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Note

Separation and quantification of ^{32}P-labeled guanine nucleotides by thin-layer chromatography

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In recent studies on the guanylate cyclase activity of rat tissues[1], we obtained indirect evidence that the substrate for the reaction, GTP*, was being rapidly utilized by other enzymes we will call collectively GTPase. In order to develop methods for inhibiting GTPase, we decided to study that activity directly under the same conditions used for the guanylate cyclase assay. We used [α-^{32}P]GTP in the guanylate cyclase assay, and it appeared that this could be used to study GTPase, if we could separate the reaction products. We had previous experience with poly(ethylene)imine (PEI) cellulose layers, however, none of the solvent systems reported by Randerath and Randerath[2] or by other authors[3] were able in a single dimension to separate all of the possible ^{32}P-labeled metabolites of [α-^{32}P]GTP. The following system was developed to effect that separation, and to enable quantification of resolved spots.

METHODS

Preparation of PEI-cellulose sheets

Layers were prepared on plastic sheets as described by Randerath and Randerath[2]. After drying, the sheet was cut up into 8-in.-wide pieces. These were washed by ascending development with 0.5 *M* formic acid, with a wick of soft paper toweling applied to the top edge of the layer in order to remove impurities. The sheets were dried overnight at room temperature. With a soft pencil, two lines were ruled across each sheet, one 3/4 in. from the bottom to mark the origin, and the other 3/4 in. from the top to mark the furthest migration of the solvent front. Each sheet was then divided into vertical channels, by scoring with a sharp pencil at $\frac{1}{2}$-in. intervals. After covering the layers with soft paper toweling, the sheets were wrapped in aluminum foil and stored in a freezer.

* Abbreviations used (only 5′-nucleotides were used): GTP = guanosine triphosphate; GDP = guanosine diphosphate; GMP = guanosine monophosphate; Pi = orthophosphate; PPi = pyrophosphate.

GTPase assay

The enzyme assay was performed in 10 × 75 mm test tubes, which were incubated at 30° in a water-bath shaker. The reaction mixture was the same as that used for assay of guanylate cyclase, except for the omission of a GTP-regenerating system[1]. It contained, in a final volume of 75 μl, 1.2 m*M* [α-^{32}P]GTP, approximately 5 × 10^5 counts/min, 6.0 m*M* $MnCl_2$, 0.2 μmole 3',5'-GMP, 50 m*M* Tris–HCl, pH 7.6, 0.1 mg bovine serum albumin and 20 m*M* caffeine. The reaction was initiated by addition of the enzyme and stopped with 20 μl of 0.5 *M* acetic acid, followed by heating at 100° for 2 min. Each tube was then immediately cooled in an ice-bath.

If the reaction mixtures were to be chromatographed on the day of the assay, the tubes were first centrifuged at 2500 *g* for 10 min in order to prepare a clear supernatant solution. Otherwise the tubes were frozen and centrifuged after thawing.

Chromatography

On each thin-layer sheet, the channels at each side were not used for analysis. They were guide channels and their use will be explained later. Two 10-μl aliquots were removed from the supernatant solution in each reaction tube. Beginning with the second channel, one of these was spotted at the origin, while the other was applied at the top of that same channel in the space above the upper pencil line. This second spot was to be used after chromatography as a reference spot to calculate the percent of the applied radioactivity recovered in each of the separated spots. Scoring the sheet into channels enabled the spotting of the 10-μl aliquots in a single application, while preventing cross-contamination during development. After all of the samples had been applied and dried, a carrier mixture of the compounds to be separated was spotted at each origin, including the guide channels. The carrier mixture contained 10 nmoles of each of the following in a 2-μl application volume: GTP, GDP, GMP, 3',5'-GMP, Pi, and PPi.

The sheets were well dried and then washed with cold absolute methanol. This was carried out in a refrigerator, each sheet in a separate tray which was periodically agitated. After 30 min, the sheets were removed, blotted with soft paper toweling, and dried in a current of cool air. Development was carried out with the solvent system 0.5 *M* formic acid–4 *M* LiCl (7:3). The solvent front was allowed to ascend until it had reached the upper pencil line. This took from 80 to 90 min, when carried out with 150 ml of solvent in a glass brick type of chromatography chamber. The developed sheets were air dried, after which the washing procedure with absolute methanol was repeated.

The carrier mixture enabled visualization of the developed nucleotides under UV light. These spots were outlined with a soft pencil. The Pi and PPi spots were revealed by spraying the guide channels with phosphate detecting reagents, after covering the remainder of the sheet with a piece of cardboard. The channels were sprayed first with 1% ammonium molybdate, and after drying, this was followed by 1% stannous chloride in 10% hydrochloric acid[4]. The locations of the Pi and PPi spots in the assay channels were determined from their positions in the guide channels.

Quantitation

The marked channels were cut from the sheets, and each channel cut up into its respective spots. Each spot was placed face up, in a separate scintillation vial,

and to each vial was added 1 ml of 0.5 *M* ammonium bicarbonate, pH 8.6 (ref. 2). After 30 min, 2 ml of water and 10 ml of Bray's scintillation cocktail was added to each vial. The vials were counted in a liquid scintillation spectrometer, using a full window in order to decrease variations in quenching.

RESULTS AND DISCUSSION

Fig. 1 shows a radiochromatogram from an experiment designed to measure the GTPase activity of the 37,000 *g* supernatant from a rat kidney homogenate. Note the minimum tailing of the spots. The solvent system used here was developed as a modification of the 1 *M* acetic acid–4 *M* LiCl (8:2) system suggested by Randerath and Randerath[2]. That system could separate all of the compounds except for GMP and 3′,5′-GMP. Substituting 1 *M* formic acid for 1 *M* acetic acid separated these two compounds, but caused GTP to overlap the PPi spot. Decreasing the formic acid concentration to 0.5 *M* resulted in a separation of all the spots. We have tried various combinations of the 4 *M* LiCl and 0.5 *M* formic acid solutions, with and without the addition of water. The recommended mixture gives the maximum resolution.

A special problem was presented by Pi and GMP. In order to separate these two reproducibly, we found that it was necessary to prewash the sheets with 0.5 *M* formic acid. This treatment removed a pH front from the final chromatogram, which interfered with Pi migration. A similar observation has been made by Lust and Sahud[5]. Another requirement for a discrete separation in this region of the chromatogram was the methanol wash. This removed the larger part of the Tris–HCl and caffeine that were present in high concentration in the reaction mixture. It is essential that the spots be completely dry before the methanol wash, in order to prevent loss of radioactivity. If this precaution is followed, we found that less than 0.5% of the applied radioactivity was solubilized by methanol.

There are two radioactive spots visible on the radioautogram which do not correspond to known compounds. The first is at the origin, and the second is between the origin and GTP. These appear to be radioactive impurities present in the [α-^{32}P]-GTP, perhaps guanosine tetraphosphate and/or other polyphosphates[3]. It can be seen from Fig. 1 that these two spots decrease in intensity with increasing incubation time. This suggests that they are being degraded to faster moving compounds, along with the GTP.

The chromatogram shown in Fig. 1 was cut up and the spots counted. The results are shown in Fig. 2. In order to avoid confusion in the diagram, we have not plotted the radioactivity found in the unknown spots slower than GTP, nor that in the PPi and 3′,5′-GMP spots. The sum of the radioactivity in these areas at zero time was 7.4% and decreased to 5.5% at the end of 25 min of incubation. This diagram shows that the rate of GTP breakdown was nearly directly proportional to GMP formation. Such a proportionality can only occur if the rate of GMP hydrolysis is comparatively slow, as is here evidenced by the slow increase in ^{32}Pi.

Because of the very rapid conversion of GTP to GMP, with little accumulation of GDP, we entertained the possibility that the reaction was proceeding directly to GMP and PPi[6]. We could not detect such PPi formation since the GTP was labeled in the α-position. However, we do not think that such a pyrophospho-hydrolysis does occur. First of all, it will be seen that there is a small increase in GDP at 2.5 min,

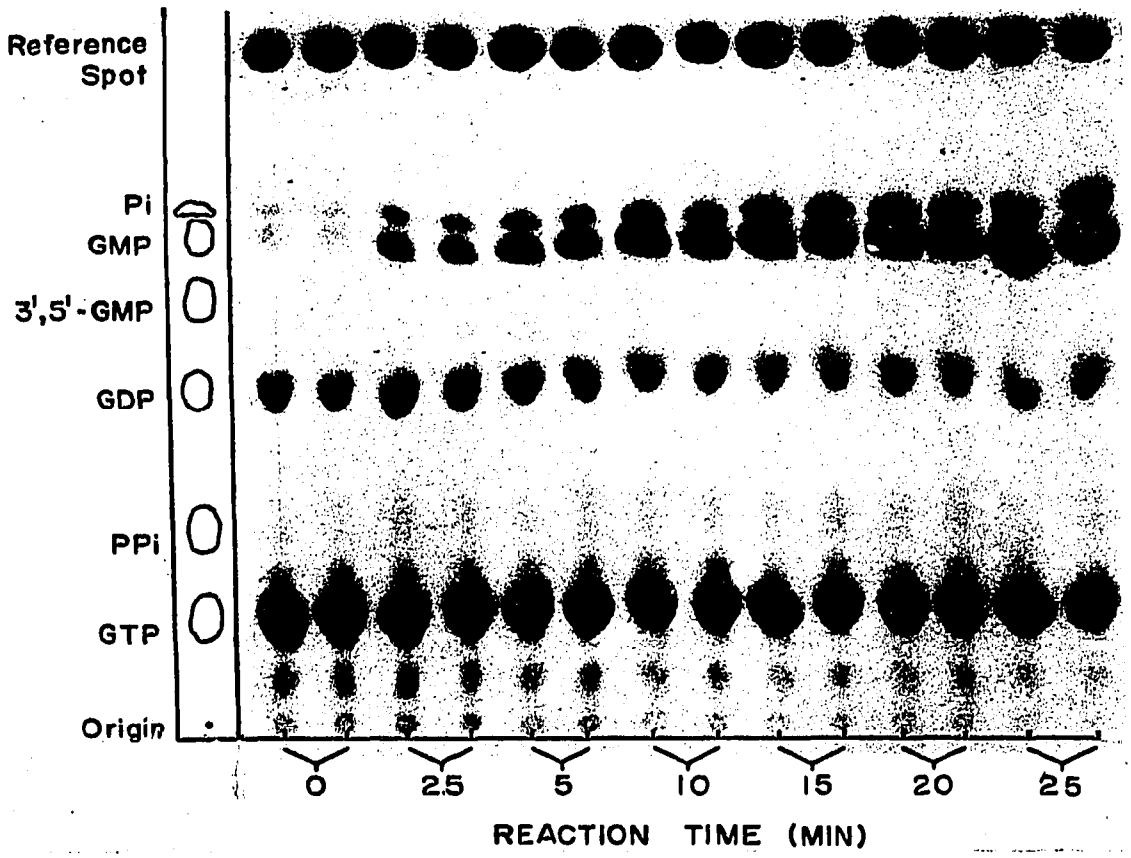

Fig. 1. Radioautogram of chromatogram developed with 0.5 *M* formic acid–4 *M* LiCl (7:3). A rat kidney (0.8 g) was homogenized in 3 ml of a solution of 10 m*M* Tris–HCl, pH 7.6, 10 m*M* 2-mercaptoethanol, 0.5 m*M* ethylenediaminetetraacetate, and the mixture was centrifuged at 37,000 *g* for 30 min. The supernatant solution was removed and diluted four times with the homogenization medium. A 25-μl aliquot of this preparation contained 88 μg protein, and this was used in the GTPase assay described in the text, for the indicated reaction times. X-ray film was exposed for 17 h to the developed chromatogram.

which we have also seen in other experiments. This suggests that GTP is being degraded to GDP, which in turn is being hydrolyzed to GMP at a rate too rapid to allow GDP accumulation. This mechanism is supported by other data we have obtained with this same system, which showed that the apparent rate of GTP hydrolysis was greatly reduced when pyruvate kinase and phosphoenolpyruvate (PEP) were present. This suggested that GDP was indeed the hydrolysis product, and that GTP was being regenerated from GDP by pyruvate kinase and PEP.

The results depicted in Fig. 2 were obtained by summing all of the radioactivity appearing in each channel, rather than using the top reference spot as 100%. We find better agreement between duplicates by this procedure. We divide each channel into the following sections, for counting purposes: Origin, Unknown, GTP, PPi, GDP, 3',5'-GMP, GMP, Pi, Solvent Front, and Reference Spot. The sum of the radioactivity in each channel usually exceeds that detected in the reference spot by some

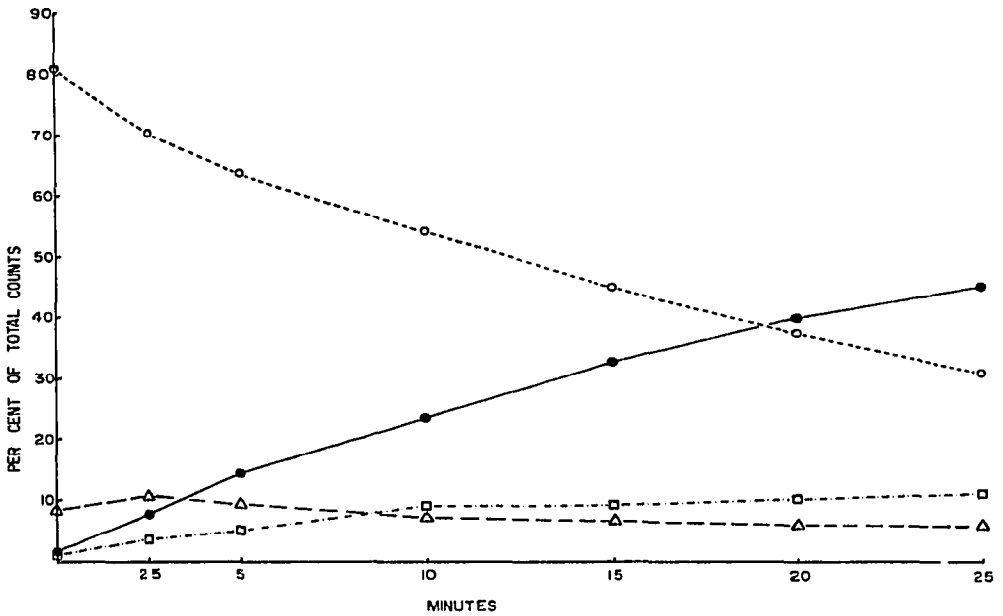

Fig. 2. Quantification of chromatogram shown in Fig. 1. After the radioautogram had been made, the spots on the chromatogram were revealed and marked, and cut from the sheet. Each was counted, and is here represented as the per cent of the total radioactivity in each channel that was recovered in that spot. ○, GTP; ●, GMP; △, GDP; □, Pi.

3 to 10%. This discrepancy is undoubtedly due to variations in quenching. We introduced the second methanol wash, after chromatography, as a means of eliminating variations in quenching by removing the LiCl from the dried sheet. The quench variations were not eliminated, however, we have retained the wash since it makes the spots appear brighter.

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