Affinity Chromatography of Thiol-Containing Purines and Ribonucleic Acid

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Evidence is presented for the specific interaction of 6-mercaptapurine with mercurated cellulose. Following from this, a new method is described for the affinity chromatography of thiol-containing molecules and of RNA containing incorporated 6-thioguanosine on columns of mercurated cellulose. This technique may find application in the study of RNA metabolism and gene expression.

Isolation of newly synthesized DNA from mammalian cells has for long been possible by treatment of cells with 5-bromodeoxyuridine and subsequent banding of dense DNA by isopycnic centrifugation in CsCl gradients (Simon, 1961). There is no equivalent method available for isolation of RNA newly synthesized in intact cells. Recently, however, a method has been developed whereby isolated nuclei can be incubated in the presence of mercurated UTP and *Escherichia coli* RNA polymerase, and the mercurated RNA synthesized *in vitro* isolated from endogenous RNA by affinity chromatography on columns of thiol-agarose (Dale & Ward, 1975). This technique would appear to have limited value for work with intact cells, owing to the low solubility of mercurated nucleosides and the potential problems with thiol exchange (Dale *et al.*, 1973).

With a view to achieving a physical separation of RNA newly synthesized *in vivo*, we have investigated the potential of affinity chromatography of thiol-containing molecules, since it is known that 6-mercaptapurine and its metabolite 6-thioguanosine can readily be incorporated into nucleic acids (Tidd & Paterson, 1974).

Experimental

Materials

6-Mercapto[8-¹⁴C]purine (3.66 mCi/mmol) was obtained from NEN Chemicals G.m.b.H., Dreieichenhain, West Germany, and [2-³H]adenosine (20 Ci/mmol) and [5-³H]uridine (28.1 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. 6-Thioguanosine was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Cell culture

BHK-21/C13 cells were grown in monolayer culture at 37°C in 5% CO₂ in air in 2.24-litre roller

bottles in Eagle's medium, Glasgow modification, containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth (medium ETC), and were checked regularly for mycoplasma contamination (Craig & Keir, 1975). Trypsin-treated cells were replated at 2 × 10⁴ cells/cm² in 10-cm-diameter plastic dishes and allowed to grow in medium ETC for 24 h before replacement with medium containing 0.5% serum and no tryptose phosphate broth (medium EC_{0.5}) for 4 h. The medium was then further changed to either EC_{0.5} medium containing 100 μM-6-thioguanosine and [5-³H]uridine (10 μCi/ml), or EC_{0.5} medium containing only [5-³H]uridine (10 μCi/ml). After a further 4 h, cell sheets were washed with ice-cold phosphate-buffered saline (137 mM-NaCl, 3 mM-KCl, 8 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.2, sterilized by autoclaving) and stored frozen until required.

Preparation of RNA

Cell sheets were dissolved in 4 ml of buffer consisting of 0.1 M-LiCl, 10 mM-EDTA, 10 mM-Tris/HCl, pH 7.4, and 0.2% (w/v) sodium dodecyl sulphate, and 4 ml of liquified phenol was immediately added, followed by 4 ml of chloroform containing 4% (v/v) 3-methylbutan-1-ol. After mixing for 30 s followed by centrifugation at 1000 g_{av.} for 2 min, the organic phase was removed (Penman, 1969).

The aqueous phase was extracted with 2 vol. of phenol/chloroform/3-methylbutan-1-ol (25:24:1, w/v/v) until no visible interphase was present. After three further extractions (each of equal volume) with chloroform/4% (v/v) 3-methylbutan-1-ol, the RNA was precipitated from the aqueous phase by addition of 2 vol. of ethanol and stored at -20°C. RNA was collected by centrifugation at 12000 g_{av.} for 12 min and dried under vacuum.

Mercurated cellulose

Mercurated cellulose was prepared as described by Shainoff (1968), packed in columns (5.5 cm × 0.7 cm)

and stored in buffer A (10mM-NaCl, 10mM-EDTA, 10mM-Tris/HCl, pH 8.0). All steps were performed at room temperature (20°C). Before use, columns were equilibrated with buffer B [60% (v/v) formamide, 40mM-LiCl, 4mM-EDTA, 4mM-Tris/HCl, pH 7.4, 0.08% (w/v) sodium dodecyl sulphate]. Samples were applied in the latter buffer; the column was washed with the same buffer and 1 ml fractions were collected. The column was then eluted with 50mM-dithiothreitol in buffer B. After completion of the operation the column was washed with buffer A before recharging by running 100mM-mercuric acetate through and again washing with buffer A.

Measurement of radioactivity

Fractions were counted for radioactivity in 10 ml of a Triton X-100/toluene (1:2 v/v) scintillation fluid containing 4g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre in an Intertechnique SL40 liquid-scintillation spectrometer with approx. 40% efficiency for ^3H and 95% efficiency for ^{14}C .

Results and Discussion

Under the conditions described [^3H]adenosine was eluted by buffer B alone, whereas [^{14}C]mercaptapurine was retained on the column until eluted with buffer B containing 50mM-dithiothreitol (Fig. 1). This suggests that the specific interaction between the thiol group and the mercury is responsible for the relative affinities to the column. Binding of RNA to the mercurated cellulose was stronger than that of adenosine, with about 0.2% of a sample of RNA normally appearing in the second peak (that is the one eluted with dithiothreitol). This may be due to an interaction between the mercury and uracil- or cytosine-rich regions, since mercury is known to interact strongly with pyrimidine rings. However, such material when rechromatographed appeared exclusively in the peak eluted with buffer B (results not shown).

Some 70% of the ^3H -labelled RNA extracted from cells treated with 100 μM -6-thioguanosine for 4h bound to the column and was eluted with buffer B containing 50mM-dithiothreitol, in contrast with the small amount of non-thiol-containing RNA bound (Fig. 2). Since the radioactivity in the second peak must be due to molecules containing both [^3H]uridine and 6-thioguanosine, it would appear that more than 67% of newly synthesized RNA can be physically isolated from older non-thiol-containing RNA, assuming a random distribution of [^3H]uridine and 6-thioguanosine in RNA. We do not know how much 6-thioguanosine is incorporated into RNA under these conditions. 6-Mercaptopurine does not

Fig. 1. Chromatography of adenosine and 6-mercaptapurine on mercurated cellulose

Adenosine and 6-mercaptapurine were applied to a column of mercurated cellulose and eluted as described in the text. The arrow indicates the change from buffer B to buffer B containing 50mM-dithiothreitol. (a) [^3H]Adenosine; (b) mercapto[^{14}C]purine.

enter RNA as readily as does 6-thioguanosine, and therefore attempts to study the number of thiol groups per RNA molecule by using mercapto[^{14}C]purine were not successful.

Since the recognition that thiopurines could be useful in cancer chemotherapy, a considerable amount of information has been obtained on the metabolic interconversions and biological effects of

Fig. 2. Chromatography of RNA on mercurated cellulose. RNA from cells labelled with [³H]uridine (10 μ Ci/ml) in the absence (a) and presence (b) of 100 μ M-6-thioguanosine was applied in buffer B to columns of mercurated cellulose. The arrow indicates the change to buffer B containing 50 mM-dithiothreitol.

these drugs. 6-Mercaptopurine is converted into 6-thioguanosine triphosphate and 6-thiodeoxyguanosine triphosphate before being incorporated into RNA and DNA respectively (Paterson & Tidd, 1975). The exact cause of the cytotoxic effect of 6-thiopurines is unknown, but it appears that the observed inhibition of synthesis of purine ribonucleotides is not capable of explaining the full effects of these drugs. Cell death from exposure to 6-thioguanine occurs only in cells containing the enzyme hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8), showing that incorporation into nucleic acids is more likely to be the principal site of the

cytotoxic effect. However, as far as the use of the drugs in physical isolation of RNA is concerned, it must be noted that, owing to the delayed cytotoxic effect of 6-thiopurines, there is little influence on cellular function at early times of administration.

Notwithstanding the cytotoxic effect of 6-thiopurines, this method of isolation of newly synthesized RNA should prove useful in the study of RNA metabolism. Physical separation of newly synthesized RNA on mercurated cellulose columns might, for example, be coupled with reverse transcription, although we have not yet tested the fidelity of thiol-containing RNA as template for reverse transcriptase. The methodology by which mercurated RNA can be purified from endogenous RNA by affinity chromatography on thiol-Sepharose has already found application in the study of the fidelity of transcription from chromatin with *E. coli* RNA polymerase (Biessman *et al.*, 1976). However, it has been shown that mercurated RNA is subject to aggregation (Konkel & Ingram, 1977). This problem should not be so great with thiol-containing RNA, since the formation of disulphides is unlikely under the conditions used. Moreover, the use of 60% formamide should help to eliminate aggregation.

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