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An improved method for the detection and radioassay of monosaccharides on thin-layer or paper chromatograms

Separation and quantitative determination of carbohydrates by thin-layer or paper chromatography usually requires the co-chromatography of these compounds with known standards. After visualizing the standards, corresponding areas are cut out of the paper or scraped off the thin-layer plate. The material is then extracted and the sugars are quantitatively determined. This technique may lead to erroneous results particularly if mixtures contain compounds with only slightly different R_F values.

It would therefore be advantageous if sugars could be visualized and then sharply separated from neighbouring compounds. However, most of the spray reagents in use at the present time cause the sugars to be washed out in the procedure or destroyed by heating during the development of color. These reagents may also form water-insoluble pigments. The strongly colored extracts may lower the efficiency of the detection of such labeled materials by liquid scintillation spectrometry because of quenching.

The present work describes techniques in which modified spray reagents have been developed which eliminate potential dilution of the saccharides. Furthermore for each of these reagents, a method of decoloration was devised, thus eliminating quenching in the subsequent liquid scintillation radioassay.

Experimental

D-Glucose-^{14}C was obtained from New England Nuclear Corp. Benzoic acid-^{14}C in toluene standard was obtained from National Bureau of Standards. Silica Gel G was a Merck A.G. product. Other reagents used in the course of this work were analytical grade materials. The solvents were U.S.P. standard quality.

Silver nitrate spray reagent. The procedure by TREVELYAN *et al.*[1] was modified in the following fashion. A solution of 1 g of silver nitrate in 4 ml of water was added to 125 ml of acetone. Thin-layer plates or paper chromatograms were sprayed with this solution and air dried. Monosaccharides appear as dark spots on yellow background when the chromatograms were sprayed with a solution of 1.2 g of sodium hydroxide in 1.2 ml of water to which 98.8 ml of absolute ethanol had been added.

*2,3,5-Triphenyl-2*H*-tetrazolium chloride spray reagent.* This reagent was also originally described by TREVELYAN *et al.*[1]. In the present work, a 0.2 % (w/v) solution of triphenyltetrazolium chloride in 100 ml of chloroform was used. To this, 5 ml of triethylamine was added in order to replace the inorganic base used previously. The sugars were visualized by heating the plates or the paper chromatograms for 10 min at 110°.

p-Aminobenzoic acid spray reagent. The reagent used in the present experiments is a modification of a mixture described by ROY[2]. Oxalic acid (2 H_2O), 75 mg, was dissolved in 15 ml of ethanol. A solution of 150 mg of *p*-aminobenzoic acid in 25 ml of chloroform and 2 ml of acetic acid was prepared. The two solutions were mixed just prior to use. The plates were sprayed with the mixture and heated for 15 min at 110°. With this reagent, as little as 0.5 μg of glucose could be detected when viewed under ultraviolet illumination at 254 mμ.

Development of the chromatograms. Glucose-1-^{14}C, 5 to 25 μg, in an 0.1 % (w/v) aqueous solution corresponding to 10–50,000 d.p.m. was spotted on a Whatman No. 1 chromatography paper or on a Silica Gel G (250 μ thick) thin-layer plate. The papers were developed with ethyl acetate–pyridine–water (3.6:1.0:1.15, v/v/v), as described by COLOMBO *et al.*[3]. Thin-layer chromatography was carried out using *n*-propanol–water (7:1, v/v) as the developing solvent as described in a previous publication[4]. Glucose migrated with an R_F 0.62 in this thin-layer system. After the chromatograms were developed, the migration of glucose was visualized with one of the spray reagents. The thin-layer plates or the papers were dried and heated as required by the various techniques. The sharply delineated glycose-containing areas were cut out of the paper chromatograms or scraped from the thin-layer plates with a scalpel.

Decolorization of the spots and liquid scintillation radioassay. The paper discs or the colored silica gel scrapings were placed in low background liquid scintillation vials. When the spots were visualized with silver nitrate or triphenyltetrazolium chloride, 10 μl of a 4 *N* nitric acid solution were applied to the samples. Decolorization was usually complete in one hour. If necessary this reaction could be accelerated by heating the vials moderately with a hot air blower. To the decolorized samples was added 0.5 ml of water and the suspension was allowed to stand for 16 h. The brown spots obtained by the *p*-aminobenzoic acid spray were decolorized and extracted by treating the samples with 0.5 ml of an aqueous solution of 5 mg of sodium borohydride[5] overnight. The aqueous extracts were then mixed with 10 ml of a scintillation solvent containing 250 g of naphthalene, 24 g of PPO (2,5-diphenyloxazole) and 0.6 g of POPOP (*p*-bis[2-(5-phenyloxazolyl)]benzene) in 2 l of dioxane.

The recovery of labeled glucose from thin-layer plates was also studied by using a thixotropic gel counting solution. The plates were sprayed with the silver nitrate reagent, the carbohydrate containing areas were identified and were decolorized with 10 μl of a 4 *N* nitric acid solution as described above. The colorless scrapings were suspended in 10 ml of a 4 % *Cab-O-Sil* (w/v)scintillation solution containing 5 g of PPO and 0.3 g of POPOP per liter of toluene according to SNYDER AND STEPHENS[6].

Radioactive measurements. The counting was carried out in a *Packard Tricarb* model 3003 liquid scintillation spectrometer equipped with an external radium source for standardization. The efficiency for ^{14}C was 82 % when the dioxane scintillation solution was used. The average background count was 15 c.p.m.

Results and discussion

The recovery of glucose-^{14}C from the two types of chromatographic procedures ranged from 75 to 82 % of the theoretical (Table I). Control experiments were performed in which the recovery of labeled glucose was determined without irrigating the chromatograms. The sugar was spotted, visualized and decolorized. The radioactive counts obtained were about 10 % higher than those found after development with the respective solvent systems. With the use of the procedures outlined in this paper no quenching occurred. The lower recovery obtained by using the *Cab-O-Sil* suspension is not due to chemical or color quenching. The radioactive material is partly concealed by the silica gel absorbent. Estimation of quenching by the external source is therefore valueless under such condition.

These values represent the average of three or more determinations. Quenching was negligible when the quantity of nitric acid used to decolorize the spots obtained

TABLE I

RECOVERY OF GLUCOSE-^{14}C

Analytical procedure	*Spray reagent*			
	Tetrazolium (%)	*Silver nitrate* (%)	*p-Amino-benzoic acid* (%)	*Silver nitrate and Cab-O-Sil* (%)
Paper chromatography	79	82	77	
Thin-layer chromatography	81	81	75	64

by spraying with tetrazolium or silver nitrate was restricted to 10–20 μl of a 4 *N* solution. However, 0.1 ml of an 8 *N* nitric acid solution caused a 20% decrease in counts. No quenching was observed with sodium borohydride which was used to decolorize the spots produced with the *p*-aminobenzoic acid solution.

All three spray reagents are useful for the detection of reducing sugars except for aminosugars. For the determination of amino- or acylaminosugars, the silver nitrate spray solution should be chosen since it gives dark spots with these monosaccharides as well. Of the reagents described, the *p*-aminobenzoic acid mixture is the most sensitive if the chromatograms are viewed under ultraviolet light.

The data presented in this paper are based on radioassays with glucose-^{14}C. Radioactivity of other reducing sugars can also be determined by the use of the same techniques. For accurate quantitative results, radioactive standards should be co-chromatographed.

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