

Artifacts in ultracentrifugal estimation of aqueous fatty acid concentration

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Abstract Ultracentrifugation for determination of isotropic concentrations of fatty acids is widely used. However, several artifacts, which would affect the isotropic concentration, could occur if care is not taken. These include sedimentation of micelles, incomplete flotation of unsolubilized oil, and uptake of labeled fatty acid by the walls of centrifuge tubes. The first artifact can be overcome by sampling large volumes from the ultracentrifuged sample, and the second by ultracentrifugation for long periods; a force-duration of 7.2×10^7 g-min is suitable. The third artifact cannot be eliminated but may be made constant if the duration of exposure of lipid mixtures to centrifuge tubes is kept constant.

Supplementary key words oleic acid · taurocholate · flotation · micellar sedimentation · partition

ULTRACENTRIFUGATION is commonly used to separate mixtures of lipids in water into an optically clear (isotropic) infranatant phase and a supernatant oil phase. This procedure has the advantage of yielding substantial volumes of isotropic phase for analysis. However, precautions are needed to avoid erroneous values. This paper reports some sources of artifacts in measuring concentrations of fatty acid in the isotropic phase. There may be sampling errors due to sedimentation of micellar lipid within the isotropic layer or, on the other hand, to incomplete flotation of very finely emulsified particles. Loss of fatty acid to the cellulose nitrate wall of the centrifuge tube may introduce a significant error.

EXPERIMENTAL PROCEDURES

Materials and methods

All solvents used were of analytical grade except for ethanol, which was redistilled in glass. For TLC, silica

Abbreviations: CMC, critical micellar concentration; TLC, thin-layer chromatography.

gel G (Merck reagents) was spread to a thickness of 0.25 mm on a 20 × 20-cm glass plate. Lipids were spotted on the plate and developed for 17 cm, using solvent systems as specified. [^{14}C]Oleic acid (Radiochemical Centre, Amersham, England) was 98% pure as determined isotopically after partitioning by TLC in the solvent system hexane-diethyl ether-glacial acetic acid 80:20:2 (v/v/v). 150 μg of unlabeled oleic acid (May and Baker, Dagenham, England) ran as one spot on TLC using the same solvent system. Glycerol-1-monooleate (Calbiochem) was purified by solvent partition. 180 μg of the purified product ran as one spot on TLC when developed in the solvent system hexane-diethyl ether-glacial acetic acid 30:70:2 (v/v/v). A stock solution containing [^{14}C]oleic acid in 100 mM unlabeled oleic acid (specific activity, 22.5 μCi/mmole) was prepared and used in all experiments.

Sodium taurocholate and sodium taurodeoxycholate were prepared according to the method of Norman as modified by Hofmann (1). A mixture of taurocholate-taurodeoxycholate 4:1 (mole/mole) was used in the experiments to be described. The CMC of this bile salt system is about 2.0 mM (2).

Solutions were prepared in phosphate buffer, pH 6.4, of composition: HPO_4^{2-} , 7.5 mM; H_2PO_4^- , 15 mM; Cl^- , 137 mM; Ca^{2+} , 1 mM; K^+ , 7.5 mM; Na^+ , 157 mM; and glucose, 1 mM.

The composition of scintillation solution used was 4 g of 2,5-diphenyloxazole and 0.05 g of dimethyl-1,4-bis-[2-(5-phenyloxazolyl)]benzene, made up in 1 l of toluene.

Lipid mixtures were prepared in the following manner. Appropriate amounts of stock [^{14}C]oleic acid and glycerol-1-monooleate in solvent were pipetted into measuring cylinders. Solvents were removed by evaporation under dry nitrogen. Appropriate amounts of bile salts and 5 ml of buffer were added, and the mixture was sonicated under atmospheric conditions using a Branson Sonifier at 40 W and 20,000 cycles/sec; probe tip

diameter was 0.5 cm. The mixture was sonicated intermittently until all lipid was solubilized or evenly emulsified, depending on the ratio of bile salts and lipid present. Care was taken not to overheat the mixtures by long periods of sonication. Buffer was then added 5 ml at a time, with sonication in between, until the final volume was reached. The entire process took less than 5 min for micellar solutions and usually less than 15 min for emulsions. The final composition was [^{14}C]oleic acid 1.0 mM and glyceryl-1-monooleate 1.0 mM, unless otherwise specified. Bile salt concentration was varied as indicated under Results. TLC of lipids before and after sonication showed no difference in partition of labeled oleic acid, indicating that no oxidation of oleic acid has occurred.

Ultracentrifugation and collection

Duplicate 9.0-ml samples of the appropriate lipid mixtures were pipetted into cellulose nitrate tubes (Beckman no. 302234, 1.2 cm diameter \times 8.9 cm). The tubes were placed in a Beckman type 30.2 fixed angle rotor prewarmed to 30°C. The samples were then centrifuged in a Beckman L2-65 preparative ultracentrifuge. Centrifugation conditions were: temperature 30 \pm 2°C, speed 25,000 rpm. Forces exerted on the top, middle, and base of the centrifuge tubes were 44,018, 55,200, and 65,681 *g*, respectively. The duration of centrifugation was varied. At the end of the run, a small hole was made in the base of the centrifuge tube, and aliquots were collected. All lipids were then extracted from the aqueous phase using the method of Blankenhorn and Ahrens (3), and radioactivities were measured in a Nuclear-Chicago liquid scintillation counter. Quenching was corrected by the channels ratio method (4).

Taurocholates in the bile salt mixture used (taurocholate-taurodeoxycholate 4:1 [mole/mole]) were esti-

mated by the method of Lee and Herman (5). All measurements were done in duplicate and from duplicate tubes; duplicates agreed within 5%.

RESULTS

Micellar solutions: sedimentation and partitioning artifacts

Lipid mixtures in 10 mM bile salts were placed in six centrifuge tubes. All the lipid was in optically clear micellar solution. Paired tubes were subjected to 1.3×10^7 , 2.6×10^7 , and 4.9×10^7 *g*-min, respectively, by centrifugation for 4, 8, or 15 hr. Three aliquots of approximately 3 ml each were collected from each tube; the percentage volume recovery was greater than 98% at all times. These aliquots were labeled "upper," "middle," or "lower" according to height above the base of the centrifuge tubes prior to collection. Radioactivity in each aliquot was determined. The tubes were then drained of all remaining fluid and washed with chloroform-methanol 2:1 (v/v). The washings were collected and the radioactivity was determined. This gave an estimate of oleic acid lost to the wall of the centrifuge tube from the aqueous phase.

Both lipid and bile salt concentrations increase toward the base of the centrifuge tube (compare "upper" and "lower" fractions, Table 1). The differences in concentration between upper and lower fractions of both fatty acids and bile salts are similar.

Increasing amounts of oleic acid label were recovered from the walls of the centrifuge tubes with increased duration of centrifugation (Table 1). This was accompanied by a corresponding decrease in amount of label recoverable from the aqueous phase. The amount of taurocholate lost to the wall of the centrifuge tube was not determined. However, the amount of taurocholate

TABLE 1. Effects of increasing gravitational forces on apparent isotropic concentrations of fatty acid and bile salt

| Average Force-Duration in <i>g</i> -min | Aqueous Concentration as % of Uncentrifuged Sample | | | Recovery | | Total |
|---|--|--------|-------|---------------|-------------------------|-------|
| | Upper | Middle | Lower | Aqueous Phase | Wall of Centrifuge Tube | |
| | | % | | | % | |
| Oleic Acid | | | | | | |
| 1.3 \times 10 ⁷ (4 hr) | 79.9 | 90.0 | 101.9 | 88.1 | 10.2 | 98.1 |
| 2.6 \times 10 ⁷ (8 hr) | 59.3 | 80.2 | 103.3 | 80.9 | 15.2 | 96.1 |
| 4.9 \times 10 ⁷ (16 hr) | 27.6 | 53.3 | 112.0 | 68.6 | 30.8 | 99.4 |
| Taurocholate | | | | | | |
| 1.3 \times 10 ⁷ | 97.5 | 102.5 | 111.3 | 101.1 | ND | 101.1 |
| 2.6 \times 10 ⁷ | 87.5 | 100.0 | 117.5 | 100.3 | ND | 100.3 |
| 4.9 \times 10 ⁷ | 72.5 | 97.5 | 135.0 | 103.5 | ND | 103.5 |

Volume of each aliquot collected was approximately 3 ml. Unspun composition of lipid mixtures was [^{14}C]oleic acid, 1 mM; glyceryl-1-monooleate, 1 mM; and bile salts, 10 mM. Samples were centrifuged in a Beckman type 30.2 fixed angle rotor. ND, not determined.

recovered from the aqueous phase did not decrease significantly with increased duration of centrifugation. This suggests that, unlike oleic acid, little bile salt was lost to the wall with increasing duration of centrifugation. A control experiment was performed in which samples of the same lipid mixture were placed in centrifuge tubes and left to stand for 12 hr at 30°C. Under these conditions, 28% of the total oleic acid, but no taurocholate, was recoverable from the walls of the centrifuge tubes.

Loss of lipid to walls of centrifuge tubes

To further investigate the loss of lipids to centrifuge tubes, mixtures of 0.25, 0.5, 0.75, and 1.0 mM [¹⁴C]oleic acid and glyceryl-1-monooleate in molar ratios of 1:1 were prepared. Bile salt concentration was either 1 or 10 mM. Duplicate aliquots were pipetted into cellulose nitrate tubes which were then capped and left to stand for 24 hr at 30°C. At the end of this period, the tubes were drained of all fluid and allowed to dry, and radioactivity in the aqueous phase and wall was determined. Volume recovery was greater than 98% at all times.

For similar concentrations of oleic acid, more lipid is lost to the wall from emulsions than from micellar solutions. This could be due to adherence of unsolubilized oil droplets to the wall of the centrifuge tubes. However, vigorous shaking of the tube prior to drainage, or drainage followed by addition of a 10 mM bile salt solution to the tube and shaking, did not improve recovery of label from the aqueous phase. Preexposure of the centrifuge tubes to chloroform-methanol 2:1 (v/v) resulted in recovery of all label from the aqueous phase for both emulsion and micellar solution. However, this treatment results in a decrease in length and diameter of the centrifuge tubes.

Effect of varying bile salt concentration on apparent isotropic concentrations of fatty acid

Lipid mixtures with bile salts varying from 0.5 to 10 mM in the final concentration were subjected to 1.3×10^7 g-min. Two aliquots of approximately 3 ml each were collected from each tube at the end of the centrifugation period. These correspond to "lower" and "middle" fractions in experiments described above (Micellar solutions).

Both fractions were optically clear and contained no oil globules visible to the naked eye at the air-water interface. Radioactivity in each aliquot was determined and is expressed as a percentage of that in the same volume of unspun lipid mixture (Fig. 1). It is apparent that above the CMC the "lower" aliquot contained more radioactivity than the "middle." The reverse is true below the CMC. The crossover point is at a bile salt concentration of approximately 3 mM, within the

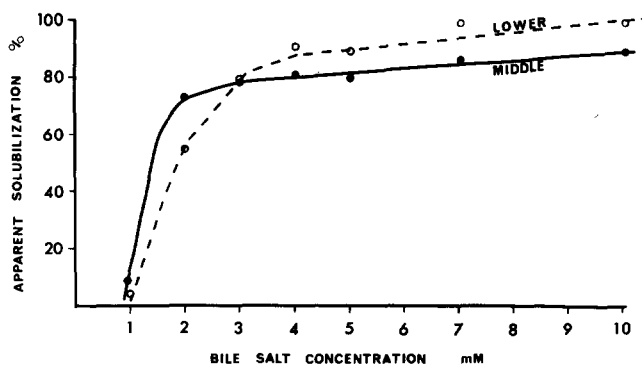


FIG. 1. Effect of incomplete flotation of unsolubilized oil, and sedimentation of micelles, on apparent isotropic concentration of fatty acid. Final composition of mixtures was [¹⁴C]oleic acid, 1 mM; glyceryl-1-monooleate, 1 mM; and bile salts, 0.5–10 mM. "Lower" and "middle" refer to positions of samples in centrifuge tubes prior to collection. Each point represents the mean of four estimations.

critical micellar range of the bile salt system used. It is also noted that the apparent isotropic fatty acid concentration below the CMC is in excess of that reported for the concentration of monomolecular fatty acid in aqueous solution (6).

These results could be explained by inadequate flotation of emulsion particles too fine to even disperse light (hence the optically clear solution noted above [7, 8]). Increasing the *g*-min should allow for more complete flotation. Therefore, lipid mixtures with a bile salt concentration of 1 or 2 mM in the final solution were subjected to 7.2×10^7 g-min (25,000 rpm for 22 hr). Two aliquots of approximately 3 ml each were collected and the isotropic fatty acid concentration was determined.

The isotropic concentrations of fatty acid were 0.59 μ M and 0.63 μ M in "middle" and "lower" fractions in emulsions containing 1 mM bile salt and were 0.60 μ M and 0.51 μ M in emulsions containing 2 mM bile salt. In contrast to Fig. 1, the isotropic fatty acid concentration in the "middle" aliquot is similar to that in the "lower" aliquot. Both are of the order consistent with monomolecular forms of oleic acid in aqueous solution (6). In addition, in these mixtures there was little difference (< 6%) in concentration of taurocholate between middle and lower aliquots. This indicates that there is no sedimentation of bile salt monomolecules.

Effects of large aliquots on gradients of bile salts

Lipid mixtures containing 1, 3, 4, or 5 mM bile salts in the final solution were subjected to 7.2×10^7 g-min.

At the end, an aliquot of approximately 6 ml was collected and the aqueous taurocholate concentration was determined. In each of the lipid mixtures, the concentration after centrifugation agreed to within 10% of that of unspun mixtures, indicating that sampling large volumes

TABLE 2. Concentration of taurocholate in lipid mixtures^a subjected to 7.2×10^7 g-min^b

| Before Centrifugation | After Centrifugation |
|-----------------------|----------------------|
| | <i>mM</i> |
| 0.79 | 0.84 |
| 2.48 | 2.62 |
| 3.34 | 3.68 |
| 4.06 | 4.46 |

^a 1 mM [¹⁴C]oleic acid, 1 mM glyceryl-1-monooleate.

^b 25,000 rpm for 22 hr.

decreases effects of micellar sedimentation caused by ultracentrifugation (Table 2).

DISCUSSION

Ultracentrifugation of micellar solutions of fatty acids and monoglyceride in bile salts results in an increasing difference in concentration of fatty acid and bile salt between the top and base of the centrifuge tube (Table 1). This difference from top to base is similar for both bile salt and fatty acid (Table 1). This suggests a sedimentation of bile salt micelles and their solubilized lipid (9, 10). Small aliquots taken from the bases of the ultracentrifuge tubes would therefore tend to overestimate the isotropic concentration of fatty acids (11). The greater the force-duration, the greater the overestimate would be.

From Table 1, there appears to be some dissociation of bile salt and fatty acid, the bile salt sedimenting faster than fatty acid. This could occur if the bile salt molecules remained in the micelle longer than the solubilized fatty acid, since only the micelles are spun down. However, whether this does indeed occur is not known. At least some of the difference is due to the loss of fatty acid onto the tube wall. Loss from the less concentrated upper layer will produce a proportionately greater change in the aqueous concentration than will equal loss from the more concentrated lower layer. Increasing duration of contact with cellulose nitrate tubes results in increased fatty acid loss to the wall of the centrifuge tube. The proportion lost is larger from emulsions than from micellar solution. Adherence of unsolubilized droplets of oil to the wall does not seem to be an explanation. A partitioning of fatty acid into a lipophilic compound in the wall is likely, as pretreatment of the centrifuge tube with a lipid solvent prevents any loss of fatty acid to the wall. This procedure is not likely to be of practical significance because the physical dimensions of the centrifuge tube are altered by this treatment.

Experiments described here give an indication of methods to reduce artifacts encountered when using ultracentrifugation for determination of isotropic concentration of fatty acid. To overcome the effect of sedi-

mentation of micelles, a maximal volume of infranate should always be collected. To overcome the effect of incomplete flotation of oil, high gravitational forces of the order of 7.2×10^7 g-min should be used. The problem of loss of lipid to the centrifuge tube wall is not easily overcome. Therefore, comparisons of isotropic concentrations of fatty acid for different lipid mixtures should be made only if the duration of contact between lipid and tube is similar for all lipid mixtures. Loss could be minimized by decreasing the exposure time between lipid and tube (Table 1). This would mean centrifugation at higher *g*'s to reach the same *g*-min. An alternative method would be to use centrifuge tubes that do not remove much lipid from the aqueous phase. From previous studies of lipid adsorption by tubing (12), Teflon or polyallomer tubes would appear to be suitable. Ideally, the experiments described in Tables 1 and 2 should be repeated for each centrifuge head and tube combination and for each lipid mixture to determine the optimal *g*-min for that particular system.

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