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ION-EXCHANGE CHROMATOGRAPHY OF TISSUE NUCLEOTIDES

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SUMMARY

A procedure has been devised for the isolation of soluble tissue nucleotides by ion-exchange chromatography on 1.0 × 90 cm columns of DEAE-Sephadex A-25, acetate form, using consecutive concave concentration gradients of the volatile salt triethylammonium acetate at pH 4.7. The procedure gives a high degree of resolution of nucleotides in tissue extracts, and eluate samples can be freed of salt and water with a minimum of manipulation, by storage at reduced pressure and room temperature over sodium hydroxide and phosphorus pentoxide. The method is applicable to the analysis of extracts from a variety of tissues.

INTRODUCTION

Many procedures have been described for the separation of tissue nucleotides by ion-exchange chromatography (see the reviews by SAUKKONEN[1], GRAV[2] AND MANDEL[3]). The materials which have been employed for such separations include the polystyrene-based ion-exchangers (see, for example, the procedures described by HURLBERT *et al.*[4], ANDERSON *et al.*[5] and INGLE[6]), DEAE-cellulose*,[7–9], TEAE-cellulose[9], ECTEOLA-cellulose[10,11], DEAE-Sephadex[12] and PEI-cellulose[13,14].

Most of the published procedures, unfortunately, possess certain inherent disadvantages. For example, most of the procedures which utilize the polystyrene ion-exchangers involve the use of acidic eluants, and some acid-labile materials could be

* The following abbreviations will be employed: A-X = an uncharacterized metabolite of adenosine; C-X_1, C-X_2 and C-X_3 = uncharacterized metabolites of cytidine; DEAE-cellulose and DEAE-Sephadex = diethylaminoethyl derivatives of cellulose and Sephadex, respectively; ECTOELA-cellulose = ion-exchanger prepared by the reaction of cellulose with epichlorhydrin and triethanolamine; FGAR = formylglycineamide ribonucleotide; FMP, FDP and FTP = the 5′-mono-, -di- and -triphosphates, respectively, of formycin; Me6MPR-P = 6-(methylmercapto)-purine ribonucleoside 5′-phosphate; 6MPR-P = 6-mercaptopurine ribonucleoside 5′-phosphate; PEI-cellulose = polyethyleneimine-cellulose; TEAE-cellulose = triethylaminoethyl-cellulose; U-X_1, U-X_2 and U-X_3 = uncharacterized metabolites of uridine. Other abbreviations used are those permitted by *J. Biol. Chem.*, 244 (1969) 2.

destroyed during chromatography. In addition, 6-thiopurine derivatives in particular are very strongly bound to polystyrene-based exchangers by non-ionic forces, resulting in unpredictable elution behavior of these substances and necessitating the use of relatively drastic elution conditions (see ref. 15); since much of the work of this laboratory involves carcinostatic thiopurine compounds, these ion-exchangers would not be suitable for our purposes.

Non-ionic interactions between ion-exchangers and the materials being separated can be minimized by the use of exchangers with a cellulose matrix, and a number of procedures using such materials have been described (see, for example, refs. 8–14). These materials, however, also suffer from a number of drawbacks; their ion-exchange capacities are limited (see PETERSON AND SOBER[16]), requiring the use of relatively large columns, and, until recently, they have been available only in a fibrous form, leading to difficulty in the preparation of uniformly-packed columns, a difficulty which is further compounded by the large column beds required.

A further disadvantage of many procedures is that relatively high concentrations of salt and/or acid are required for the complete elution of the nucleotides; the removal of salts from column eluates in order to facilitate characterization and detailed analysis of the eluted compounds involves the time-consuming manipulation of large numbers of samples. This difficulty has been minimized in some procedures by the use of volatile eluants such as ammonium or triethylammonium salts (see, for example, refs. 8, 12, 17 and 18).

Another problem which frequently arises is that while some procedures can achieve excellent resolution of mixtures of authentic nucleotides, they give much less satisfactory separations of nucleotides in tissue extracts, presumably as a result of the presence in these extracts of salts, sugar phosphates, lipids, etc.

In view of the problems inherent in the use of the best of the available procedures, a study was undertaken to devise a method which would achieve the resolution of most if not all of the soluble tissue nucleotides in a single chromatographic run; would employ an ion-exchange material which did not exhibit the disadvantages of the polystyrene and cellulose materials as noted above; and would employ an eluant salt which could be removed from column effluents with a minimum of manipulation.

DEAE-Sephadex A-25 was chosen as the ion-exchanger, since it possesses a high exchange capacity, exhibits minimal non-ionic interaction with both naturally-occurring and synthetic purine and pyrimidine derivatives, and is available in the form of uniform beads, thus facilitating the preparation of uniformly packed columns. For reasons that have been discussed elsewhere[6,7,19,20], concave concentration gradients at constant pH were employed; initial studies showed that maximal resolution of tissue nucleotides could be achieved at pH 4.70. The volatile salt triethylammonium acetate was selected as the eluant, since it is easily prepared as needed from triethylamine and acetic acid, both of which are readily available in high purity; it buffers well at the pH employed; it exhibits minimal absorption of UV light; and it is readily removed from eluant samples at room temperature and reduced pressure[12,17].

Results which have been obtained with this procedure have been included in a number of earlier reports[21–25], and a brief description of the method has been published[26]. This report presents the details of the procedure.

EXPERIMENTAL

Materials

DEAE-Sephadex A-25 was purchased from Pharmacia (Canada) Ltd. Triethylamine (reagent grade) was purified within four weeks of use by refluxing for 3 h with 2,4-diaminophenol dihydrochloride ("Amidol") (3 g/l), followed by distillation at atmospheric pressure (see PARISH[27]). [8-^{14}C]-6-Mercaptopurine was purchased from New England Nuclear Corp., and was stored frozen in saline saturated with hydrogen sulfide at pH 8; before use, the suspensions were acidified to pH 2, hydrogen sulfide was removed with a stream of nitrogen, and the pH was then adjusted to 7.5–8.0 with sodium hydroxide. (Under these conditions, no decomposition of the drug could be detected after two years, *cf.* ref. 28.) Other radioactive materials were obtained from Schwarz BioResearch Co. Other chemicals were obtained from commercial suppliers, and were of the highest available purity.

Procedures for the maintenance and propagation of the Ehrlich ascites carcinoma have been described elsewhere[15].

Preparation of the ion-exchanger

DEAE-Sephadex A-25 (purchased in the chloride form) was allowed to hydrate overnight in distilled water, and was then washed by decantation ten times with 0.5 *N* sodium hydroxide and three times with water. The ion-exchanger was then suspended in 2 *M* sodium acetate pH 4.5–5.5, packed into a column and washed with the same buffer until the effluent was free of chloride ion. The material was then transferred to a large beaker and suspended in 0.04 *M* triethylammonium acetate, pH 4.70 ± 0.05 ("starting buffer"); the suspension was allowed to settle for 15 min, and the milky supernatant fluid was siphoned off. The procedure was repeated until all of the "fines" had been removed. The ion-exchanger was prepared shortly before use; it can be regenerated and re-used, but has generally been discarded after one use.

Preparation of the columns

Columns were made from 1 × 120 cm Pyrex tubing, and were "siliconized" by treatment with a solution of dimethyldichlorosilane in benzene* according to the supplier's instructions. (The use of coated columns is not required for optimal resolution of tissue nucleotides, but greatly facilitates the cleaning of the columns.) A short section of gum rubber tubing was attached to the bottom of the column, and closed off with a clamp. The column was then filled with starting buffer, and a column support was prepared by inserting a 2 cm square of surgical gauze into the column with a long glass tube, to form a "pocket" at the bottom of the column, and dropping eight to ten glass beads (diameter, 5 mm) into the "pocket" through the tube. A slurry of prepared ion-exchanger was added to a reservoir fitted to the top of the column, and the outlet was then opened. The exchanger was allowed to settle to a height of 90 to 93 cm, while the flow rate through the column was maintained at 50 ml/h by means of a metering pump**. Excess ion-exchanger was then removed, and starting buffer (at least 300 ml) was pumped through the column at the same flow rate to ensure complete equilibration. Immediately before application of the sample, the top

* "Column coating", Cat. No. 81900, BioRad Laboratories, Richmond, Calif., U.S.A.
** Instrument MiniPump, Milton Roy Instrument Ltd., Philadelphia, Pa., U.S.A.

5–6 cm of the column bed were stirred up with a glass rod, and allowed to settle by gravity; this ensured a uniform bed surface. A disc of filter paper was placed on top of the column bed to minimize disturbance of the surface during sample application.

Tissue extracts

Liver. Mice were killed, and readily-accessible portions of the liver were rapidly excised and frozen by compression between blocks of dry ice. The frozen livers were extracted with 0.4 *M* perchloric acid (1.5 ml/g of tissue), using a glass homogenizer fitted with a Teflon pestle; the homogenate was centrifuged, and the residue was re-extracted with perchloric acid (1.0 ml/g of tissue). The two extracts were combined and neutralized with potassium hydroxide; the neutralized extract was stored in ice for 60 min and centrifuged to remove potassium perchlorate. The final extract was used immediately, or stored at −20° for up to four days.

Tumor cells. For studies of the metabolism of 6-mercaptopurine, the drug (6 μmoles per mouse, 1.1 μC per μmole) was administered to mice, seven days after implantation of the Ehrlich ascites carcinoma, by intraperitoneal injection. Two hours after administration, the animals were killed, the tumors were rapidly drained through an abdominal incision into chilled saline and the cells were collected immediately by centrifugation at 2°. The cells were extracted with perchloric acid by the general procedure described for liver.

In other experiments with tumor cells, the cells were collected, washed and incubated with the appropriate precursors in FISCHER's medium[29] as described elsewhere[30]. At the completion of the incubation period, the cells were collected by centrifugation, and extracts were prepared as described above.

Chromatography

Samples were applied to the column by means of a syringe and a length of polyethylene tubing. Material adhering to the sides of the column was washed into the column bed with several small portions of starting buffer, and elution was then begun.

The elution scheme employed is presented in Table I. Gradient 1, which elutes nucleotides through GMP, and 2, which continues elution through ADP, were run to completion. Gradient 3 was run until the elution of ATP was complete, and was then discontinued; "gradient" 4 was then pumped through the column to elute GTP as a sharp band. For the preparation of a stock 2 *M* triethylammonium acetate solution, glacial acetic acid (230 ml) and distilled water (1200 ml) were stirred at 2°, and cold purified triethylamine (210 ml) was added in portions; the mixture was stirred until homogeneous, and diluted to 2 l. Working solutions were prepared by dilution of the stock solution to 90 % of the desired volume, dropwise addition of triethylamine to pH 4.70 ± 0.05 and dilution to volume. Fractions of approximately 5 ml were collected at an initial flow rate of 45 to 50 ml/h, maintained with the pump; the flow rate was decreased at intervals to compensate for the increased resistance to flow which developed during chromatography, in order to prevent undue compaction of the column bed.*

* The resistance to flow and the flow rate fluctuate considerably during the first 3–4 h of operation of the column, and the column must be watched carefully during this period. During the balance of the chromatography, however, changes in flow rate are slight and gradual.

TABLE I

ELUTION SCHEME FOR CHROMATOGRAPHY OF NUCLEOTIDES

Gradient No.	*Mixing chamber*	*Reservoir*
1	600 ml 0.04 *M* buffer[a] (1000 ml bottle[b])	450 ml 0.35 *M* buffer (500 ml conical flask[d])
2	440 ml 0.35 *M* buffer (500 ml bottle[c])	260 ml 1.00 *M* buffer (250 ml conical flask[d])
3	700 ml 1.00 *M* buffer (1000 ml bottle[b])	260 ml 1.40 *M* buffer (250 ml conical flask[d])
4	200 ml 1.40 *M* buffer containing 1.00 *M* triethylammonium formate	

[a] "Buffer" refers to triethylammonium acetate, pH 4.70 ± 0.05.
[b] Cat. No. 1240, Corning Glass Works, Corning, N.Y.; I.D., 9.5 cm.
[c] Cat. No. 1550, Corning Glass Works Corning, N.Y.; I.D., 7.5 cm.
[d] Cat. No. 4980, Corning Glass Works, Corning, N.Y.

Recovery of eluted materials

The appropriate eluate fractions were pooled, transferred to a petri dish, and frozen to a slush at −20°. Water and salt were removed from the frozen samples by storage in vacuum desiccators over solid sodium hydroxide and phosphorus pentoxide at reduced pressure (20–50 μ) at room temperature for 16–24 h. Residual salt was removed by dissolving the residue in water and repeating this procedure.

Identification of eluted materials

Nucleotide materials in column eluates were identified on the basis of a minimum of three of the following criteria: (1) UV absorption spectra, (2) coincident elution from the column with authentic samples added to the tissue extracts, (3) co-chromatography with authentic compounds by paper chromatography in at least two solvent systems, (4) incorporation of radioactivity into the eluted materials following exposure of the tissue to appropriate radioactive precursors, (5) identification of the purine or pyrimidine moiety after treatment of the eluted material with *Crotalus terrificus* venom, and (6) specific enzymic assays, where applicable.

RESULTS

The chromatographic profiles presented in the accompanying figures are typical of the results obtained in more than 150 chromatographic experiments.

Fig. 1 depicts the chromatographic analysis of an extract of tumor cells which had been exposed to [^{14}C]cytidine, C-X_1, C-X_2 and C-X_3 are all cytidine-containing compounds, but have not been characterized as yet. Similarly, U-X_1, U-X_2 and U-X_3 are uridine-containing compounds; although they have not been characterized in detail, the presence of uridine and the apparent net charge of these metabolites suggest that the first two may be UDP-sugar and/or UDP-aminosugar derivatives, while U-X_3 may be UDP-glucuronate.

Figs. 2 and 3 represent the analysis of extracts of tumor cells which had been incubated with [^{14}C]adenine and [^{14}C]guanine, respectively. Peak A-X is an un-

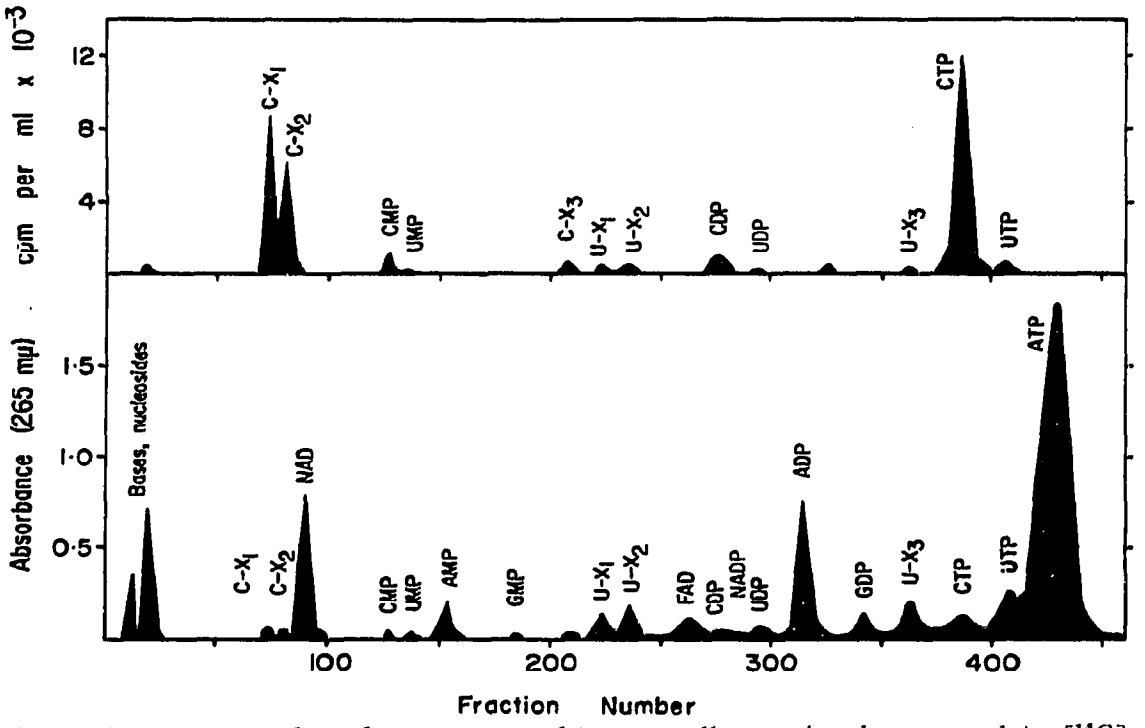

Fig. 1. Chromatography of an extract of tumor cells previously exposed to [^{14}C]cytidine. Tumor cells (5 g, wet weight) were incubated with [2-^{14}C]cytidine (23 μC per μmole, 0.04 μC per ml) for 30 min. The cells were extracted and the extracts were analyzed as described in the section EXPERIMENTAL. (GTP was not eluted in this experiment.)

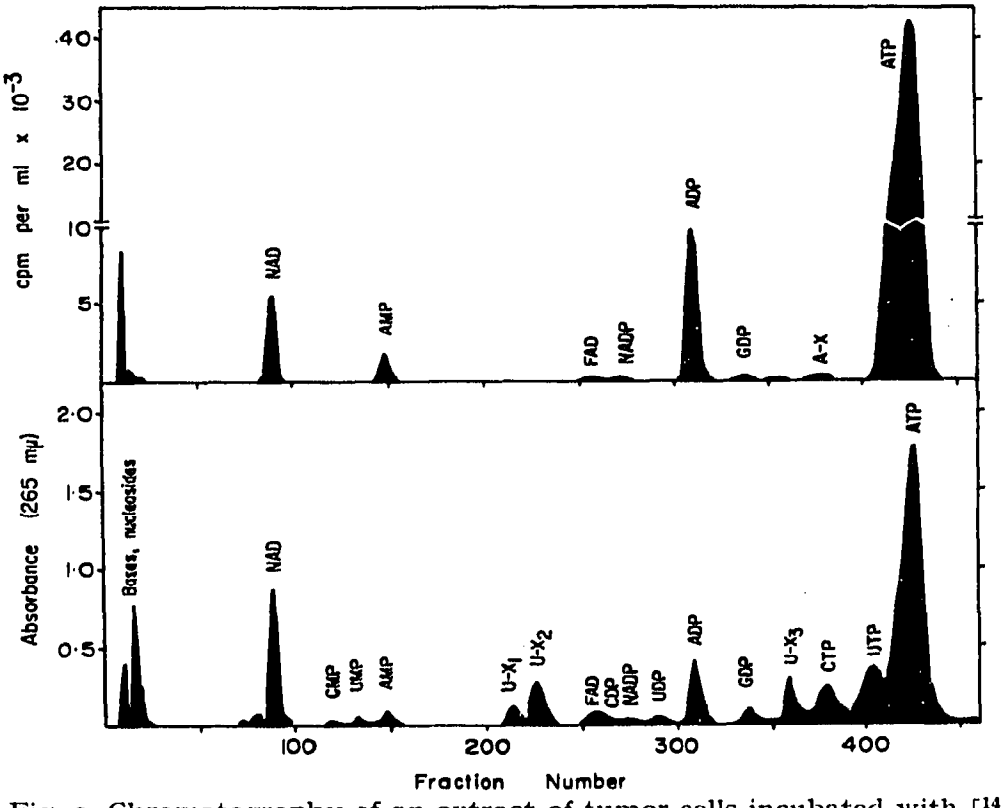

Fig. 2. Chromatography of an extract of tumor cells incubated with [^{14}C]adenine. The conditions were the same as for Fig. 1, except that the cells were incubated with [8-^{14}C]adenine (53 μC per μmole, 0.1 μC per ml) for 60 min. (GTP was not eluted in this experiment.)

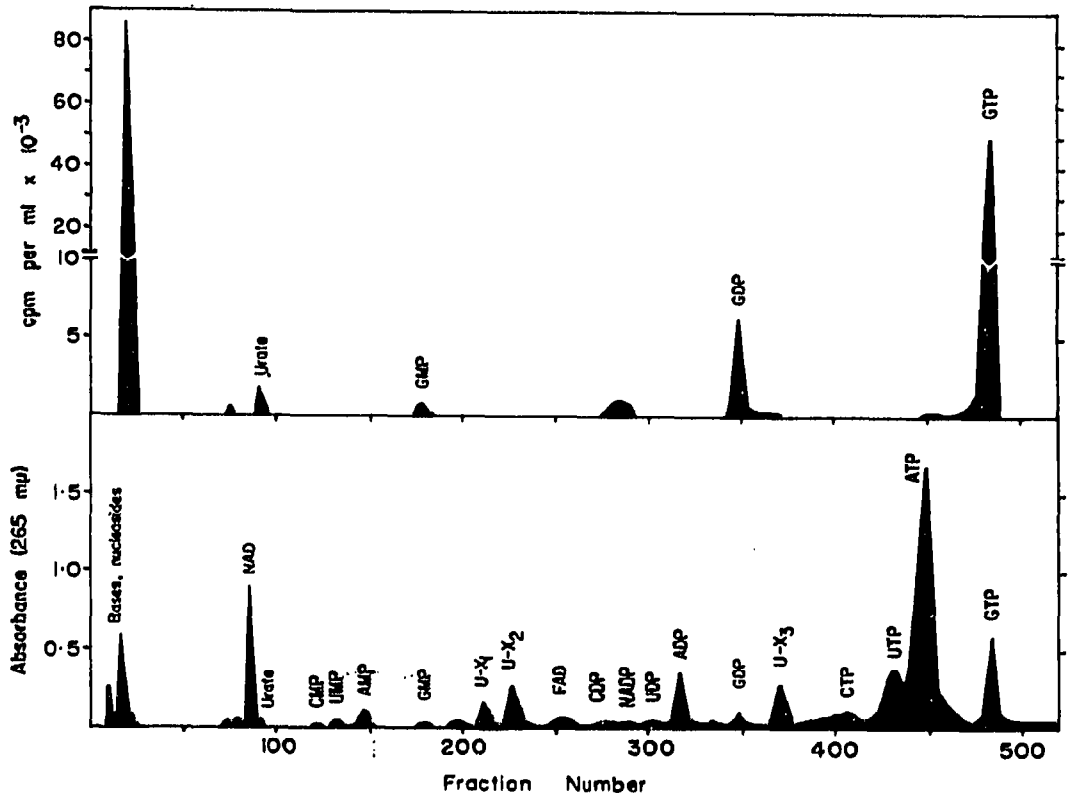

Fig. 3. Chromatography of an extract of [^{14}C]guanine-labeled cells. The conditions were the same as for Fig. 1, except that the cells were incubated with [8-^{14}C]guanine (29 μC per μmole, 0.1 μC per ml) for 2 h.

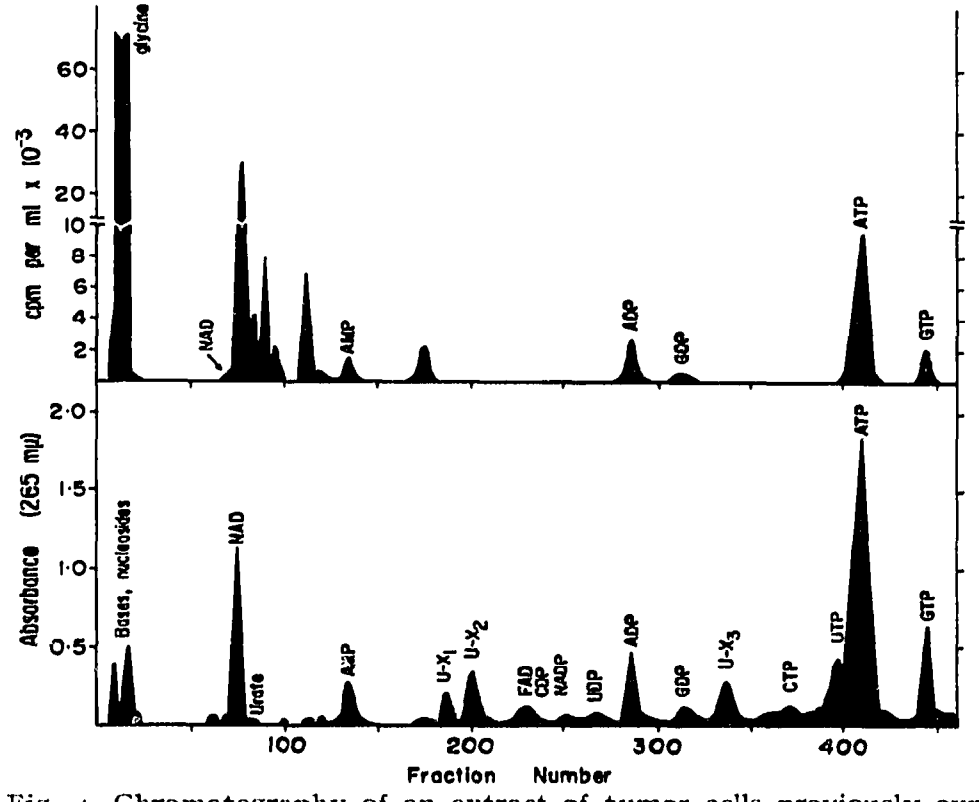

Fig. 4. Chromatography of an extract of tumor cells previously exposed to [^{14}C]glycine. The conditions were the same as for Fig. 1 except that the cells were incubated with [1-^{14}C]glycine (33 μC per μmole, 0.2 μC per ml) for 90 min.

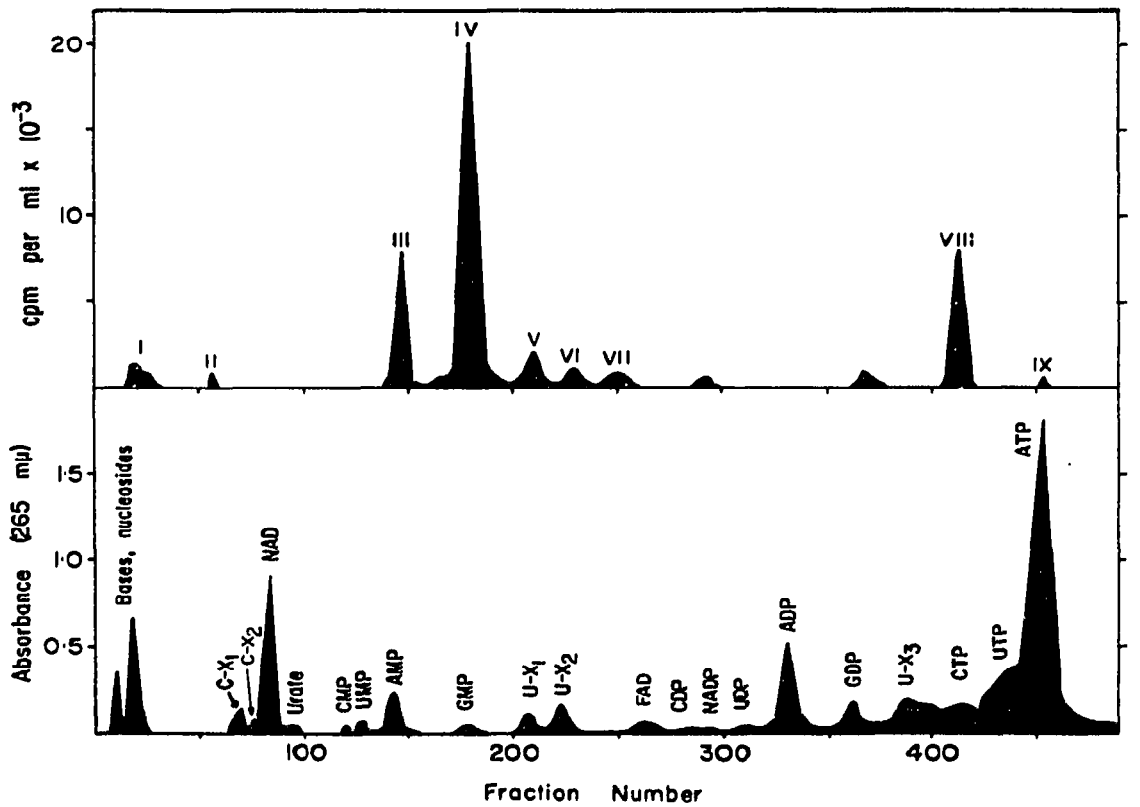

Fig. 5. Chromatography of an extract of tumor cells after treatment with [^{14}C]6-mercaptopurine. The details of this experiment are presented in the section EXPERIMENTAL. The standard chromatographic procedure was employed, except that the column was washed with 100 ml of 0.35 *M* buffer between gradients 1 and 2. (GTP was not eluted in this experiment.)

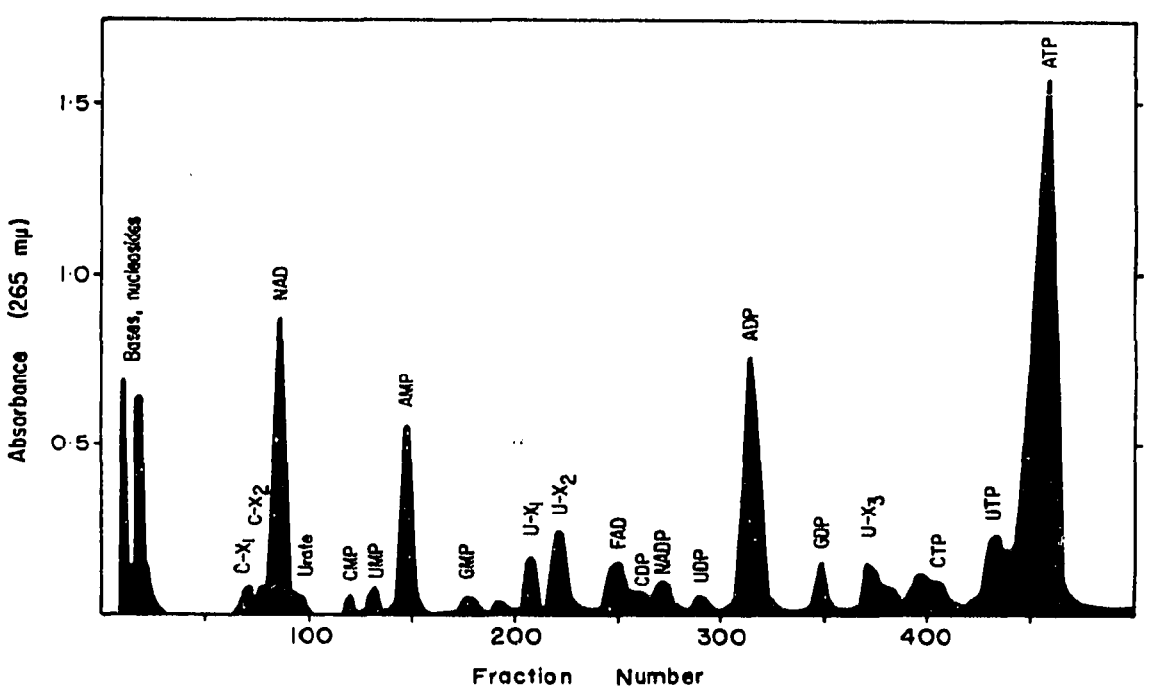

Fig. 6. Chromatography of an extract of mouse liver. For details, see the section EXPERIMENTAL.

characterized adenosine derivative; the peaks which are not identified represent metabolites which have not yet been examined in detail.

Fig. 4 shows the results of the chromatography of an extract of tumor cells which had been incubated with [^{14}C]glycine, and indicates the degree of resolution which can be achieved with this procedure. Most of the peaks which are eluted between fractions 75 and 130 have not been characterized; some may be intermediates in purine synthesis *de novo*, since many of these peaks are diminished or absent when cells which have been treated with 6-(methylmercapto)purine ribonucleoside, a known inhibitor of this pathway[31], are treated in the same manner[32].

Fig. 5 presents the results of an experiment in which [^{14}C]mercaptopurine was administered to tumor-bearing mice 2 h before the collection and extraction of the cells. Peak I consist of at least three compounds, including unchanged 6-mercaptopurine; peaks III and IV have been identified by procedures which have been described previously[15,33], as 6-(methylmercapto)purine ribonucleoside 5'-phosphate and 6-mercaptopurine ribonucleoside 5'-phosphate, respectively; and peak VIII has been identified as 2-hydroxy-6-mercaptopurine ribonucleotide[34] ("thioXMP"). The other peaks have not been characterized at the present time*. (One or more of these other metabolites may be derivatives of 2-amino-6-mercaptopurine ("thioguanine")—see SCANNELL AND HITCHINGS[35].) The "shoulder" on the leading edge of peak IV and the two peaks between VII and VIII have been observed in some, but not all, experiments, and their status as metabolites of 6-mercaptopurine is uncertain.

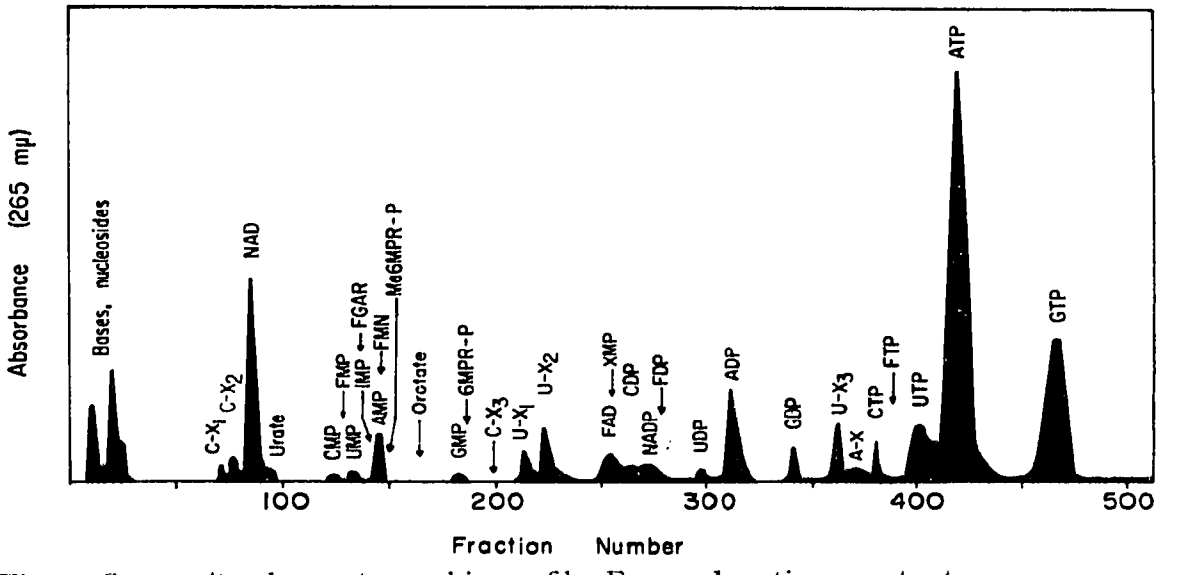

Fig. 7. Composite chromatographic profile. For explanation, see text.

Fig. 6 presents a chromatographic profile obtained with an extract of mouse liver. The procedure has also been applied successfully to the analysis of extracts of blood cells[36] and skin fibroblasts[24]; extracts of other tissues have not been examined in this laboratory, but studies in other laboratories have shown that the method can be employed successfully in the analysis of extracts of rat uteri[37] and bacterial cells[38].

* Peak IX coincides precisely with the ATP peak and may well be the result of a trace contamination of the 6-mercaptopurine preparation with [^{14}C]hypoxanthine.

Fig. 7 presents a composite chromatographic profile, summarizing the results of studies performed in this laboratory over the past four years, and showing the elution positions of a number of purine and pyrimidine derivatives.

DISCUSSION

The chromatographic procedure described in this report achieves a high degree of resolution of soluble nucleotides in extracts of a variety of tissues, although the original objective, that of achieving complete resolution of these nucleotides in a single chromatographic run, has not been realized. For most purposes, the degree of resolution achieved with this method will be adequate; in some instances, however, rechromatography of selected portions of the column eluates may be required.

The procedure as described here has been successfully employed in the analysis of up to 30 ml of extract, derived from up to 10 g of tumor cells or 5 g of liver, without significant impairment of resolution. The chromatography of larger volumes of tissue extracts, or of extracts containing very high levels of salt, leads to some loss of resolution (see ref. 24). The analysis of extracts derived from larger amounts of tissue can also lead to diminished resolution; this is observed primarily in the nucleoside triphosphate region, and is mainly a consequence of broadening of the ATP peak.

Most nucleotides are eluted in volumes of 50–75 ml, and column eluates can be freed of salt and water with a minimum of manipulation. The procedure gives highly reproducible results; the elution position of a given nucleotide rarely varies by more than five fractions, and in most instances chromatographic profiles obtained in experiments carried out under comparable conditions are virtually superimposable. Tissue nucleotides appear to be very stable during the chromatographic procedure; the absence of an increase in background radioactivity in the later stages of the experiments depicted in Figs. 1–5 strongly suggests that little or no breakdown of nucleotides occurs under the conditions employed.

This procedure, which was originally devised for studies of the metabolism of carcinostatic purine analogues by tumor cells, offers a number of advantages over the existing procedures, and should be of considerable value in studies of many aspects of nucleotide metabolism.

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