A rapid method for determining the specific activity of methylene-^{14}C citric acid in cell extracts

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The microchemical spectrophotometric method for the determination of citric acid has been found to be sufficiently specific to permit its use for the measurement of specific activity, provided the labelling occurs in the noncarboxyl carbon atoms of the molecule. The simplicity of the method permits determinations to be carried out simultaneously on large numbers of samples especially if liquid-scintillation techniques are employed for the measurement of radioactivity.

The determination of specific activity of a compound such as citric acid in a cellular extract is a time-consuming procedure largely because the citric acid must be isolated from the extract before its radioactivity can be measured. This is usually done by conventional chromatographic techniques which do not readily lend themselves to processing large numbers of samples.

On the other hand, the measurement of citric acid concentration by microchemical techniques is relatively simple because no isolation procedures are necessary. The microchemical spectrophotometric method based upon an oxidative-bromination procedure appears to have first been described by Pucher *et al.* (1) in 1936. These authors drew attention to the highly specific characteristics of the reactions. In the years that followed several modifications were made, intermediates and final products were identified, and improvements in reagents were introduced (2–5).

The Taylor (4) modification of the procedure involves the oxidative decarboxylation of citric acid to acetonedicarboxylic acid followed by bromination to pentabromacetone (PBA). The oxidative step removes the carboxyl carbon atoms 1, 5, and 6 leaving the atoms 2, 3, and 4 in the PBA. In the microchemical procedure, the PBA is extracted from the aqueous reaction mixture into petroleum ether. After phase separation the petroleum ether solution is shaken up with an aqueous sodium sulfide reagent with which the PBA develops a yellow color. Since the sulfide reagent used by Taylor yields a color with PBA which fades with time, we have routinely used McArdle's (5) thiourea–borate–sulfide reagent (TBS) which does not show this undesirable characteristic. The specificity of the method derives from the fact that most compounds that are encountered in a cell extract are destroyed in the oxidative step which is carried out in 9 *N* H_2SO_4, and even if they did survive they would have to be soluble in petroleum ether and capable of reacting with the sulfide reagent. This high degree of specificity suggested to us that the petroleum ether extract might be used to measure radioactivity as well as concentration if it could be shown that a cell extract would contribute nothing but the citrate-derived PBA to the petroleum ether. Inability of extraneous material to react with sulfide would not preclude the possibility of contributing to radioactivity if such materials were to survive the oxidation and be soluble in petroleum ether. This paper is concerned with the validation of the specificity of this method for citric acid in a cell extract.

Materials

All chemicals were of reagent grade, either British Drug Houses or Baker's. Labelled citric acid was obtained from New England Nuclear Corporation and Mallinckrodt Nuclear. Radioactivity was measured in a Unilux II liquid-scintillation spectrometer using butyl-PBD (Nuclear-Chicago) as the scintillator.

Methods

The analytical method involves the addition of 5 ml of 27 *N* H_2SO_4 to a 5-ml volume of the sample to be analyzed. After mixing, 5 ml of the oxidative-bromination reagent (4) are added, and the mixture is allowed to react for 30 min. Excess of the bromination reagent is destroyed by the addition of 3 ml 22% aqueous $FeSO_4$. The PBA formed is now extracted by 30 s vigorous shaking with 6 ml petroleum ether, 5 ml of which is removed to a clean container. Aliquots (20–20 µl) of this are added directly to the liquid-scintillation fluid for radioactivity determinations. Four milliliters of the TBS reagent (5) are added to the remaining petroleum ether solution and, after phase

separation, the supernatant layer is discarded. The underlying aqueous phase is used for optical density measurements read at 450 mμ.

During the course of this investigation, certain of these steps were modified for specific purposes but the above description applies to the method when used for analysis. In those cases where citric acid concentration or radioactivity is expected to be low, 3 ml rather than 6 ml petroleum ether may be used to extract the PBA.

Reference standards consisted of 5 ml of a solution containing 100 or 200 μg citric acid in 1 *N* H_2SO_4.

For most of the chromatographic analyses, thin-layer chromatography (t.l.c.) plates were prepared from silica gel D-5 (Camag) spread at 300 μ thickness. These were activated by heating to 120° for 15 min just prior to using. In other experiments, factory-coated silica gel D-A plates (Camag) were employed. Aliquots of the petroleum ether solutions of PBA were spotted directly on the plates which were allowed to dry at room temperature. Following development, the plates were air-dried before spraying with TBS reagent or with Tollen's reagent (6) which detects compounds possessing a carbonyl group in the molecule.

Experimental

Microchemical Analysis of Authentic Citric Acid-^{14}C

Before proceeding to an analysis of cell extracts, it was necessary to establish the precision with which citric acid could be recovered and the theoretical specificity for the noncarboxyl carbon atoms of the molecule. Solutions of 'cold' citric acid (20 μg/ml) were enriched by the addition of citric acid labelled in the 1,5, 2,4, or 6 positions. Five milliliters of each solution were put through the standard microchemical analysis as described previously, modified only in that 3 ml rather than 6 ml of petroleum ether were used to extract the PBA from the oxidation-bromination mixture. Twenty-five microliters of the petroleum ether extract were added directly to 10 ml of the scintillation fluid. This amount of petroleum ether was found to exert no quenching. Twenty-five microliters of the original citric acid solution were used to determine the initial radioactivity, 0.75 ml of absolute ethanol being added in order to maintain solubility in the scintillation fluid. The results shown in Table I establish the high recovery and specificity for the noncarboxyl carbon atoms of citric acid.

TABLE I

Recovery of citric acid labelled with ^{14}C in various positions

Position of label	Initial count	Counts recovered	Percentage recovery
2,4	38 009	37 520 ± 424*	99.7
1,5	46 985	1 590 ± 148	3.4
6	44 358	1 250 ± 132	2.8

*Standard error of mean.

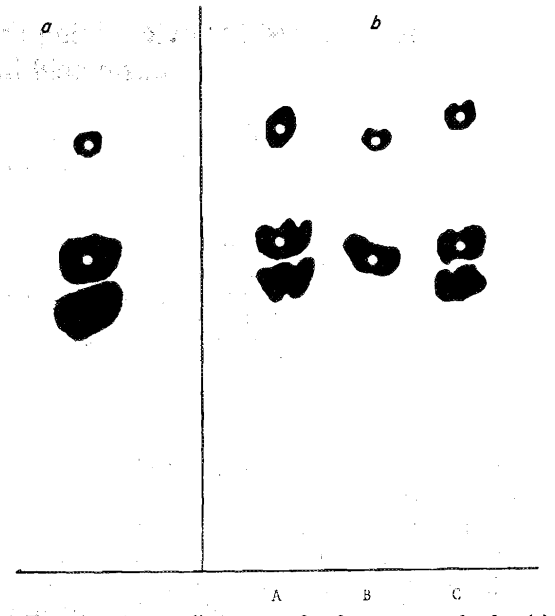


FIG. 1. Autoradiograms of reference standards. (*a*) 100 μg citric acid; (*b*) 100 μg citric acid processed in 9 *N* H_2SO_4 (A), 1 *N* H_2SO_4 (B), and 15 *N* H_2SO_4 (C). Open circles indicate TBS-positive areas of chromatograms.

Analysis of Reference Standards

The solvent system adopted for all work reported herein consisted of *n*-pentanol – glacial acetic acid – water (45:15:10) (7). When reference standards were developed in this system and the chromatogram was sprayed with TBS reagent, two spots appeared; but autoradiograms showed there to be three areas of radioactivity (Fig. 1*a*). Goldberg and Bernheim (2) reported that both temperature and sulfuric acid concentration in the oxidative-bromination reaction may lead to mixtures of bromacetones. At low acid concentration, the products are tri-, tetra-, and penta-bromacetone, whereas, in high acid concentration, hexabromacetone predominates. The ability of the bromacetones other than PBA to react with TBS reagent is not mentioned.

On the assumption that the entities in the reference standard did in fact represent these other bromacetones, the oxidative-bromination procedure was carried out in 1 *N*, 9 *N*, and 15 *N* sulfuric acid (final concentrations). The petroleum ether extracts were reduced in volume from 5 ml to 0.2 ml, and 80 μl were spotted on the t.l.c. plates. Autoradiograms were made from the

chromatograms prior to spraying them with TBS reagent. A tracing of the autoradiogram is reproduced in Fig. 1*b* where the TBS-positive spots are marked with a circle. In sample B where the acid concentration was weakest, the hexabrom derivative should be missing and this would correspond with the lowest of the three spots in samples A and C. It should be noted that this spot is radioactive but is TBS-negative. Since in theory all three bromacetones should be radioactive, failure of the hexabrom species (if this is in fact its identity) to react with TBS might be due to a hinderance effect of extra bromine atom. Unless it could be shown that these three radioactive products were always produced in the same relative proportion, estimates of specific activity based on the content of the petroleum ether extract would be in error.

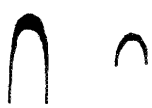
Analysis of a series of reference standards varying in citric acid concentration from 40 to 120 μg, but in which the specific activity was the same throughout, showed a mean ratio of radioactivity/optical density of 11.18 ± s.e. 0.069 $\times 10^{-4}$. This means that even though the TBS reagent does not detect all the citrate-derived material in the petroleum ether, it does detect a constant proportion of it (this fact is also accounted for by the linear relation between optical density and concentration).

Chromatograms visualized with Tollen's reagent revealed the presence of four spots, three of which corresponded in mobility to those of Fig. 1*a*. This would indicate that a fourth carbonyl-containing component is produced in the reaction mixture but since this is neither TBS-positive nor radioactive, its presence in the petroleum ether does not influence the measurement of specific activity.

Occasionally while preparing some of the above chromatograms, a sample of cell extract was spotted and developed with a reference standard. These samples yielded only one comet-shaped radioactive spot. Subsequent investigations showed this to be due to the presence of TCA in the cell extract. When the citric acid standards were made up in 8% TCA instead of 1 *N* H_2SO_4, the TBS-sprayed chromatograms derived from them were identical with their autoradiograms. It was also shown that although TCA changed the chromatographic pattern, its presence did not influence the measurement of radioactivity or of optical density of a series of standards containing from 40 to 120 μg citric acid of the same specific activity (mean specific activity in 1 *N* H_2SO_4, 418.3 ± s.e. 3.55; in 8% TCA, 414.4 ± s.e. 2.97).

Analyses of Cell Extracts

Cell extracts containing biosynthetic citric acid-^{14}C were prepared as follows. A suspension of cells from an 18-h culture of *Saccharomyces cerevisiae* was suspended in McIlvaine's buffer containing 10 m*M* acetate enriched with acetate-2-^{14}C. After 90 min aeration, the cells were harvested and washed thoroughly with distilled water. The cell paste was alternately frozen and thawed before adding 8% TCA to precipitate protein and extract the citric acid. Without further purification, 5 ml of the cell extracts were put through the oxidative-bromination procedure and aliquots of the petroleum ether solutions of PBA were chromatographed. This invariably yielded chromatograms showing the 'comet' effect due to the presence of TCA. When the reference standard as well as the cell extract contained 8% TCA, the results were as shown in Fig. 2. This is a reproduction of an autoradiogram



A B

FIG. 2. Autoradiogram of reference standard and cell extract both dissolved in 8% trichloroacetic acid. (A) 100 μg citric acid; (B) cell extract.

in which A represents 100 μg reference standard and B represents extract of cells exposed to acetate-2-^{14}C for 60 min. A second t.l.c. plate was prepared in an identical manner and after drying was sprayed with TBS reagent. The yellow reactions obtained were found to coincide exactly in position and shape with the darkened areas of the autoradiogram.

Discussion

The experimental data are fairly good evidence for the specificity of the analytical procedure.

What one does not know is whether radioactive substances other than citric acid are capable of being converted to radioactive PBA and therefore appearing to originate from citric acid. Were this to be a possibility, such compounds should yield a color with the TBS reagent. Pucher *et al.* (1) report that 100–120 mg amounts of acetone, glycogen, creatine, creatinine, cholesterol, urea, allantoin, and uric, hippuric, lactic, oxalic, tartaric, succinic, maleic, and fumaric acids all yield a negative color test. In our experience glutamic, glyoxylic, isocitric, oxaloacetic, α-ketoglutaric, pyruvic, and acetic acids as well as dihydroxyacetone and dihydroxyacetone phosphate fail to produce a color reaction with TBS reagent. Hydroxybutyric acid and the diethylester of acetone dicarboxylic acid are known to yield about 1% of the color reaction of an equal amount of citric acid (1), and therefore, the likelihood of contamination from these sources is small. With this reservation in mind the work reported herein indicates that a combination of the microchemical procedure of Taylor and the TBS reagent of McArdle can be used to determine the specific activity of methylene-labelled citric acid with a high degree of precision.

It is also clear from this work that authentic citric acid-2,4-^{14}C is converted to four carbonyl-containing petroleum-ether-soluble products, three of which are radioactive, but only two of these are capable of reacting with the sulfide reagent to produce the yellow color that forms the basis of the colorimetric estimation of citrate concentration. The data of Table I leads one to conclude that these compounds represent residues derived from the methylene carbon atoms of citric acid.

Acknowledgment

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