Journal of Chromatography, 153 (1978) 247–252
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CHROM. 10,655

HIGH-VOLTAGE PAPER ELECTROPHORETIC ASSAY FOR GUANYL CYCLASE

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(Received October 17th, 1977)

SUMMARY

A simple, sensitive and rapid technique is described, permitting separation of cGMP* from GMP, GDP and GTP by the use of unidirectional high-voltage paper electrophoresis. The recovery of labeled cGMP in the assay of guanyl cyclase, by this procedure is 85–90%; the blank values (no enzyme) are negligible.

INTRODUCTION

Guanyl cyclase catalyzes the formation of cGMP* from GTP. cGMP has been demonstrated in several mammalian tissues[1–3] and also in bacteria[4]. It has been suggested that cGMP and cAMP "provide opposite regulatory influences in some bi-directionally controlled systems"[5]. cGMP levels in several cell types can be elevated by a number of hormones, neurotransmitters and other agents[6]. Thus, accurate *in vitro* assays of guanyl cyclase are essential for studies on regulatory role of cGMP in various metabolic processes.

The main technical difficulty in the guanyl cyclase assay is the recovery of labeled cGMP from *in vitro* incubations with labeled GTP. The incubation mixture usually contains a high concentration of GTP (0.5–2.0 m*M*) and other degradation products of GTP that might be formed during incubation. Since the conversion of labeled GTP into cGMP is low due to the relatively low activity of guanyl cyclase, even small amounts of contamination recovered as cGMP might introduce a large error in determining the amount of cGMP formed.

The procedures, currently available for the measurement of guanyl cyclase activity, consist of either Dowex chromatography coupled with thin-layer chromatography[6,7] or enzymatic cycling methods[1,8]. These available methods are laborious and limited in sensitivity to some extent because of relatively high and fluctuating blank values. Recently, Nakazawa and Sano[9] have reported a sequential alumina–Dowex chromatographic procedure for the separation of cGMP. But this method also

* Abbreviations for nucleotides: AMP = adenosine 5′-monophosphate; cAMP = cyclic AMP. GDP = guanosine 5′-diphosphate; GMP = guanosine 5′-monophosphate; cGMP = cyclic GMP; GTP = guanosine 5′-triphosphate.

requires the setting up of a series of columns for processing a certain number of samples.

We report here a rapid and highly sensitive method for the separation of labeled cGMP in the assay of guanyl cyclase.

MATERIALS AND METHODS

^{14}C- and ^{3}H-labeled nucleotides were purchased from New England Nuclear (Boston, Mass., U.S.A.). Bovine serum albumin (BSA), creatine phosphate, creatine phosphokinase, cyclic 3′,5′-guanosine and adenosine monophosphates were supplied by Sigma (St. Louis, Mo., U.S.A.). Whatman No. 3MM chromatography paper and all other chemicals were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.).

Electrophoresis

The electrophoresis was carried out in a Savant high-voltage electrophoresis apparatus consisting of high-voltage generator (0–8000 V), and electrophoresis tank ($24 \times 46 \times 50$ cm) provided with a refrigerating system. Larger tanks may be used for 45×60 cm Whatman No. 3MM paper. The temperature of the electrophoresis tank was maintained at 4–5° during electrophoresis. The buffer solution used to fill the electrode vessels and to wet the paper was prepared by adding a mixture of 50 ml glacial acetic acid and 5 ml pyridine (reagent quality) to 945 ml of distilled water. The buffer contained 10 mM EDTA and the final pH was 3.5. Whatman No. 3MM paper (30×60 cm) was generally used for electrophoresis. A horizontal starting line was drawn 6 cm from the cathode-end of the paper. A drop of marker dye[10] was applied 2 cm from one end of the starting line. A 5-μl volume of samples containing ^{14}C- or ^{3}H-labeled nucleotides were applied on the marked positions at the starting line by means of capillary pipettes. The distance between two consecutive spots was 2 cm. The spots were dried by blowing cold air. To locate the positions of nucleotides after electrophoresis, 5 μl of each of the nucleotides (about 3–5 μg) were applied to the respective spots. For the guanyl cyclase assay of the samples, 5 μl of a mixture of GMP and cGMP (3–5 μg of each of the authentic standards) were routinely applied to each spot. The paper was sprayed with pyridine acetate buffer, the excess of buffer was removed by blotting. The paper was then hung on vertical rack; the rack was placed in the electrophoresis tank noting the cathode and anode chambers. Electrophoresis was carried out at 4000 V. After 1 h run, the rack was taken out. The paper was dried in air for 20 min and then dry autoclaved at 124° for 10 min to remove all traces of pyridine acetate. Positions corresponding to the nucleotides were located by a UV lamp, cut out from the paper, and radioactivity was determined in a Nuclear Chicago liquid scintillation counter using POP (4 g), POPOP (50 mg) and scintillation grade toluene (1 l).

Homogenization

Brain and other tissues (as noted in Table III) were removed from Sprague–Dawley rats and chilled in buffer containing 0.25 *M* sucrose, 0.02 *M* Tris–HCl (pH 7.5), 1 m*M* EDTA and 5 m*M* dithiothreitol (DTT)[9]. The tissues were blotted with filter paper, minced and homogenized in 10 volumes of buffer in a Potter–Elvehjem glass homogenizer. The homogenates were centrifuged 10 min at 4° at

1000 *g* and the supernatants were further centrifuged for 30 min at 4° at 105,000 *g*. The final supernatant was employed to measure guanyl cyclase and cGMP phosphodiesterase activity.

Particulate fractions containing adenyl cyclase activity were prepared from different tissues according to the method of Sweat and Wincek[11]. Crude cAMP phosphodiesterase extracts were prepared from different tissues according to the method of Butcher and Sutherland[12]. Proteins were determined by the method of Lowry *et al.*[13] using BSA as standard.

Assay of guanyl cyclase

The incubation volume of 50 μl contained 25 m*M* Tris–HCl, pH 7.5; 10 m*M* $MgCl_2$; 10 m*M* theophylline; 0.03 mg BSA; 20 m*M* creatine phosphate and 0.2 mg/ml creatine phosphokinase; 1 m*M* cGMP; 5 m*M* KCl; 1 m*M* DTT; 1 m*M* GTP containing $6.8 \cdot 10^5$ cpm and 0.75 to 1 mg/ml of enzyme preparation. Incubation was carried out for 15 min at 37° and terminated by adding 5 μl of 10% trichloroacetic acid. The incubation tubes were kept in ice for 10 min, centrifuged for 10 min at 3000 rpm in International Refrigerated Centrifuge. A 5-μl volume of each sample was used for paper electrophoresis. Each reaction was carried out in duplicate. Adenyl cyclase assay was carried out in a similar manner[14]. Labeled cAMP was also separated by paper electrophoresis.

Phosphodiesterase assay

The incubation volume of 250 μl contained 50 m*M* Tris–HCl, pH 8.3; 5 m*M* $MgCl_2$; 5 m*M* DTT; 100 μg of BSA; 100 μ*M* of cGMP containing $1 \cdot 10^5$ cpm and 1 to 2 mg/ml of crude enzyme preparation. The incubation procedure is the same as described above. cAMP phosphodiesterase assay was carried out in a similar manner. Labeled GMP and AMP were also measured by paper electrophoresis[15].

RESULTS

The electrophoretic technique clearly separates cGMP from other guanine nucleotides. The separation of different guanine nucleotides are illustrated in Fig. 1. The results of electrophoretic mobilities of different guanine nucleotides are shown in Table I. It is found that both GDP and GTP move faster than cGMP. As shown in Table II, the recovery of labeled cGMP is 85–90%. The lower recovery of labeled GTP and GDP might be due to lability of these nucleotides in electrophoresis. The higher recovery value of labeled GDP in the mixture might result from partial hydrolysis of GTP as indicated in Fig. 1.

The cGMP spot after electrophoresis was eluted with cold water. The eluate was lyophilised and the material was confirmed as cGMP by thin-layer chromatography[10]. Fig. 2 shows the time course study of rat brain guanyl cyclase. The enzyme activity is linear up to 20 min under our assay conditions. Table III shows the distribution of adenyl and guanyl cyclases and the corresponding phosphodiesterases in various organs of rat. Of the tissues examined, the highest guanyl cyclase activity was found in rat brain and the lowest activity was in the kidney. Adenyl cyclase activity was found to be extremely high in brain and ranked second in pancreas. The lowest adenyl cyclase activity was in the heart tissue. The brain exhibited the highest

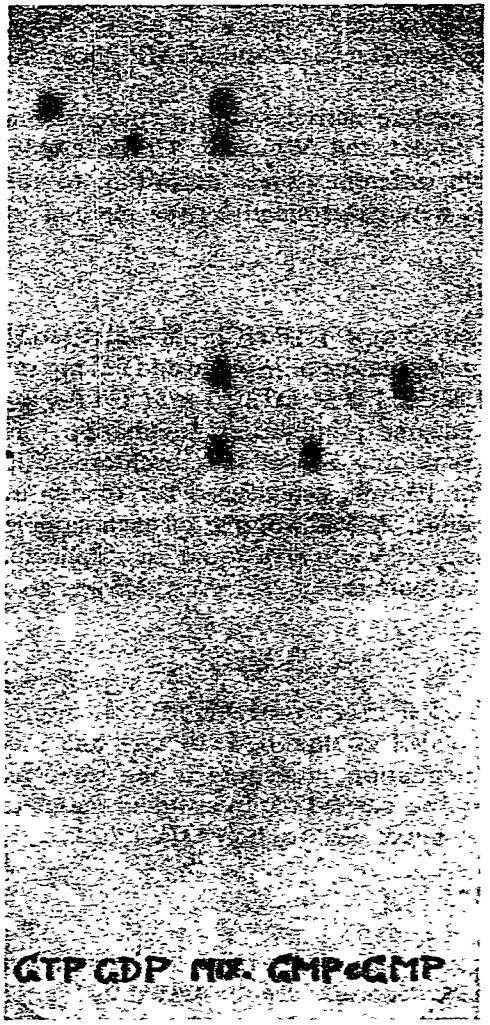

Fig. 1. Autoradiography of a typical electrophoretic run. 5 μl of each of ^{14}C-labeled nucleotides were applied on Whatman No. 3MM paper. Electrophoresis was carried out for 1 h as described in the text.

TABLE I

RELATIVE ELECTROPHORETIC MOBILITIES OF GMP, cGMP, GDP AND GTP

1-h run.

Compound	*Distance moved from origin (cm)*	*Relative mobility**
GMP	23.6	59
cGMP	27.2	68
GDP	36.8	92
GTP	40	100

* Relative mobility $= \frac{\text{distance moved by the compound}}{\text{distance moved by GTP}} \times 100.$

TABLE II

RECOVERY OF ^{14}C-LABELED GMP, GDP, GTP AND cGMP AFTER ELECTROPHORESIS*

1-h run. A, From individual spot application; B, from mixture of nucleotides.

Compound	*cpm applied*	*Recovery (cpm)*		*Recovery (%)*	
		A	*B*	*A*	*B*
GMP	76.900	66.900	64.590	87	84
GDP	65.000	52.200	56.700	79	86
GTP	88.000	70.000	67.500	79	77
cGMP	98.900	89.000	85.000	90	86

level of both, cAMP and cGMP phosphodiesterase activity. cAMP phosphodiesterase activity appeared to be lowest in pancreas, whereas cGMP activity was at the lowest level in the heart tissue. The existence of cAMP phosphodiesterase in great excess over adenyl cyclase has been observed by a number of investigators[12,16,17]. Nakazawa and Sano[10] have also reported much higher activity of cGMP phosphodiesterase than that of guanyl cyclase in various organs of rat.

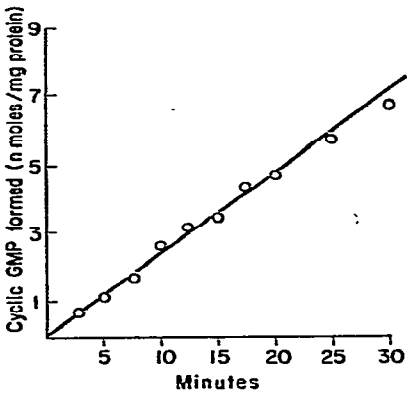

Fig. 2. Time course of the formation of cGMP. Incubations were performed using reaction components described in the text. A concentration of 0.80 mg/ml of enzyme preparation was used.

TABLE III

DISTRIBUTION OF GUANYL AND ADENYL CYCLASE AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN DIFFERENT ORGANS OF RAT

Each value is the average of five determinations (Mean ± S.D.).

Organs	*Guanyl cyclase (pmoles of cGMP/mg·min)*	*cGMP phosphodiesterase (pmoles of GMP/mg·min)*	*Adenyl cyclase (pmoles of cAMP/mg·min)*	*cAMP phosphodiesterase (pmoles of AMP/mg·min)*
Liver	101 ± 9	1090 ± 100	205 ± 12	3000 ± 260
Brain	220 ± 15	2460 ± 180	2800 ± 210	7000 ± 420
Heart	85 ± 7	170 ± 16	154 ± 17	2100 ± 198
Lung	205 ± 14	244 ± 21	265 ± 22	620 ± 54
Kidney	66 ± 6	560 ± 52	358 ± 31	3020 ± 280
Pancreas	117 ± 10	1460 ± 130	493 ± 42	800 ± 72

DISCUSSION

The electrophoretic method described is simple, sensitive and relatively rapid. It permits simultaneous determination of labeled cGMP and other nucleotides (guanine and guanosine), that might be formed in the assay mixture from the degradation of GTP. Guanosine and guanine (not shown in the chromatogram) move towards the cathode. A large number of samples can be processed within a relatively short time by this procedure. No heating of the sample, after incubation, is necessary and a small aliquot (5 μl) of the sample is sufficient to determine labeled cGMP so that the incubation volume may be maintained at a minimum.

The separation of cGMP by electrophoresis appears to have advantages over previous chromatographic methods. Guanyl cyclase assays involving two-step chromatographic procedures[6–9] appear to be tedious and time consuming for processing a large number of samples. In the electrophoretic method a large number of samples can be spotted on a single sheet of paper and can be processed under identical conditions and column to column variation associated with chromatographic procedures may be avoided. Since a high recovery of cGMP (85–90%) can be achieved by electrophoresis, this method would be suitable for measurement of low levels of guanyl cyclase activity in various tissues.

REFERENCES

1 N. D. Goldberg, S. B. Dietz and A. G. O'Toole, *J. Biol. Chem.*, 244 (1969) 4458.
2 E. Ishikawa, S. Ishikawa, J. W. Davis and E. W. Sutherland, *J. Biol. Chem.*, 244 (1969) 6371.
3 A. L. Steiner, A. S. Pagliara, L. R. Chase and D. M. Kifnis, *J. Biol. Chem.*, 247 (1972) 1114.
4 N. D. Goldberg, R. F. O'Dea and M. K. Haddox, *Advan. Cyclic Nucleotide Res.*, 3 (1973) 155.
5 R. W. Bernlohr, M. K. Haddox and N. D. Goldberg, *J. Biol. Chem.*, 249 (1974) 4329.
6 E. Bohme, *Eur. J. Biochem.*, 14 (1970) 422.
7 A. A. White and G. D. Auerbach, *Biochem. Biophys. Acta*, 191 (1969) 686.
8 J. G. Hardman and E. W Sutherland, *J Biol. Chem.*, 244 (1969) 6363.
9 K. Nakazawa and N. Sano, *J. Biol. Chem.*, 249 (1974) 4207.
10 F. Sanger, G. G. Brownlee and B. G. Barrell, *J. Mol. Biol.*, 13 (1965) 373.
11 F. W. Sweat and T. J. Wincek, *Biochem. Biophys. Res. Commun.*, 55 (1973) 522.
12 R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.*, 237 (1962) 1244.
13 O. N. Lowry, N. J. Rosebrough, A. L. Farr and R. I. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 H. Mukerjee and A. Goldfeder, *J. Cell. Biol.*, 64 (1974) 469.
15 H. Mukerjee and A. Goldfeder, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 34 (1975) 2701.
16 G. I. Drummond and S. Perrott-Yee, *J. Biol. Chem.*, 236 (1961) 1126.
17 A. S. Rajakovik and R. B. March, *Biochem. Physiol.*, 43 (1972) 209.