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An artefact in the chromatography of sugar nucleotides using solvents containing ammonium acetate

The solvents (A) 95% ethanol–1 *M* ammonium acetate pH 7.5 (5:2) and (B) 95% ethanol–1 *M* ammonium acetate pH 3.8 (5:2) are widely used in the analysis and preparation of sugar nucleotides. These solvents were originally described by PALADINI AND LELOIR[1] and were used to assist in studies on the properties of uridine diphosphoglucose (UDPG). In one respect solvent A is the more useful of the two because it allows an easier separation of sugar nucleotide from the related nucleoside 5'-phosphates which often accompany it in biochemical preparations. Some $R_{adenosine}$ values in this solvent are given in Table I. It is the purpose of this communication, however, to show that unreliable analytical results are obtained with solvent A unless certain precautions are taken.

Experimental

Analytical standards were obtained from Sigma, London Ltd. Chromatographic

TABLE I

SOME $R_{adenosine}$ VALUES AT 22° IN SOLVENT A

Compound	$R_{adenosine}$
Adenosine 5'-triphosphate	0.05
Adenosine 5'-diphosphate	0.09
Adenosine 5'-monophosphate	0.24
Adenosine 5'-diphosphoglucose	0.32
Adenosine	1.00
Uridine 5'-triphosphate	0.09
Uridine 5'-diphosphate	0.14
Uridine 5'-monophosphate	0.32
Uridine 5'-diphosphoglucose	0.44
Glucose-1-phosphate	0.39
Glucose-1:2-cyclic phosphate	0.86

solvents of highest purity were freshly prepared prior to use. Ammonium acetate, Analar grade, was purchased from BDH Ltd., Poole, Dorset. Descending chromatography was carried out in sealed tanks using Whatman No. 1 paper. For analysis of uridine diphospho[^{14}C]glucose (76 mCi/mmole) and adenosine diphospho[^{14}C]glucose (217 mCi/mmole), samples (0.1 μCi) were spotted on paper accompanied by 10 μg of pure carrier. Spots were dried using a stream of cold air. After chromatography (16 h), radioactive areas were located by preparing autoradiograms with "Kodirex" X-ray film. The radiochemical purity was determined by cutting up the chromatogram strip, placing the pieces in toluene/2,5-diphenyloxazole and counting in a liquid scintillation spectrometer[2]. The ^{14}C in sugar nucleotide was expressed as a percentage of the total radioactivity along the solvent track. Nucleosides and nucleotides were detected on paper chromatograms by viewing under UV light and phosphates by spraying with ammonium molybdate reagent[3].

TABLE II

STABILITY OF UDPG IN STERILISED AQUEOUS SOLUTION AT 25° AT VARIOUS pH VALUES

Figures refer to % radiochemical purity. At time 0 this was 98%. The ^{14}C-labelled UDPG was dissolved in 0.05 *M* buffer at a radioactive concentration of 5 μCi/0.2 ml and the solution sterilised by filtration. At appropriate time intervals analyses were carried out using solvent C. Buffers employed were sodium phosphate (pH 4.6, 7.6 and 8.0), glycine–NaOH (pH 9.2) and Na_2CO_3–$NaHCO_3$ (pH 10.7).

pH	*Time (h)*					
	1.5	*4*	*24*	*48*	*72*	*96*
4.6	—	—	97.5	98.0	97.5	97.7
7.6	—	—	97.0	97.4	97.1	96.6
8.0	—	—	97.6	97.0	96.5	95.7
9.2	97.2	96.7	90.4	83.8	—	—
10.7	93.0	80.9	29.7	14.5	—	—

Results and discussion

During the determination of the radiochemical purity of various sugar nucleotides including UDPG, adenosine diphosphoglucose (ADPG), uridine diphosphogalactose and uridine diphosphoglucuronic acid labelled with ^{14}C in the hexose moiety, it was observed that solvent A gave apparently anomalous results. A sample of ADPG, for example, gave a purity of 99% when analysed in the following systems: isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* EDTA (5:3:0.1) (solvent C), solvent B, and by paper electrophoresis in 0.2 *M* KH_2PO_4. However, a purity of only 90% was indicated in solvent A. The apparent impurity was a single, faster running component ($R_{adenosine}$ 0.86) which upon further investigation was found to be glucose-1:2-cyclic phosphate. Cyclic phosphates of this type are typical alkaline degradation products of sugar nucleotides in which position 2 of the sugar is not blocked. ADP-N-acetylglucosamine, for instance, is quite stable under alkaline conditions[4]. Had the labelled cyclic phosphate originally accompanied the ^{14}C-labelled ADPG as a contaminant, its presence would have been detected using solvents B and C. Furthermore sugar nucleotides are normally quite stable in solution at the

pH value (~7.5) of solvent A and it is not until the pH becomes considerably more alkaline that rapid decomposition occurs at room temperature. Table II, for example, gives some indication of the stability of UDPG at various pH values.

Both the glucose-1:2-cyclic phosphate and the sugar nucleotide appeared as compact spots (Fig. 1). There was no evidence for the streaking which is usually indicative of continuous decomposition during chromatography. We suggest that the decomposition is caused by the equilibrating vapour of solvent A. It is essential with this solvent to pre-equilibrate the tanks before use. Ammonium acetate is, of course, considerably hydrolysed in solution with free acetic acid and ammonia present. Ammonia, however, predominates in the vapour phase of solvent A, rendering it sufficiently alkaline (roughly pH 9–10 with wet pH paper) to cause decomposition of the sugar nucleotide before irrigation of the spot by the solvent. To test this hypothesis, chromatograms loaded with 10 μg ^{14}C-labelled ADPG were hung for various times in tanks pre-equilibrated with solvent vapour before addition of developing solvent to the trough (Table III). Time 0 in Table III represents the period of about

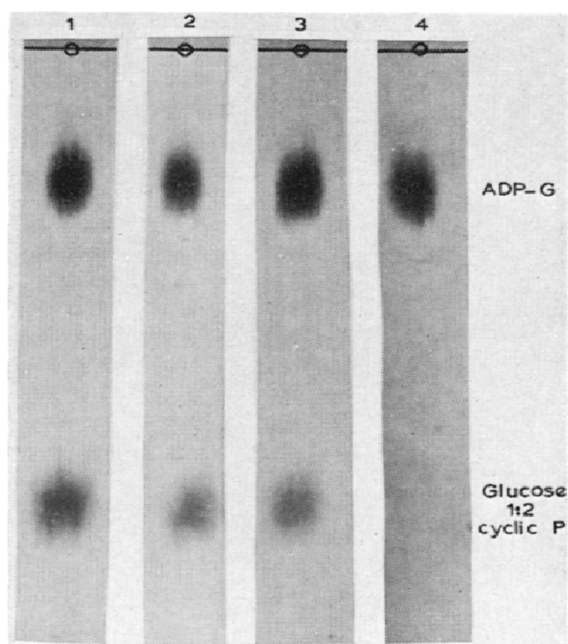

Fig. 1. Chromatography of ^{14}C-labelled ADPG in solvent A. Each strip was loaded with ADPG (0.1 μCi; 10 μg). Strips 1, 2 and 3 were hung in tanks equilibrated with solvent A for 3, 1.5 and 0.5 h, respectively. Strip 4 was hung for 5 h in a tank equilibrated with ethanol–water (5:2). Each chromatogram was then developed overnight (16 h) with solvent A and autoradiograms were prepared to locate radioactive areas.

20 min for the solvent front to reach the origin of the chromatograms. The developing solvent was used to saturate the atmosphere in all tanks except in system A′. In this case, the developing solvent was ethanol–1 *M* ammonium acetate pH 7.5 (5:2) as in A, but the solvent poured into the bottom of the tank for equilibration purposes was ethanol–water (5:2).

It is apparent from Table II that the nucleotide sugar is very sensitive to hydrolysis during equilibration in the vapour of solvent A and extensive degradation can occur within a few hours. An incorrect analytical result for the purity of the

TABLE III

THE RADIOCHEMICAL PURITY (%) OF ADPG AS MEASURED IN CERTAIN SOLVENT SYSTEMS AFTER VARYING THE PRE-EQUILIBRATION TIMES OF THE PAPERS IN SOLVENT VAPOUR

Solvent	*Time (h)*			
	0	*1.5*	*3*	*4.5*
A	90.0	74.5	67.4	67.2
A'	98.9	99.0	98.8	98.7
B	99.4	99.1	99.0	99.3
C	98.9	99.2	99.0	98.8

compound is obtained and, with preparative chromatograms, a reduction in yield will be encountered, particularly if the papers are hung in the tank for some hours prior to irrigation by the solvent, a practice which is frequently recommended. The simple expedient of pre-equilibrating the tank with ethanol–water alone is sufficient to prevent any decomposition of sugar nucleotide.

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