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CONTRIBUTIONS OF ANION-EXCHANGE THIN-LAYER CHROMATOGRAPHY TO THE STUDY OF FREE NUCLEOTIDES IN TISSUES

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SUMMARY

Purification by anion-exchange resin of a rat liver trichloroacetic acid extract is described. This permitted the separation of twenty-two compounds by two-dimensional anion-exchange thin-layer chromatography for nucleotides. Conditions of chromatography developments were studied to correct artifacts of the spots. Maps were constructed which included nucleotides that were unstable during extraction and purification; degradation products of these nucleotides were observed as well circumscribed spots with well defined positions on the map. The usefulness of these chromatograms for nucleotide identification of small amounts of tissue extracts is discussed.

INTRODUCTION

A large number of nucleotides** were discovered in the last fifteen years, primarily by chromatography of tissue acid extracts on anion-exchange columns. The composition and levels in these nucleotides were found to vary between tissues having different metabolic activities[1-3]. Sensitive procedures of chromatography were introduced in more recent years for smaller amounts of biological material. Inorganic and organic phosphate compounds were labeled and detected by autoradiography[4-8].

Thin-layer chromatography (TLC) is a well known technique[9]; however, the procedure of anion-exchange TLC with a high degree of resolution and sensitivity

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** Abbreviations: AMP, GMP, IMP, CMP, UMP = adenosine, guanosine, inosine, cytidine, and uridine 5'-phosphates; ADP, GDP, CDP, UDP = the corresponding diphosphates; ATP, GTP, ITP, CTP, UTP = the corresponding triphosphates; CAMP = cyclic AMP; GDPM = guanosine diphosphate mannose; CDPG = cytidine diphosphate glucose; UDPG = uridine diphosphate glucose; UDPGA = uridine diphosphate glucuronic acid; UDPAG = uridine diphosphate N-acetylglucosamine; CDPC and CDPE = cytidine diphosphate choline and ethanolamine; FMN = flavin monophosphate nucleotide; FAD = flavin-adenine dinucleotide; NADH = nicotinamide-adenine dinucleotide and NADPH = nicotinamide-adenine dinucleotide phosphate

was devised by RANDERATH AND RANDERATH[10]. Other investigators showed that autoradiography of labeled nucleotides gave greater sensitivity than the technique of Randerath[11]; however, anion-exchange TLC proved to be more specific for nucleotide identification since it detects the compounds by quenching of ultraviolet (UV) light.

This report describes the purification and concentration procedures of trichloroacetic acid (TCA) extracts of nucleotides. The separation of twenty-two compounds on a single chromatogram is shown when the extract was prepared from 1 g of rat liver. Products of degradation of some nucleotides are observed as well circumscribed spots with respect to their position on construction maps and on chromatograms of tissue extracts.

EXPERIMENTAL

Materials

AG1 X4 resin, 200–400 mesh, in Cl^- form and columns with glass barrels, measuring 10 × 7 cm were supplied by Bio-Rad, Richmond, Calif. TLC sheets were prepared with poly(ethyleneimine) (Polymin P), Cellulose MN 300, Rigid Vinyl sheets and a Stahl spreader manufactured by companies previously mentioned by other investigators[12]. The instruments used were as follows: The homogenizer Type C from A. H. Thomas, Philadelphia, Pa., the ultraviolet lamp, Model UVS11 from Ultraviolet Products, Inc., San Gabriel, Calif., and the lyophilizer from Virtis Co., Gardiner, N.Y. All nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo., except for CAMP which was from California Corporation for Biochemical Research, Los Angeles, Calif.

Methods

Preparation of liver extract. The liver of a male rat was quickly removed under ether anesthesia and pieces measuring approximately 1.5 cm in their greatest dimensions were immediately frozen in a mixture of dry ice and acetone. All subsequent manipulations and centrifugations were at 2°. One gram of the frozen tissue was suspended in 7.0 ml of 12% (w/v) precooled TCA solution, then homogenized with ten strokes of the homogenizer. This suspension and a 3.0-ml rinsing of the homogenizing cup were centrifuged for 10 min at 16,000 × *g*. TCA was removed from the supernatants by five extractions, each with 20 ml of diethyl ether. The residual diethyl ether was evaporated by flowing nitrogen through the solution.

Purification and concentration of the liver extract. A size No. 0 büchner funnel was fitted with a Whatman filter paper No. 42. 75 ml of 1 *N* HCl, 350 ml of water, and 75 ml of 1 *M* LiCl with 350 ml of water were used to clean 2.5 g of AG1 X4 resin. Each column utilized a volume of the suspended resin equivalent to 0.5 g of dry product. After loading the TCA extract on the column, the resin was washed with 20 ml of water. The content of the column was eluted with 10 ml of 0.4 *M* LiCl, 0.01 *N* HCl and neutralized with 0.1 *N* LiOH in the presence of phenol red. The purified extract was lyophilized twice to obtain a small residue. The lithium chloride was then removed from the residue by vigorous shaking with 2.0 ml of absolute ethanol for 3 min. Centrifugation of the suspension followed at 2,000 r.p.m. for 10 min. The resulting sediment was further washed with 1.0 ml of ethanol–diethyl

evaporation of the residual diethyl ether, the sediment was dissolved in 1.0 ml of water and lyophilized. A suspension was made of the lyophilized material and 0.08 ml of water, then clarified by centrifugation at 2,000 r.p.m. for 10 min. Two-fold serial dilutions of the supernatant were prepared for further analysis.

Chromatography of extracts. A procedure described by RANDERATH AND RANDERATH[13] was modified. The solution of poly(ethyleneimine) (PEI) was neutralized with formic acid to give pH values ranging from 5 to 6. After immersion of the coated plastic sheets in 10 % (w/v) NaCl and water, washing was completed by an ascending irrigation in 1 *N* formic acid. The sheets were then dried overnight at room temperature.

Varying amounts ranging from 10 μl to 2 × 1 μl of purified extract were applied in the left hand corner of the sheet at a distance of 2 cm from the two edges. The separations of the nucleotides were obtained in three stepwise two-dimensional developments with ammonium formate buffers. In the first dimension 0.5, 1.2, and 1.8 *M* ammonium formate solutions, pH 6.5–7.0, were allowed to rise to a height of 3 cm, 7 cm, and 17 cm, respectively, above the lower margin of the sheet for the stepwise development. The chromatogram was examined in UV light, its solvent removed by bathing the sheets with methanol for 15 min and the finishing line of its first development cut away. The second development was in perpendicular direction to the first one and 0.5, 1.6, and 3.2 *M* ammonium formate buffers, pH 3.5, were allowed to rise from the lower edge of the chromatograms for the same distances as the ones used in the first dimension. In other studies, chromatograms were developed in the two dimensions in the presence of buffer solutions with the same pH. In these chromatographic developments the buffers at pH 3.5 were used in the first dimension and the buffers at pH 6.5–7.0 in the second dimension. Development times were approximately 150 min before methanol washing and 60 min after this washing. Quenching of UV light by compounds on the chromatograms was observed in a dark room.

Mapping of commercial nucleotides. 2 × 1 μl of 0.2 % (w/v) solutions with twenty-three different nucleotides were developed on three groups of 20 × 20 cm in sheets each containing nucleotides with R_F values close to each other. One group of sheets was developed at pH 6.0–7.0 and the other group at pH 3.5, following the technique described previously. A two-dimensional map, which combined the developments at the two pHs, was then constructed with tracing paper.

Solutions of FMN, FAD, NADH, and NADPH with nucleotide markers were subjected separately to the same purification and concentration procedure as the one used with liver tissue. The processed solutions were then chromatographed in two dimensions, using first a pH 6.0–7.0 buffer, then pH 3.5 buffer. As a control to the above study, unprocessed solutions of nucleotides were used in some chromatographic runs.

Low temperature phosphorescence of nucleotides. Sheets prepared with a liver extract were immersed in liquid nitrogen and illuminated by UV light as reported by RANDERATH[14]. The UVS11 light source was used without filter.

RESULTS

Fig. 1 shows the degree of resolution achieved by chromatography of a purified

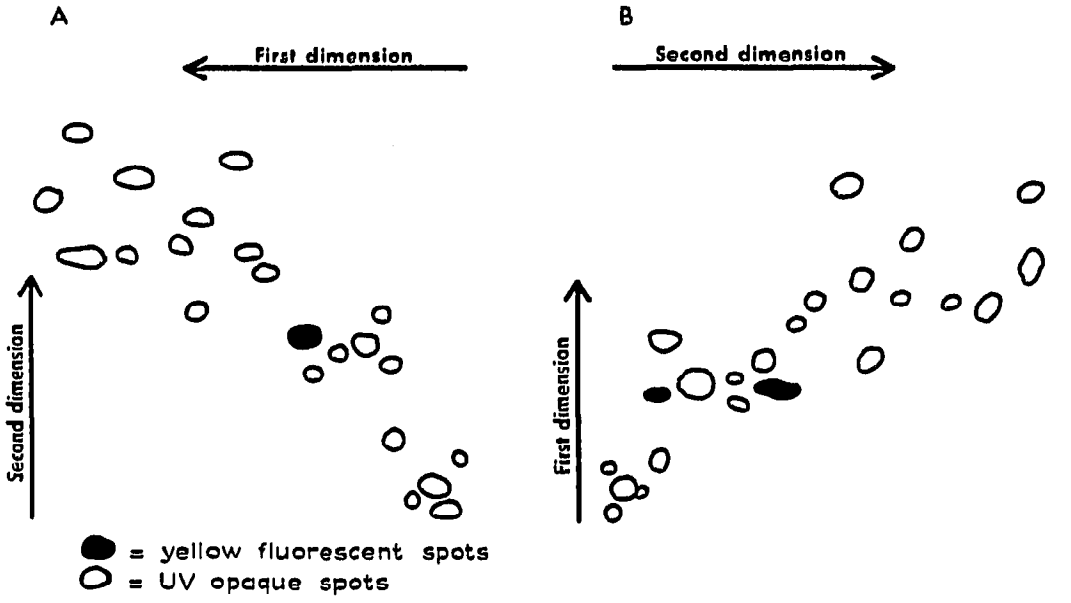

Fig. 1. Two-dimensional anion-exchange TLC of purified rat liver extract. A, first dimension: ammonium formate buffer, pH 6.5; second dimension: ammonium formate buffer, pH 3.5. B, first dimension: ammonium formate buffer, pH 3.5; second dimension: ammonium formate buffer, pH 6.5.

When the purification and concentration of the TCA extracts on anion-exchange columns were omitted, smears of UV quenching material accumulated on the chromatogram, thus preventing the separation of well circumscribed compounds. Sorption of the nucleotides on the resin and their subsequent elution eliminated impurities.

It was necessary to apply an optimum of extract to obtain reproducible results; either 5 μl of a diluted extract (50:50) or 2 × 1 μl of the undiluted extract was found to be satisfactory. An overload produced wide bands in the upper part of the sheet. This was observed after the first dimension. A variable arrangement of fast migrating spots was also seen after the second dimension; however, there was no gain in overall resolution. Good quality diagonals on chromatograms were developed in two dimensions with a solvent of one pH only. This indicated that the solvent was removed between the two runs (see Fig. 2). When the two solvents with different pH were reversed in an additional two-dimensional development, there was good agreement between both mirror images (see Fig. 1). No deterioration of most of the spots was observed. Reports have been postulated showing the many factors influencing deterioration during chromatography[15].

A closer examination of the diagonals in Fig. 2 shows a flavin-containing substance, identified by its yellow fluorescence[16], trailing below its spot on the line. This trail was larger below the diagonals prepared at pH 3.5 than at pH 6.5. This anomaly, however, was less distinct when the chromatograms were developed first at pH 6.5 and in the second dimension at pH 3.5. Under the same conditions, a fast moving component, probably a sugar nucleotide, was observed to have a less elongated profile. This technique of development was therefore adopted for further studies.

Fig. 2 shows the position of twenty-three nucleotides on the map. These

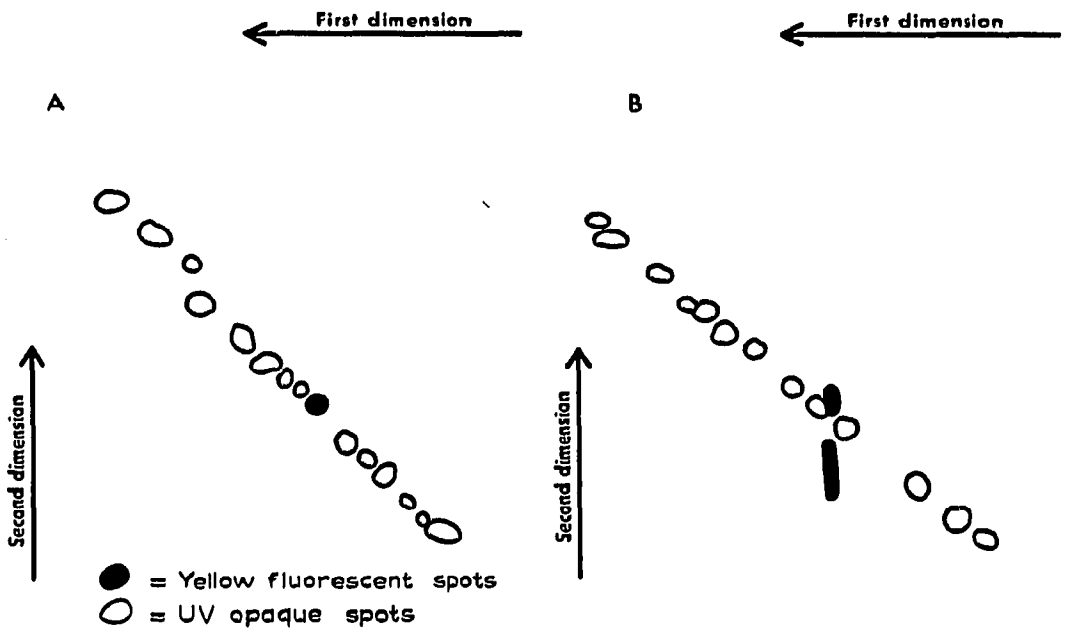

Fig. 2. Diagonal studies by TLC of purified rat liver extract. A, first and second dimension: ammonium formate buffer, pH 6.5. B, first and second dimension: ammonium formate buffer, pH 3.5.

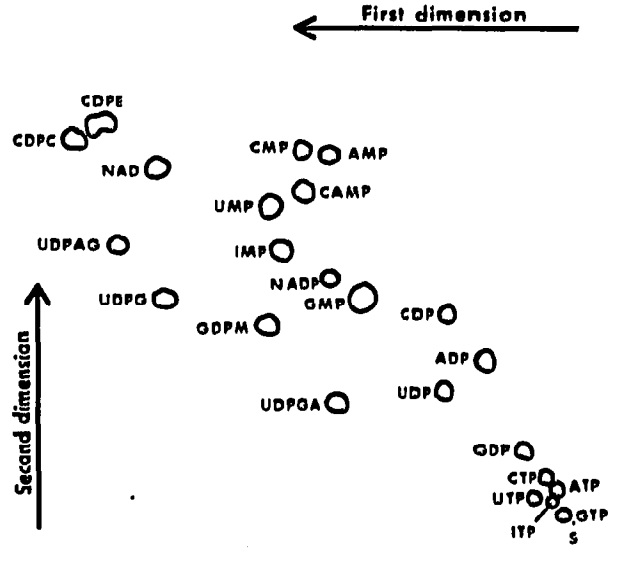

Fig. 3. Map of commercial nucleotides. First dimension: ammonium formate buffer, pH 6.5. Second dimension: ammonium formate buffer, pH 3.5. S = starting point.

correlation of the separation of the nucleotides, when this work is compared to that of previous investigators[13]. In addition, it was found that CAMP, CDPE and CDPC had distinct positions on the map. CDPC nevertheless moved with the front of the solvent in the first dimension.

Three groups of nucleotides were readily distinguishable—the nucleotide triphosphates, diphosphates and all the others—their relative rates of migration were the same as reported by RANDERATH AND RANDERATH[17]. In each group, the com-

those which had very close R_F values in both dimensions. On the other hand, the sugar nucleotides and UDPGA were below and on the left of the more centrally located substances.

Fig. 4 is the result of a concentration of several experiments. It shows a map of commercial nucleotides—NAD, NADPH, FMN, and FAD with their markers. These nucleotides were processed with the same manipulations as with the liver TCA

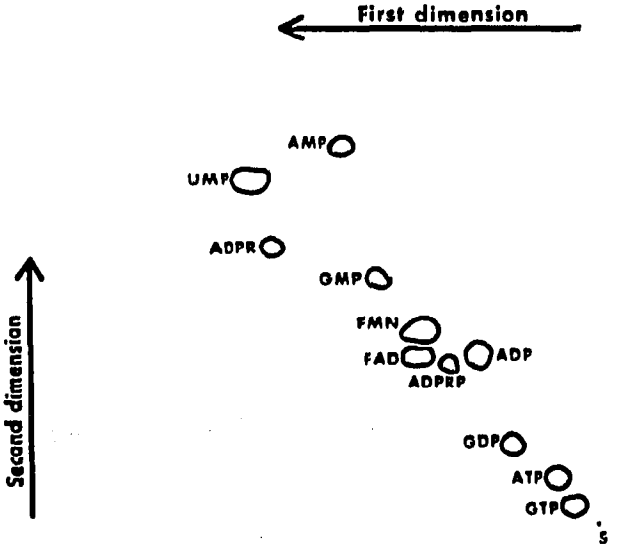

Fig. 4. TLC of commercial FMN, FAD, NADH, and NADPH after same treatment as purified liver extract. Nucleotide markers: UMP, AMP, GMP, ADP, GDP, ATP and GTP.

extract. With or without this treatment, there was trailing behind spots formed by FMN and FAD when they were run separately, but the contrast between these spots and the delayed yellow fluorescence was increased by washing in methanol. After treatment, the fluorescence of the upper spot decreased in intensity and a new spot of UV opaque material appeared, which migrated with added AMP. This was attributed to a hydrolysis of FAD with liberation of AMP during processing of the nucleotide solutions. Although liver tissue is known to contain large amounts of FAD[18], only the upper spot corresponding to FMN was present on the chromatograms of liver extracts. Presumably the same hydrolysis of liver FAD occurred as the one observed with the commercial product.

PAPENBERG[19] showed that NADH and NADPH were eluted from anion-exchange columns loaded with samples of an acid tissue extract produced by ADPR or ADPRP. In this study, thin-layer chromatograms revealed a UV opaque spot for NADH in the region of the nucleotide monophosphates and for NADPH in the region of the nucleotide diphosphates (see Fig. 4).

Exposure of nucleotides containing sheets under liquid nitrogen to UV light results in the phosphorescence of adenosine and guanosine phosphates[14]. The pattern obtained by UV illumination of liver extract sheets coincided with the labeling given to the same spots by comparison of the maps. This indicated that the maps were useful to give an orientation in the identification of nucleotides on chromatograms

CONCLUSION AND DISCUSSION

Reproducible nucleotide separations by TLC were accomplished after adsorption on an anion-exchange resin. A high degree of resolution was achieved after purification of a liver TCA extract and concentration of the eluates by lyophilization. Centrifugation of this purified extract at 2,000 r.p.m. produced a precipitate which after washing revealed no UV quenching spots by TLC. Washing of the sheets with 1 *N* formic acid also prevented the appearance of artifactual second fronts. These fronts appeared as nucleotides, consisting of an accumulation of UV absorbing material at the level of NAD.

After extraction and purification, the degraded nucleotides from FAD, NADH and NADPH also showed good separation. This gave indirect information as to their presence in the acid extracts of tissues. Smearing of FMN was minimized in adopting the best order of development of the two-dimensional chromatography.

Because of its sensitivity, this technique could be most useful for the identification of small amounts of nucleotides available from tissues. Our studies showed that the constructed map was useful in giving an orientation for identifying nucleotides of liver. A complete direct identification of nucleotides from eluates of TCA extracts is not always possible and this sensitivity is not regarded as adequate since a complete fingerprinting of all nucleotides in the sample is lacking. An increase in the quality of their identification has been obtained in other ways[11].

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