

Use of a filter-paper disk assay in the measurement of lipid biosynthesis

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ABSTRACT The precipitation by trichloroacetic acid (TCA) of radioactive lipids on disks of filter-paper, followed by extraction with TCA and water to remove soluble radioactive precursors, provides the means for a convenient assay for lipid biosynthesis. Large numbers of disks can be washed in the same vessel, dried, and assayed for radioactivity with liquid scintillation counting equipment. Extraction of disks with lipid solvents provides a convenient control for the possible presence of TCA- and water-insoluble, nonlipid products. The application of the method to the assay of cyclopropane fatty acid synthetase is shown.

KEY WORDS determination · radioactive · lipids · biosynthesis · precipitation · trichloroacetic acid · filter-paper disk · cyclopropane fatty acid synthetase

INVESTIGATIONS IN THE FIELDS of nucleic acid and protein biosynthesis have been greatly facilitated by rapid techniques for the assay of macromolecular synthesis. Separation on membrane filters of the macromolecular products of enzymatic reactions may be effected by the use of precipitating agents followed by filtration and washing, usually with the aid of suction. In another general method, first applied by Bollum to nucleic acid synthesis (1), and later to protein synthesis by Mans and Novelli (2), the macromolecule is precipitated directly on a disk of filter-paper, which is subsequently washed and extracted to remove both precursor and interfering macromolecules. The latter procedures lend themselves to batch treatment of large numbers of filter-paper disks. The method of Bollum has now been adapted to investigations of lipid biosynthesis. The techniques and an application are described in the present report.

Abbreviation: TCA, trichloroacetic acid.

EXPERIMENTAL PROCEDURES

The general method described below is applicable to studies involving the biosynthesis of lipids from acid-soluble precursors.

Assay

An aliquot of the enzymatic reaction mixture, generally 0.05–0.1 ml, is pipetted onto a 24 mm diameter disk of Whatman No. 3 MM filter paper mounted on a pin (2). After exposure (15 sec) to a stream of warm air, the disk with its mounting pin is plunged into an ice-cold bath of 10% (w/v) trichloroacetic acid (TCA). A number of disks may be collected in the same bath in the course of an experiment and, as will be shown below, there is no cross-contamination from disk to disk. After at least 30 min, the 10% TCA is decanted and drained and ice-cold 5% TCA (w/v) is added. A volume of approximately 10 ml is used per disk, but it should be possible to decrease this (2). After 15 min the 5% TCA is decanted and replaced with the same volume of ice-cold distilled water. After 10 min with occasional gentle stirring the first water wash is decanted and a second wash is similarly performed in an ice bath. The water is then decanted and the disks are mounted with their pins in a cork ring. If the pins are horizontal the wet disks will not slide down the pins and touch the surface of the cork. The disks are then dried either overnight at room temperature or under an ordinary (250 w) heat lamp at a distance of 30 cm. If a stream of air is also blown over the disks, drying can be accomplished in 30 min. The dried disks are placed in scintillation counting vials with 5 ml of a toluene scintillator solution containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per liter.

Materials

³H- and ³²P-labeled *Clostridium butyricum* lipids were obtained by growing 2-liter cultures of the organism on a

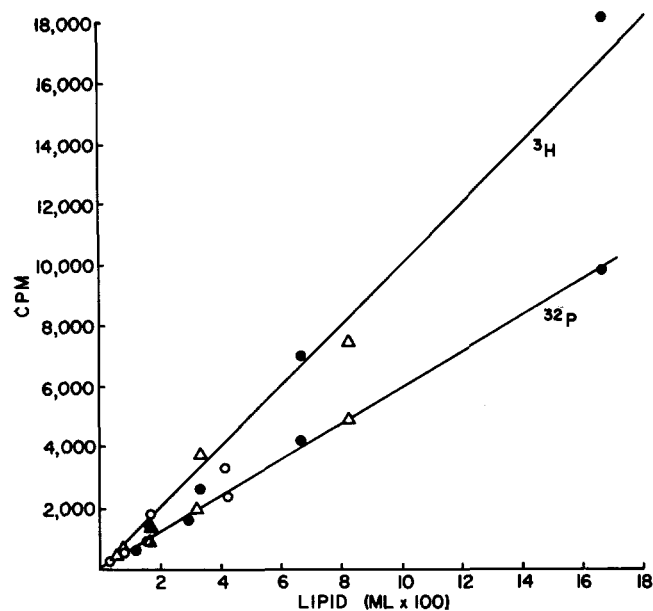


FIG. 1. Linearity of the filter-paper disk method with ^3H - and ^{32}P -labeled lipids. ● = 1.2 mg of protein per disk, Δ = 0.6 mg of protein per disk, ○ = 0.3 mg of protein per disk. All points in the experiment with ^{32}P -labeled lipids fall on a straight line. There is some scatter of the points obtained with ^3H -labeled lipids.

Casamino acid medium containing either 110 μC of ^3H -glycerol (71 $\mu\text{C}/\mu\text{mole}$) or 350 μC of ^{32}P -inorganic phosphate (3). ^3H -Glycerol was purchased from Nuclear-Chicago Corp. and $^{32}\text{P}_i$ from Isoserve, Inc. (Cambridge, Mass.).

The lipids were extracted with chloroform-methanol 2:1 (v/v) (4). Neutral lipids were separated from phospholipids on columns of silicic acid. The composition of these lipid mixtures has been described (3).

Partially purified cyclopropane fatty acid synthetase from *C. butyricum* and [methyl- ^{14}C] S-adenosylmethionine were kindly provided by Drs. J. H. Law and P. J. Thomas.

RESULTS

Linearity with Amount of Radioactive Lipid

Various amounts of chloroform solutions of ^3H -labeled and ^{32}P -labeled lipids from *C. butyricum* were evaporated in a Potter-Elvehjem homogenizer tube. To the lipids was added 0.3 ml of a crude extract of *Escherichia coli* (11.7 mg of protein per ml) and the lipid was suspended by homogenization with a motor-driven pestle. Of the resulting homogenate 0.1 ml was transferred to a small test tube and 0.1 ml was put on a filter-paper disk. The remaining 0.1 ml was diluted with 0.1 ml water, and 0.1 ml of the diluted suspension was put on another filter-paper disk. By repeating this procedure 4-fold dilutions were also obtained. The disks were dried and washed (together) as described under Experimental Procedures. As shown in

Fig. 1 the amount of ^3H and ^{32}P detected on each disk was proportional to the amount of the original lipid solutions transferred to it. No effect of the amount of protein, in the range of 0.3–1.2 mg per disk, was observed with either isotope. No cross-contamination was observed.

It was possible that the lipids were binding to the protein precipitated on the disks. To test for this, 0.02 ml of a chloroform solution of ^3H -labeled *C. butyricum* lipids was evaporated directly on a number of disks followed by 0.1 ml of serial 2-fold dilutions in Tris buffer [tris(hydroxymethyl)amino methane], pH 7.5, 0.05 M, of a crude sonic extract of *C. butyricum* (27 mg of protein per ml). Ten dilutions were made (range of protein 2.7 mg–2.6 μg per disk) and one disk had only Tris buffer in addition to the lipid. There was no noticeable effect of the cell extract on the recovery of lipid radioactivity after the standard washing procedure, except for a 6% reduction below the mean with the largest amount of cell extract. The mean deviation was $\pm 2.9\%$ when the disks were counted for 10 min (2560 cpm).

Efficiency of Counting

The efficiency of scintillation counting of various isotopes on the disks ranges from a minimum of 8% with ^3H , to 40% with ^{14}C , to a maximum of approximately 65% with ^{32}P . This compares with 20, 62, and approximately 65% when the same radioactive lipids are completely dissolved in the scintillation fluid at the settings routinely used with our Model 703 Nuclear-Chicago Corp. counter. The loss of counting efficiency with ^3H may militate against the filter-disk assay in studies involving low levels of this isotope or precious ^3H -labeled precursors.

Elution of Lipids from Disks

With disks containing ^3H -labeled or ^{32}P -labeled mixed *C. butyricum* lipids plus crude *E. coli* extract (0.5 mg of protein), replacing the two water washing steps (after TCA treatment) by extraction with warm ethanol-ether mixtures (2) resulted in a 90% loss of radioactive lipid (Table 1). This procedure can therefore serve as a control for

TABLE 1 EXTRACTION OF LIPIDS FROM FILTER-PAPER DISKS BY ETHANOL-ETHER

	Normal Washing Procedure	Ethanol-Ether Extraction after TCA*	Loss
	cpm	cpm	%
^3H -Labeled total <i>C. butyricum</i> lipids	2180	163	93
^{32}P -labeled <i>C. butyricum</i> lipids	1310	149	89

* The disks were extracted with ethanol-ether 1:1 (v/v) for 30 min at 37°, twice. They were then washed in ether for 15 min.

TABLE 2 EXTRACTION OF LIPIDS FROM FILTER-PAPER DISKS BY TOLUENE SCINTILLATION SOLUTION

	Disk Radioactivity before Transfer	Radioactivity Transferred with Disk		Radioactivity Left in First Scintillation Solution	
	cpm	cpm	%	cpm	%
³ H-Labeled "neutral" lipids	211	53	25	160	76
	4130	743	18	3492	85
³ H-Labeled phospholipids	415	387	93	46	11
	1935	1920	99	152	8
³² P-Labeled phospholipids	4950	4540	92	104	2
	2430	2190	90	39	2

lipid biosynthesis in crude systems or where precursors of other macromolecules are used.

Toluene scintillation fluid was found to extract 80% of ³H-labeled *C. butyricum* "neutral" (nonpolar) lipids from the filter-paper disk. Removal of the disk from the first scintillation vial, containing 15 ml of toluene scintillator, followed by radioassay in a fresh vial containing toluene scintillation solutions, gave a distribution of 80% of the radioactivity in the first vial and 21% carried over on the disk (Table 2). When the same experiment was performed with ³H-labeled phospholipids, 96% of the radioactive lipid remained on the disk and only 9% was extracted into toluene scintillator (Table 2). With ³²P-labeled *C. butyricum* lipids 91% of the label remained on the disk.

Washing and Drying of Disks

The procedure used was based on that of Mans and Novelli (2). The original treatment of the disk consisted of 1 hr in 10% TCA, 15 min in 5% TCA, and 30 min in water, twice. Reduction of the time in 10% TCA to 0.5 hr and reduction of the time of the water washes to 10 min each with intermittent stirring resulted in no change in recovered radioactivity in tests with ³H-labeled lipids. The possibility of further reducing the washing time has not been tested. Drying of the disks in air overnight at room temperature is used routinely; however, rapid drying of the disks can be obtained under an infrared heat lamp in a stream of warm air. With these aids the drying time can be reduced to 30 min with no change in recovered radioactive lipid.

Test of the Method with Cyclopropane Fatty Acid Synthetase

The activity of this enzyme has previously been measured by the transfer of a labeled methyl group from S-adenosylmethionine to ester-linked unsaturated fatty acids in phospholipids. The phospholipids were saponified and the fatty acids were extracted and assayed for radioactivity (5). In Fig. 2B it can be seen that the incorporation of radioactivity from [methyl-¹⁴C] S-adenosyl L-methionine into TCA- and water-insoluble material

precipitated on filter-paper disks parallels the transfer of methyl groups into fatty acids as measured by the method of Zalkin, Law, and Goldfine (5). In the absence of phospholipid acceptor, incorporation as measured

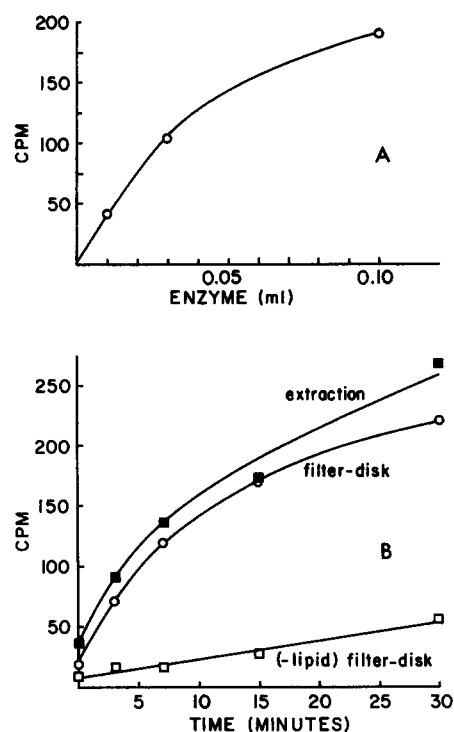


FIG. 2. Use of the filter-paper disk method for assay of transfer of the methyl group of S-adenosylmethionine to the fatty acids of a phospholipid.

A. Each reaction mixture (at 33°) contained Tris-HCl buffer, pH 7.5, 50 μ moles; [methyl-¹⁴C] S-adenosylmethionine, 0.05 μ mole, 0.023 μ c; a dispersion of purified phosphatidyl ethanolamine from *Azotobacter agilis* (6), 0.58 μ mole of P; sodium dodecyl sulfate, 0.2 μ mole; purified extract of *C. butyricum* (6) as indicated, in a total volume of 0.50 ml.

B. The reaction mixtures (at 33°) contained Tris-HCl buffer, pH 7.5, 50 μ moles; [methyl-¹⁴C] S-adenosylmethionine, 0.10 μ mole, 0.046 μ c; 1.16 μ moles of phosphatidyl ethanolamine; sodium dodecyl sulfate, 0.4 μ mole, 0.20 ml of 5-fold purified cyclopropane fatty acid synthetase solution (6) in a final volume of 0.95 ml. A portion (0.08 ml) of the reaction mixture was pipetted onto a filter-paper disk at the times indicated and 0.08 ml was pipetted into 0.05 ml of 10% KOH in 50% methanol for saponification and extraction (5). Cpm per disk (O), and per combined extractions (■) are shown. □, incorporation, according to the filter-disk assay, when the phospholipid acceptor was omitted.

by the filter-paper disk method was much slower than in the presence of phospholipid. This observation has also been made when incorporation into fatty acids is measured directly (5). Incorporation of the methyl group of S-adenosylmethionine is also shown to be dependent on the amount of enzyme added (Fig. 2A).

DISCUSSION

The usefulness of the filter-paper disk assay in studies of protein synthesis (2, 7) and nucleic acid synthesis (1) has been amply demonstrated. The experiments reported here indicate the potential utility of this assay in the field of lipid biosynthesis. Lipids are quantitatively precipitated and recovered on disks of filter-paper, and the yield is independent, in the range studied, of the amount of either lipid or other substances found in crude cell extracts added to the disks. The method provides a means for following the kinetics of incorporation of water-soluble precursors into lipids, even if the reactions are quite rapid. With no special equipment it is not difficult to take samples at 1 min intervals.

A potential drawback is the possibility of interfering reactions leading to the synthesis of nonlipid products that are insoluble in TCA and water. Methods involving liquid-liquid extraction with lipid solvents and counting of the lipid-soluble phase are more specific and should always be used as a check before the filter-paper disk assay is accepted for routine work. However, once the products of incorporation of the water-soluble precursor have been established as lipid, the filter-paper disk assay offers the advantages of multiple sampling from the same reaction mixture to establish the kinetics of a given reaction and the extreme ease of extracting and washing large numbers of disks in the same vessel with very little working time consumed in manipulation. With the exception of experiments involving few samples, we have repeatedly found the filter-paper disk assay to be less time-consuming than assays involving liquid-liquid extractions or acid precipitation of individual samples in centrifuge tubes.

We have also applied the assay to a rather complex system involving the de novo synthesis of lipid from a nonlipid radioactive precursor, L- α -glycerophosphate. The results obtained with the filter-paper disk assay are not completely satisfactory because radioactive glycerol

is incorporated in this system into nonlipid TCA- and water-insoluble products.

The results obtained with cyclopropane acid synthetase, a purified enzyme, show the assay to better advantage. It is currently being used for the routine assay of this enzyme.¹ The filter-paper disk assay has also been found to give results similar to those with a liquid-liquid extraction assay, for the synthesis of glycolipids catalyzed by bacterial enzyme systems.²

Brossard, Erbland, and Marinetti (8) have briefly described the use of filter paper loaded with silica gel in assays of lipid biosynthesis. In their procedure the nonlipid precursor, ¹⁴C-glycerol, is incubated with the enzyme mixture, and the reaction mixture is spotted on silica gel-loaded filter paper and chromatographed. The precursor remains at the origin and the lipid products migrate at different rates. The products can thus be identified and assayed. The method described in the present report involves less manipulation and yields quantitative data more directly, but does not permit one to identify the products of the reaction at the same time.

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¹ J. H. Law and P. J. Thomas, personal communication.

² W. J. Lennarz, personal communication.