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Note

Separation by thin-layer chromatography of nucleotides from bases and nucleosides in trichloroacetic acid extracts of cells

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Trichloroacetic acid (TCA) is commonly used to precipitate nucleic acids and to extract bases, nucleosides and nucleotides from cells after they have been grown with a radioactive nucleic acid precursor. Analysis of the acid-soluble fraction for radioactivity in the phosphorylated and nonphosphorylated derivatives is sometimes required [1]. Thin-layer chromatography (TLC) has been used but TCA interferes with the chromatographic separation and must be removed; this can lead to the loss of nucleotides [2]. Recently, K_2CO_3 has been used to neutralize TCA extracts prior to TLC [3] and because this approach would appear to overcome the problems associated with TCA removal, we have developed TLC systems that separate common bases and nucleosides from nucleotides when these compounds are in either TCA or TCA neutralized with K_2CO_3.

EXPERIMENTAL

The compounds were dissolved in 5% (w/v) TCA which in some cases was neutralized immediately afterwards with K_2CO_3. The final concentration of K_2CO_3 was between 0.10 and 0.20 *M*. The radioisotopes, [2,8-^{3}H]adenosine-3′,5′-cyclic monophosphate or [8-^{3}H]guanine, were added to 5% (w/v) TCA to give 5 μCi/ml; some solutions were neutralized immediately with K_2CO_3.

Acid-soluble fractions were prepared from human or Chinese hamster fibroblasts that were labelled in one chamber of a two-chamber LabTek slide (Miles Laboratories, Naperville, IL, U.S.A.) or on a Thermanox (Lux, Newbury Park, CA, U.S.A.) plastic coverslip (15 mm diameter) that was held in a well of a Costar (Cambridge, MA, U.S.A.) 24-well plate. [G-^{3}H]Hypoxanthine or [8-^{3}H]-adenine was added to the cultures to give 5 μCi/ml. Four hours later the

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cultures were rinsed three times with cold phosphate-buffered saline and extracted with 500 μl of ice cold 5% (w/v) TCA for 30 min. Microliter aliquots of 2.5 *M* K_2CO_3 were added to the extract and on each addition the pH of the solution was tested on wide-range B Accutint indicator paper (Anachemica Chemicals, Champlain, NY, U.S.A.). When the paper turned light green, which indicated a pH of between 7 and 8, the extract was suitable for chromatography. Generally 20 μl of 2.5 *M* K_2CO_3 neutralized 250 μl of extract.

Precoated TLC sheets (20 × 20 cm) of polyethyleneimine (PEI) cellulose

TABLE I

R_F VALUES OF NUCLEOTIDES, NUCLEOSIDES AND BASES ON PEI SHEETS

Chromatography conditions: in A and B the compounds were in neutralized TCA whereas in C they were in TCA. The solvent was methanol—water (7:3) in A. In B and C the first solvent was methanol and this was followed by development in the same direction with methanol—water (7:3). The mean and standard deviation are given in A where $n=3$ except for cAMP ($n=10$), NAD^+ ($n=6$), $NADP^+$ ($n=4$) and adenine ($n=4$). Chromatography was done only once in B and C.

	Chromatography conditions		
	A	B	C
Nucleotides			
cAMP	0.10±0.04	0.02	0.10
AMP	0.02±0.01	0.00	0.08
dAMP	0.02±0.01	0.00	0.05
ADP-ribose	0.05±0.05	0.00	0.00
ADP	0.01±0.01	0.00	0.02
ATP	0.01±0.00	0.00	0.00
NAD^+	0.08±0.06	0.00	0.00
$NADP^+$	0.01±0.01	0.03	0.06
GMP	0.02±0.01	0.00	0.06
dGMP	0.03±0.01	0.00	0.05
GDP	0.01±0.00	0.00	0.00
XMP	0.02±0.01	0.03	0.05
dTMP	0.01±0.00	0.10	0.05
UMP	0.00±0.00	0.05	0.08
Nucleosides			
Adenosine	0.48±0.02	0.60	0.79
Deoxyadenosine	0.49±0.03	0.61	0.76
Guanosine	0.26±0.02	0.23	0.63
Deoxyguanosine	0.50±0.03	0.58	0.70
Inosine	0.47±0.01	0.50	0.62
Xanthosine	0.29±0.01	0.29	0.60
Thymidine	0.76±0.01	0.78	0.95
Uridine	0.66±0.01	0.69	0.83
Bases			
Adenine	0.46±0.02	0.63	0.66
Hypoxanthine	0.46±0.01	0.57	0.59
Guanine	0.36±0.01	0.29	0.70
Xanthine	0.23±0.03	0.33	0.53
Thymine	0.70±0.02	0.72	0.88
Uracil	0.63±0.02	0.67	0.84

were run in distilled water and dried prior to use. Aliquots of 10 and 20 μl of marker compound (1 mg/ml) and 20 and 40 μl of neutralized extract were spotted 2.5 cm from the bottom of the chromatogram. The chromatograms were developed in either distilled water, methanol, methanol—water (1 : 1) or methanol—water (7 : 3). The solvents were run approximately 12 cm above the origin and this generally took less than 2 h. Detection of the compounds has been described previously [4].

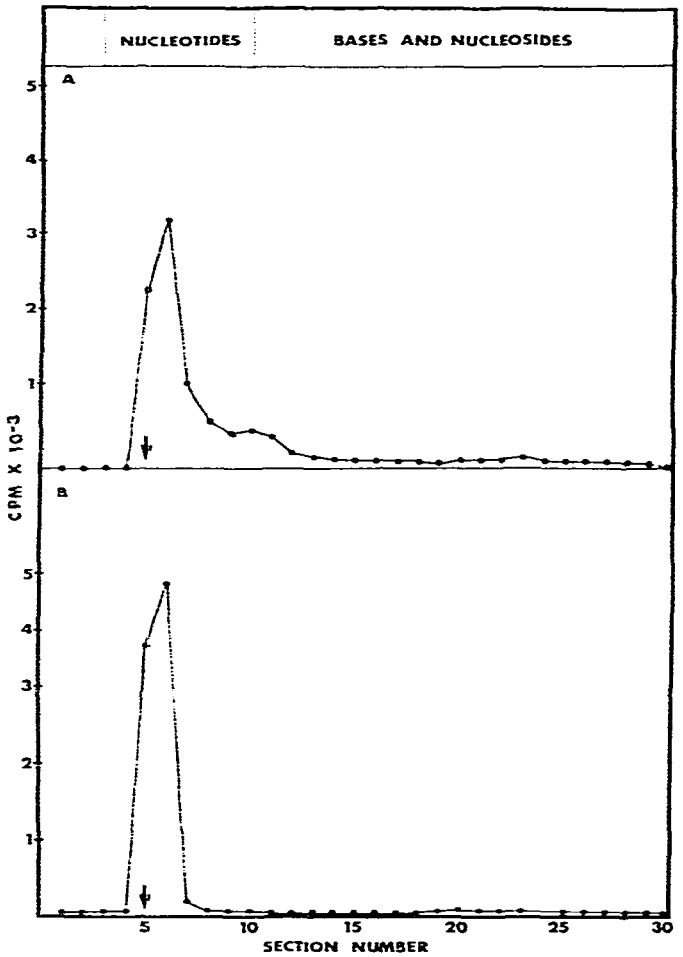

Fig. 1. Chromatography of TCA cell extracts. Human fibroblasts were grown with [^{3}H]-hypoxanthine for 4 h and then extracted with 5% TCA for 30 min at 4°C. In A a PEI strip (2.5 x 20 cm) was spotted with 20 μl of extract whereas in B the extract first was brought to a pH of between 7 and 8 with 2.5 *M* K_2CO_3 prior to the application of 20 μl to a PEI strip. The chromatograms were run in methanol, dried, developed again in the same direction with methanol—water (7 : 3), and then cut into 0.5 x 2.5 cm segments. These were counted in a liquid scintillation counter. The regions where known nucleotides (segments 4—10) and known bases and nucleosides (segments 11 to solvent front) migrated are outlined at the top of the figure. The arrows indicate the origins.

RESULTS AND DISCUSSION

Solvents were tested for their ability to separate nucleotides from bases and nucleosides when these compounds were in neutralized TCA or in neutralized TCA cell extracts. Only methanol—water (7 : 3) gave the desired separation. The nucleotides remained at the origin while the bases and nucleosides migrated well away (Table I). The success of this solvent was established with radioactive standards and cell extracts. When [^{3}H]cAMP that was in neutralized TCA was chromatographed, 96.3% of the radioactivity recovered on the chromatogram was found in the nucleotide region, whereas when [^{3}H]guanine was chromatographed, 99.5% of the radioactivity was found in the base and nucleoside region. Fibroblasts were labelled with [^{3}H]adenine and extracted under conditions that would preserve the nucleotides and under conditions that would hydrolyze them. These extracts were compared by chromatography. When the cells were extracted with 5% TCA for 30 min at 4°C, 93.1% of the radioactivity was found in the nucleotide region, whereas when the cells were extracted with 5% TCA for 6 h at 70°C, 88% was found in the base and nucleoside region. If the pH of an extract was above 8 due to an excess of K_2CO_3, separation was not achieved but could be achieved in the solvent system described below.

Inasmuch as acetic [5], formic [6], and perchloric [7] acid cell extracts have been chromatographed directly, solvent systems were sought for the separation of nucleotides from bases and nucleosides in TCA or TCA cell extracts. If the chromatograms were run in absolute methanol, dried and developed again in the same direction with methanol—water (7 : 3), separations were achieved whether the compounds were in TCA or neutralized TCA (Table I). When [^{3}H]cAMP that was dissolved in 5% TCA was chromatographed, 94.7% of the radioactivity was found in the nucleotide region. The chromatographic behaviour of TCA and neutralized TCA cell extracts was compared immediately after preparation (Fig. 1). In the first case the nucleotide region contained 88.3% of the radioactivity found on the chromatogram while in the second instance this region contained 97.8%. This suggests some breakdown of nucleotides in TCA. In contrast, nucleotides appeared quite stable in neutralized TCA cell extracts as a sample stored for 10 months at −20°C showed no change.

In summary TLC methods for separating nucleotides from bases and nucleosides in TCA extracts of cells have been described that unlike previous methods do not require the removal of TCA.

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