4. It can be seen that a substantial proportion of the lipid can be extracted with *n*-heptane alone, i.e., without the addition of SDS or ethanol, although in this case it is more difficult to obtain a separation of the aqueous and organic layers. (This problem was even more severe when an attempt was made to extract plasma lipid with *n*-heptane alone.) If the SDS extraction procedure is used

TABLE 3

Effect of SDS Concentration upon Recovery of Lipid from Rat Liver Homogenate Comparison with Bligh and Dyer Extraction[a]

Method	α-Tocopherol (nmol g^{-1})	Cholesterol (μmol g^{-1})	O-Acyl lipid: Total fatty acid (μmol g^{-1})	Composition (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
Bligh-Dyer (1)	27 ± 12	4.56 ± 0.10	62.4 ± 0.7	20	18	10	25	21	7
Bligh-Dyer (2)	20 ± 6	3.52 ± 0.10	52.2 ± 5.1	19	19	8	25	24	5
SDS (mM)[b]: 12.5	36 ± 9	5.49 ± 0.10	57.3 ± 3.4	19	20	8	22	26	5
20.0	35 ± 10	6.00 ± 0.31	67.9 ± 4.1	18	20	8	23	26	6
25.0	34 ± 8	5.80 ± 0.21	65.1 ± 4.1	18	19	8	22	25	7
50.0	35 ± 9	5.90 ± 0.31	59.3 ± 1.7	18	20	8	24	26	4
100.0	37 ± 11	5.49 ± 0.10	24.9 ± 0.7	17	16	11	30	21	5
150.0[c]	40 ± 15	4.45 ± 0.16	9.9 ± 0.3	24	3	21	34	11	6
			Change	+5	−17	+13	+12	−15	+1

[a]Lipid concentrations refer to 1 gm of liver (wet weight). The aqueous homogenate contained 0.25 gm liver per ml. α-Tocopherol, cholesterol and O-acyl lipid were determined in the usual way (see Materials and Methods).

[b]Final SDS concentration after the addition of ethanol. The homogenate (1 ml) was extracted with SDS solution (1 ml; 0.05-0.40 M)/ethanol (2 ml)/*n*-heptane (2 ml) (see Materials and Methods). The final liver concentration was 63 mg ml^{-1}.

[c]1 ml of homogenate was extracted with 0.4 M SDS (3 ml)/ethanol (4 ml)/heptane (2 ml). The final liver concentration was 31 mg ml^{-1}.

TABLE 4

The Effect of Omitting Ethanol or SDS, or Both, upon Recovery of Lipid from MLV[a]

	SDS: Volume (ml)	SDS: Final concentration (mM)	Ethanol (ml)	Water (ml)	α-Tocopherol (nmol ml^{-1})	Cholesterol (μmol ml^{-1})	O-Acyl fatty acid (μmol ml^{-1})
Control[b]	–	–	–	–	94.0	3.79	20.0
MLV[c]	1	2	2	0	86.6	3.18	19.0
MLV[c]	0	0	2	1	91.0	3.47	20.3
MLV[d]	0	0	0	0	79.2	3.68	16.8
MLV[e]	1	40	0	0	–[e]	–[e]	–[e]

[a]Volumes of SDS solution, ethanol and water indicate the amounts of each that were added to 1 ml of aqueous MLV suspension containing α-tocopherol, cholesterol and egg lecithin. Lipid was extracted into 2 ml of *n*-heptane (see Materials and Methods). Lipid concentrations refer to 1 ml of aqueous MLV suspension.

[b]Lipid values were determined directly from a 1 ml aliquot of the dichloromethane stock solution (see Materials and Methods).

[c]Layer separation was not readily obtained. See footnote "f," Table 1.

[d]Required 15 min centrifugation.

[e]Lipid values could not be determined because the emulsion did not separate into distinct aqueous and organic layers during centrifugation.

on the MLV, then ethanol cannot be omitted since, without ethanol, the lipid/SDS/*n*-heptane emulsion does not separate clearly into two phases on centrifugation (Table 4).

Although plasma lipids can be successfully extracted without SDS using only ethanol and *n*-heptane, our attempts to extract crude RBC ghosts in the same way were unsuccessful.

For quantitative work one must know the volume of the organic phase containing the extracted lipid. In systems employing equal volumes (2 ml) of aqueous solution, ethanol and *n*-heptane, the recovery of the organic layer was found to be 95-97.5% (1.90-1.95 ml).

DISCUSSION

General Considerations: SDS Method vs. Folch or Bligh and Dyer Methods

The control experiments conducted with plasma and MLV, as well as the comparisons with the extraction results obtained by the

traditional methods of Folch for RBC ghosts and Bligh and Dyer for liver homogenate, show very clearly that the SDS method as outlined herein provides an extremely rapid (≤5 min), mild, clean and efficient method for extracting lipid.

Within certain upper and lower limits, the values for which depend upon both the type of lipid and the nature of the lipid extracted, the recovery of lipid is independent of the SDS concentration.

These limits are widest for rat liver (0.25 g of liver suspended in 4 ml of aqueous ethanol, see Table 3). For α-tocopherol no upper or lower limits were established, and the yield of cholesterol decreased slightly at final SDS concentrations greater than 100 mM, while the yield of O-acyl lipid decreased fairly precipitously at SDS concentrations greater than ca. 50 mM. Although the recovery of O-acyl lipids from RBC imposes narrower limits on the concentration of SDS, these limits are nevertheless sufficiently broad to be useful for routine extraction of total lipid.

As a practical guide, RBC may be extracted by successively adding and mixing SDS (1 ml; 40-80 mM), ethanol (2 ml) and *n*-heptane (1-2 ml; vortex stir for 30-60 sec) with crude ghosts suspended in 5 mM phosphate/5 mM ascorbate (ca. 1 ml; pH 7; packed RBC volume originally 1 ml), followed by brief centrifugation. Rat liver homogenate (1 ml; 1 part liver to 3 parts water) is extracted in the same way using SDS (1 ml; 40-200 mM), ethanol (2 ml) and *n*-heptane (1 ml).

The "cleanness" of the SDS method is apparent not only in the significant reduction of contaminants in the HPLC analyses for vitamin E and by the absence of TLC-detectable amounts of SDS in the *n*-heptane extracts, but also from the fact that the *n*-heptane extracts of RBC ghosts heavily contaminated with hemoglobin are colorless, i.e., the iron-containing porphyrins that normally contaminate the RBC lipids extracted with chloroform/methanol are excluded from the SDS/ethanol/*n*-heptane lipid extract.

Preliminary experiments indicate that vitamin E can be extracted directly from either fresh or frozen RBC by the SDS method without first making ghosts. For example, rat RBC (1.0 ml; hematocrit ca. 50%) suspended in 5 mM ascorbate/5 mM phosphate-buffered saline (1 ml) were treated with SDS (5 ml; 0.1 M), ethanol (7 ml), and *n*-heptane (1-2 ml). The recovery of vitamin E was found to be the same for both fresh RBC and RBC stored frozen in the ascorbate/phosphate-buffered saline.

The amount of *n*-heptane used is not critical. This offers the potential for a modest, initial concentration of lipids by using a volume of *n*-heptane which is less than the volume of the original tissue. Other alkanes can be substituted for *n*-heptane, and it seems likely that certain other combinations of solvents also could be employed successfully. However, the dependence of lipid recovery upon the proportion of ethanol used and the effect of ethanol substitution by other alcohols have not been investigated.

The successful application of the SDS method to rat liver homogenate strongly suggests that the method has general applicability. A preliminary confirmation of the generality, speed and efficiency of the method has been obtained using aqueous homogenates of lung, heart, muscle, kidney, testes and brain tissue from a rat. The extractions of all six of these homogenates were accomplished in 20 min (1 ml homogenate/1 ml 0.1 M SDS/2 ml ethanol/2 ml *n*-heptane), and the results for α-tocopherol, cholesterol and O-acyl fatty acid were all at least equal to the results obtained by the method of Bligh and Dyer. Details of this work will be published at a later date.

It seems likely that the SDS method also could be used for the extraction and analysis of other minor, but important, lipid components such as β-carotene, ubiquinone and vitamins A, D and K.

We also have explored the use of Triton X-100 but found it to be much less satisfactory than SDS both because of poor phase separation and because there were a multitude of unidentified peaks in the HPLC chromatogram.

Factors Affecting Recovery and Composition of Lipid

Studies of the solubilization of pure PC vesicles and of biological membranes with detergents have provided evidence for differential complexation of the various components of membranes and for the eventual formation of soluble mixed lipid-detergent micelles and lipid-protein-detergent complexes (29-36). These observations help explain some of our experimental results. For example, the recovery of total O-acyl lipid from MLV and RBC declined to zero at the higher concentrations of SDS (Figs. 1 and 3), whereas its recovery from plasma declined by only ca. 30-40% and then reached an approximately constant value (Fig. 2). We suggest that these results are due to the fact that when there is sufficient SDS present to produce mixed lipid-SDS micelles, the more polar phospholipid is preferentially retained in these micelles upon addition of ethanol and *n*-heptane, whereas the less polar lipids (e.g.,

tocopherol, cholesterol, cholesteryl esters and triglycerides) are partitioned predominantly into the *n*-heptane. Since plasma contains triglycerides and cholesteryl fatty acid esters but RBC and MLV do not, the total O-acyl fatty acid value does not decline to zero for plasma but does for the RBC and MLV. Presumably the change in composition of the plasma-derived fatty acids with changing SDS concentration (Table 1) reflects differences in the fatty acid composition of the phospholipids, triglycerides and cholesteryl esters.

The dependence of phospholipid recovery upon SDS concentration shows a considerable variation with the nature of the material being extracted. MLV were found to be the most sensitive and the rat liver homogenate the least sensitive. This phenomenon appears to be related to the relative amount of protein present in each material. This is not too surprising since it is known that SDS binds strongly to proteins (29,30,37-40). We suggest that the protein acts as a kind of "buffer" or "sponge" which allows a considerable quantity of SDS to be added before the concentration of free, monomeric SDS reaches the CMC.[3] This will occur only after complete saturation of the protein. Rat liver shows the greatest range of acceptable SDS concentrations because it contains the most protein, while the "buffer" effect is inoperative in MLV because they contain no protein.

It will be clear from the foregoing that the upper SDS concentration limit at which phospholipid recovery begins to decline will depend to some extent on the tissue extracted, since it depends on the concentrations of both lipid and protein.

The marked changes that occur in the total fatty acid composition at the higher SDS concentrations that are associated with declining phospholipid recovery from plasma, RBC and rat liver (Tables 1, 2 and 3) show that the nature of the phospholipid affects its partitioning between the mixed micelles and the *n*-heptane. This effect can be attributed, in part, to differences in the nature of the phospholipid head groups, since it has been shown (36) that there are differences in the rates of SDS solubilization of PC, PE, PS and SP from RBC ghost membranes. Results obtained with MLV at concentrations of SDS associated with rapidly declining O-acyl lipid recovery (data not shown) indicate that the nature of the fatty acid tail must also play some role since the MLV contain only one type of phospholipid, PC. These latter results indicate that retention in the mixed micelles of the aqueous phase is favored for PC containing palmitic acid and is disfavored for PC containing arachidonic acid.

CONCLUSION

The results presented here indicate that the combination of the well-known property of detergents to dissociate and solubilize membrane proteins (29,30,37,38,44,45) with the extraction capabilities of aqueous alcohol/alkane mixtures[4] provides a promising alternative for general lipid extraction. The mildness and speed of the new method are important for the recovery of small quantities of labile compounds such as vitamin E. Provided the concentration of SDS is kept within certain rather broad limits, excellent recoveries of O-acyl lipid are obtained.[5] Higher concentrations of SDS appear to offer a way of separating phospholipids from less polar compounds such as cholesterol, cholesteryl esters, triglycerides, tocopherols, etc., as indicated by the results obtained for plasma and MLV.

ACKNOWLEDGMENT

This work was supported by the National Foundation for Cancer Research and the Association for International Cancer Research. The Ottawa Red Cross Donor Center provided blood. Dr. Alvin Chan of the University of Ottawa provided the homogenates of rat tissue and Mr. D. A. Lindsay helped with many of the analyses.

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[3] Even in the absence of protein and lipid, the CMC will not be the same as in water since it is influenced by ethanol (41,42) and by ions from buffers and dissolved salts (29,42,43).

[4] We have shown by a comparison of vitamin E, O-acyl fatty acid, cholesterol and phosphorus values that extraction of plasma with ethanol/*n*-octane is at least as efficient as the Folch method. The latter method has been reported to provide near-quantitative yields of lipid (23,24).

We have found that ethanol/hexane quantitatively extracts α-tocopherol dissolved in aqueous SDS micelles.

Alcohol/alkane mixtures have been used previously for the quantitative recovery of neutral lipid from liver (46) and microsomes (47) and also have been found to be suitable for replacement of chloroform/methanol in the Folch method (48).

[5] Although a rapid procedure for specifically measuring vitamin E in tissue and plasma using acetone has been reported recently (49), the insolubility of phospholipids in acetone precludes the use of this method for determining levels of peroxidizable lipid.

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[Received July 25, 1984]