Methanol Solubility of Acid-Precipitated Proteins: A Possible Source of Error in Incorporation Studies[1] (34140)

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Trichloroacetic acid (TCA) is used commonly to precipitate proteins in studies of synthesis of protein using radioactive precursors. The precipitated proteins are usually washed with TCA followed by washes with a polar organic solvent or a combination of solvents to remove lipids which may be present in the precipitated protein. This general procedure is used even though it has long been known that albumin (1–3) and some other proteins (4) are soluble in polar organic solvents under these conditions. Some investigators have utilized the alcohol solubility of acid-precipitated albumin to study its isolation (5) and synthesis (6, 7), but other investigators studying other aspects of protein synthesis continue to overlook the possibility that significant losses of protein into polar solvents can occur in the preparation procedure.

The present experiments showed the distribution of radioactivity, in various cell fractions, between the TCA-precipitated methanol-soluble and TCA-precipitated methanol-insoluble material. This material is considered to be primarily protein since leucine, the tracer employed, participates in relatively few metabolic reactions other than protein synthesis and is known to be incorporated into proteins largely as leucine (8–12). The effect of temperature on the amount of radioactivity extracted by methanol is also described. The results of the following experiments suggest that studies of incorporation of precursors into lipids could also be in error if the precursors used were of a kind that could be incorporated into both protein and fatty acids, *e.g.*, glycine, as the extraction of lipids with polar solvents would potentially also extract some of the labeled proteins.

Materials and Methods. Mice were injected intraperitoneally with 3.3 μCi of leucine-^{14}C-UL (New England Nuclear, $>$ 200 mCi/mmole), and 500 mg of each of the livers fractionated, after 45 min, by the method described by Hogeboom (13). The cell fractions of two livers were pooled for each experiment. The solvent extraction procedures are summarized in Table I.

Results and Discussion. The results in Table I show the amount of methanol-soluble material obtained after precipitating proteins with TCA or PCA (perchloric acid). These experiments were done at room temperature (26°). The precipitated proteins were washed twice with acid to remove free radioactive leucine. The first wash (less than twice background), and the last TCA wash (A, extract) contained very little radioactivity, as is to be expected. The TCA-precipitated, methanol-soluble radioactivity (B) was 57% of the recovered radioactivity for the microsome fraction and 44% for the supernatant fraction. This is not to say that this radioactivity represents albumin entirely. Although microsomes have rather large quantities of albumin (5), the amount of albumin is small relative to the amount of protein extractable with methanol (4). Also, the ratios between methanol-insoluble and methanol-soluble should not be considered as absolute, as considerable variation may be expected under various conditions. Nevertheless, the data are representative of the distribution that may be expected. Using a less polar solvent combination, chloroform–methanol–ether (2:1:

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TABLE I. Distribution of Radioactivity in Methanol-Insoluble, and Methanol-Soluble Precipitated Proteins of Mouse Liver.[a]

Set	Cell fraction	Precipitating acid	Extracting solvent	(dpm × 10^{-3}) in: Sediment	Extract
A	Microsome	TCA	TCA	21.6	0.04
	Supernat.	TCA	TCA	10.8	0.00
B	Microsome	TCA	MeOH	8.0	10.7
	Supernat.	TCA	MeOH	5.5	4.3
C	Microsome	TCA	C-M-E	9.2	6.7
	Supernat.	TCA	C-M-E	7.2	2.6
D	Microsome	PCA	MeOH	16.2	1.5
	Supernat.	PCA	MeOH	8.9	0.7

[a] The precipitation and initial washing procedures were identical in sets A, B, and C. A microsome pellet equivalent to 250 mg of liver was suspended in 1 ml of water and precipitated with 1 ml of 10% trichloroacetic acid (TCA) and this precipitate was washed twice with 1 ml of 5% TCA. The supernatant was treated similarly, except that 0.3 ml of 60% TCA was used for the initial precipitation of 5 ml of supernatant. In set A, the TCA-washed precipitates were dissolved in 1 ml of Hyamine hydroxide (Packard Instruments) and counted in a scintillation counter; an aliquot of the second TCA wash was also counted to obtain the radioactivity in the TCA extract. In set B, the TCA-washed precipitate was washed once with 5 ml of methanol (MeOH) at 25° and the activities remaining in the precipitate and appearing in the methanol wash were counted. Treatment in set C was identical to that in B except that chloroform:methanol:ether (2:1:1) (C-M-E) was used in place of MeOH. In set D, 0.5 ml of 26% perchloric acid (PCA) was used for precipitating the microsomal and supernatant fractions, and the precipitates were washed twice with 2.6% PCA. Thereafter the procedure was the same as in set B. An internal standard was added to each sample to obtain disintegrations per minute (dpm).

1), resulted in less radioactivity being extracted (C), but the amount was still considerable; 42% for the microsome fraction and 27% for the supernatant fraction. The use of PCA as the precipitating agent (D) resulted in less radioactivity extracted by methanol, but it was still of significant amount; 9 and 7% for microsomal and supernatant fractions, respectively. The greatly decreased extractibility of labeled material from PCA precipitates demonstrates that the activity in methanol extracts to TCA precipitates is not attributable to a substantial lipid contamination.

The effect of temperature on the methanol solubility (%) of TCA-precipitated proteins of mitochondrial, microsomal and supernatant fractions from mouse liver is shown in Table II. The procedures shown are similar to those commonly employed in the literature. Not included is the procedure of heating one of the TCA washes to 90° to eliminate nucleic acids (15), as this heat denaturing process results in no radioactivity in the methanol wash. The greatest solubility was obtained when both the sample and the methanol were kept in an ice bath. Other work has shown that 25 to 30% of the total RNA could be extracted by cold ethanol from a TCA-precipitated liver homogenate (14). In the present experiments, solubility was markedly decreased when the samples were removed from the refrigerated centrifuge and placed in test tube racks at room temperature and mixed with methanol at room temperature. The alteration in solubility with respect to temperature is presumably due to changes in the conformation of the protein.

When the mitochondrial and microsomal pellets obtained after centrifugation were solubilized with 0.5% Triton prior to precipitation with TCA, the solubility in methanol was low, Table II. The relatively low methanol solubility of the precipitated proteins in these experiments was also presuma-

TABLE II. The Effect of Temperature on the Percent of Radioactivity in Trichloroacetic Acid-Precipitated Protein that is Soluble in Methanol.[a]

				Triton	
	Mit.	Mic.	Sup.	Mit.	Mic.
Sample and methanol kept at 2–4°	82	68	76	6	14
Sample at room temp., MeOH refrigerated (4°)	49	64	56		
Sample and methanol at room temp. (25°)	30	48	36	9	11
Sample neutralized, MeOH refrigerated (4°)	4	6	3		

[a] Mitochondrial (Mit.), microsomal (Mic.) and supernatant (Sup.) fractions were treated as described under B in Table I. Triton refers to mitochondrial and microsomal pellets solubilized with 0.5% Triton (Foley and Co., Foley, Alabama) prior to precipitation with TCA.

bly due to changes in protein structure brought about by solubilization and change in pH. Samples that were neutralized with potassium hydroxide prior to extraction with methanol showed relatively little radioactivity in the methanol-soluble fraction. It has been shown previously that TCA-precipitated protein exhibits low solubility in alcohol buffered with potassium acetate (4, 16). In this case, the authors conclude that the proteins form salts insoluble in alcohol, and thus prevents the electrostatic interaction between ionized macromolecules which would increase their solubility in alcohol (16).

Cohn and co-workers (17) applied low pH and cold alcohol to fractionate plasma proteins almost 30 years ago. However, this classic method of separating proteins has been superseded by methods that yield proteins that are purer and not denatured. Thus, the effects of low pH, polar solvents, and temperature on different proteins are now often overlooked. The present results reinforce the importance of this information when polar solvents are used to wash acid-precipitated proteins, especially so in experiments using isotope-labeled precursors.

Summary. The methanol solubility of acid-precipitated proteins was investigated. Cell fractions obtained from mouse liver, were precipitated with trichloroacetic acid, and in perchloric acid. The extent of the solubility of these precipitated proteins in methanol under various conditions was determined by radioactivity measurements. The results indicate the magnitude of error that may result in incorporation experiments wherein unheated acid-precipitated proteins are washed with a polar solvent.

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