PRO EXPERIMENTIS

A source of error in the fluorometric determination of protein in human placental microsomes

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Summary. When protein is assayed in human placental microsomes by the fluorescamine procedure, 25–30% of the observed fluorescence results from labeling of phospholipids.

The fluorometric method for determining protein of Böhlen et al.[1], based on the formation of stable fluorophores by reaction of fluorescamine (4-phenylspiro (furan-2(3H)-1-phthalan)-3,3′-dione) with primary amino groups has found wide application.

It has the advantages of being highly sensitive and is not subject to interference by detergents, 2-mercaptoethanol, sucrose and various buffers which can affect other methods[2,3]. Amino acids and peptides react with fluorescamine and could be a source of error in assays of protein in tissue homogenates. However, such effects can be obviated by the incorporation of a gel-filtration step in the assay procedure[4]. We report here on another source of error in the estimation of protein in microsomes due to the reaction of membrane phospholipids with fluorescamine.

Placental microsomes were prepared at 4 °C by homogenization of 80 g of fresh tissue in 80 ml of 0.25 M sucrose:10 mM 2-mercaptoethanol:0.1 M Bicine, pH 9.0, and centrifugation of the 10,000×g, 10 min, supernatant fraction at 105,000×g for 60 min. The pellet from the second centrifugation was washed twice and suspended finally in 6 ml of buffer. To prepare phospholipids 0.5 ml aliquots of microsomes were extracted twice with 2.5 ml of 2:1 $CHCl_3$:CH_3OH. The combined extracts were washed with 1.0 ml of aqueous salt solution[5] and taken to dryness under nitrogen. The residue was suspended by sonication in a volume of H_2O or 0.05 M potassium phosphate, pH 8.0, equal to that of the original microsomes.

Aliquots (0.005–0.10 ml) of microsomal phospholipids or microsomes were diluted to 1.5 ml with 0.5 M potassium phosphate, pH 8.0, and combined with 0.5 ml of fluorescamine (Fluram, Roche Diagnostics) at 0.3 mg per ml in acetone. Fluorescence was read at 475 nm in an Aminco-Bowman spectrophotofluorometer (390 nm excitation, 10×10 mm cuvettes). With ovalbumin as a protein standard microsomal phospholipids yielded a fluorescence equivalent to 25 to 27% of the total estimated protein of the whole microsomes (table).

When microsomes were labeled with fluorescamine first and then extracted with $CHCl_3$:CH_3OH the fluorescence emission spectrum of the resuspended phospholipid fraction was identical in shape to that of the whole microsomes (figure). Emission intensity at 470–480 nm was 25% of that of the whole microsomes.

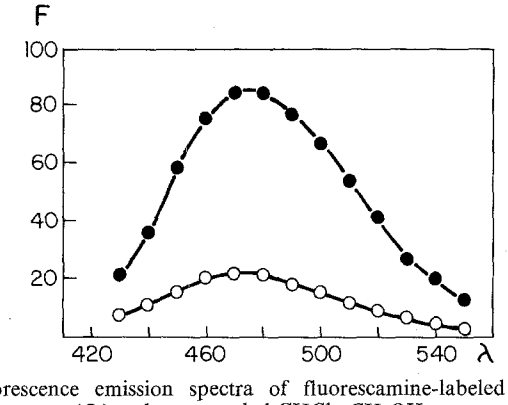
When equal amounts of individual, purified phospholipids were suspended by sonication and labeled with fluorescamine the relative fluorescence intensities at 475 nm with phosphatidylserine as 100% were phosphatidylethanolamine 71%, sphingomyelin 2% and phosphatidylcholine 1%. No protein was detected on amino acid analysis of the $CHCl_3$:CH_3OH extracts[6]. The only ninhydrin-positive materials were ethanolamine and serine. The estimated amounts agreed well with published levels of phosphatidylethanolamine and phosphatidyl serine in placental microsomes[7].

In the case of placental microsomes, the phospholipids can make a significant contribution in the fluorometric estimation of total protein. The significance of this for protein determinations in general would be expected to vary with membrane phospholipid composition and content and each particular membrane preparation will have to be characterized individually.

Fluorescence of fluorescamine-labeled microsomes and microsomal phospholipids

Sample	Volume (ml)*	Fluorescence at 475 nm	Est. protein** (mg× ml^{-1})
Microsomes	0.005	60.0	45.0
Phospholipids	0.010	30.3	11.4
Phospholipids	0.020	68.7	12.9

*Volume of aliquot diluted to 1.5 ml. **Estimated protein content of sample based on fluorescence with ovalbumin as a protein standard. Data have been corrected for dilution and the fluorescence of reagent blanks.



Fluorescence emission spectra of fluorescamine-labeled whole microsomes (●) and resuspended $CHCl_3$:CH_3OH extracts of fluorescamine-labeled microsomes (○). Excitation was at 390 nm. Fluorescence intensity (F) is in arbitrary units, emission wavelength (λ) is in nm.

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