SIMPLE AND QUICK ASSAY OF GUANYLATE CYCLASE*

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A simple and quick procedure for the separation of radioactive cyclic 3',5'-GMP from other guanine derivatives and metabolites is described. The technique is rapid enough to assay 50 or more samples in a few hours and is sensitive enough to detect 0·005% conversion of GTP to cyclic 3',5'-GMP. The results presented provide a justification for the use of this separation technique for the determination of radioactive cyclic 3',5'-GMP formed by guanylate cyclase.

Methods for the determination of cyclic guanosine 3',5'-monophosphate[1-6] (cyclic GMP) and for the estimation of guanylate cyclase[5,7-10] have been described recently. Separation of cyclic GMP from other nucleotides by these methods is time consuming and difficult to apply on a large scale routine basis. Krishna and coworkers[11] described a simple procedure for the purification of cyclic adenosine 3',5'-monophosphate (cyclic AMP) which was found unsuitable for cyclic GMP purification. However, modification of this method, as described in this report, makes possible to use it for the separation of radioactive cyclic GMP formed by guanylate cyclase.

EXPERIMENTAL

Materials

Cyclic GMP, GTP, GDP, GMP, guanosine and guanine were purchased from Sigma. Cation exchange resin AG 50 W — X2 (200—400 mesh, hydrogen form) was obtained from Calbiochem. All other chemicals were commercial preparations and were used without further purification.

Guanosine 5'-triphosphate-[α-^{32}P], trisodium salt (250-350 m Ci/mmol) and guanine-[8-^{14}C] hydrochloride (52·5 m Ci/mmol) were purchased from International Chemical Corporation. Inorganic [^{32}P] phosphate was obtained from New England Nuclear Co. Cyclic guanosine-[8-^{3}H] 3',5'-monophosphate, ammonium salt (4·4 Ci/mmol); guanine-[8-^{3}H]sulfate (211 m Ci/mmol), guanosine-[8-^{3}H] 5'-triphosphate, ammonium salt (13·3 Ci/mmol) and guanosine 5'-triphosphate-[α-^{32}P], trisodium salt (850 mCi/mmol) were products of Amersham. Another sample of cyclic guanosine 3',5'-monophosphate-[^{3}H] (2·6 Ci/mmol) was a product of Calbiochem. Radioactive cyclic GMP was purified on AG 50 W ion exchange resin as described in results for the isolation of cyclic GMP. Chrom AR sheet 1000 were purchased from Mallincrodt and Silica Gel_{254} for thin layer chromatography was from Merk.

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Guanylate Cyclase Extracts

Rat lung was frozen on solid carbon dioxide, crushed and homogenized in 150 mM Tris-HCl buffer (pH 7·5). The homogenate was centrifuged for 30 min at 25000 *g* and the supernatant fraction containing guanylate cyclase activity was diluted to contain 50—200 μg protein per 20 μl sample.

Assay of Guanylate Cyclase

The assay is based upon detection of radioactive cyclic GMP which is formed from the labelled GTP. Except where stated otherwise, 20 μl of the supernatant (equivalent to 50—200 μg of proteins) were added to 30 μl of incubation mixture containing 1 μCi GTP-[α-^{32}P] and incubated at 37°C for 10 min. Composition of the incubation mixture was similar to that of White and Aurbach[8]: Tris-HCl buffer, 30 mM (pH 7·5); theophylline, 10 mM; cyclic GMP, 0·1 mM and GTP, 0·4 mM. The reaction was terminated by adding 1 ml of 0·05 M-HCl which contained 50 μg of

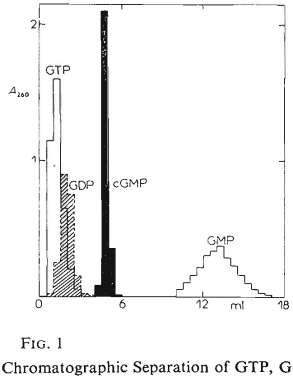

FIG. 1

Chromatographic Separation of GTP, GDP, GMP, cyclic GMP, Guanosine, and Guanine on AG 50 W-X 2 Ion Exchange Resin Columns equilibrated with 0·05M-HCl

Compounds (50—100 μg per 1 ml of 0·05M-HCl) were applied in two 0·5 ml portions and then eluted with 3·5 ml of 0·05M-HCl, followed by 1·0 ml of distilled water and then again with 0·05M-HCl. Fractions of 0·5 ml were collected and UV absorbance at 260 nm was determined.

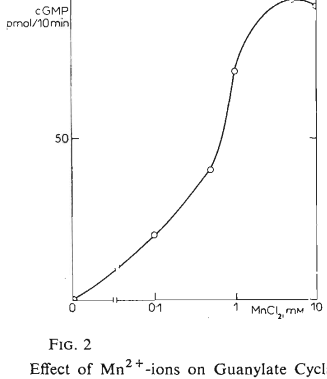

FIG. 2

Effect of Mn^{2+}-ions on Guanylate Cyclase Activity in Rat Lung Supernatant

Supernatant fraction (120 μg of proteins) was incubated for 10 min at 37°C as described in Methods.

cyclic GMP or cyclic GMP-[^{3}H] for the calculation of recovery and the tubes were immersed in the boiling water for 5 min. Cyclic GMP formed during the incubation was separated from the other labelled compounds either by the method described in results or by the procedure used by White and Aurbach[8]. Protein content was estimated according to Lowry and coworkers[12], bovine albumin served as a standard.

Degradation of Cyclic GMP by Phosphodiesterase

Phosphodiesterase prepared from the rat brain according to Brooker and coworkers[2] was used for testing the product of guanylate cyclase reaction which was isolated by the method described. Samples from the assay of guanylate cyclase were passed through a short column of AG 50 W exchange resin (0·6 . 2·0 cm) which removes all theophylline and cyclic GMP elutes by distilled water in the first 2 ml. This fraction was divided in two portions. One portion was exposed to the action of phosphodiesterase for 60 min. Then both control and phosphodiesterase samples were purified by the procedure described in results and the difference in the radioactivity indicated cyclic GMP destruction. The product of guanylate cyclase reaction was also isolated using thin layer chromatography on tetraborate impregnated silica gel layers with developing solvent[13] n-butanol–methanol–ethyl acetate–ammonium hydroxide (1 : 1 : 1 : 1).

Measurement of Radioactivity

^{32}P and ^{3}H radioactive samples were counted by liquid scintillation using dioxane solvent. The amount of cyclic GMP formed during the incubation with guanylate cyclase was calculated from the specific radioactivity of GTP-[α-^{32}P] in the incubation mixture and cyclic GMP-[^{32}P] recovered minus reaction blank without enzyme preparation. Recovery was assessed by using cyclic GMP-[^{3}H] or cyclic GMP which was measured at 260 nm. All determinations were performed in duplicates or triplicates and the results are meaned.

RESULTS AND DISCUSSION

Isolation of Cyclic GMP

Separation of cyclic GMP from other nucleotides was achieved by the combination of column chromatography on cation exchange resin and subsequent negative precipitation with barium sulfate. Cyclic GMP, GTP, GDP, GMP, guanosine or guanine (0·05 – 0·1 mg/ml of 0·05M-HCl) were applied in 1 ml sample on a 0·5 . 9·0 cm column of AG 50 W (H^+) X 2 cation exchange resin (200 – 400 mesh). The column was prepared by pipetting 4 ml of a mixture of the resin and distilled water (1 : 1, v/v) and equilibrating with 0·05M-HCl. The pattern of nucleotide elution was estimated by measuring the optical density at 260 nm.

When column was eluted only with 0·05M-HCl, then 70 – 80% of cyclic GMP was recoverd between 5 – 10 ml. When the compounds were eluted first with 3·5 ml 0·05M-HCl, followed with 1 ml of distilled water and again with 0·05M-HCl, GTP and GDP was eluted in the first 3·5 – 4·0 ml and about 90% of cyclic GMP was eluted in 0·7 ml from 4·5 to 5·2 ml. GMP was eluted between 10 – 17 ml whereas guanosine

TABLE I

Percentage Recovery of Various Nucleotides and Inorganic Phosphate after one Zinc Sulfate and Barium Hydroxide Precipitation

Acidic solutions containing cyclic GMP, GTP, GDP, GMP, guanosine, guanine (0·35 to 0·5 mg/ml 0·05M-HCl) or ^{32}P inorganic phosphate were precipitated with zinc sulfate and barium hydroxide at two different pH. At pH 7·5, 0·5—0·7 ml of acidic solutions were mixed with 0·2 ml of 0·25M zinc sulfate and then with 0·2 ml of 0·25M barium hydroxide. At pH 6·2 only 0·1 ml of barium hydroxide was used. The recovery was measured at 260 nm and by scintillation counting.

Compound	Recovery, %	
	pH 7·5	pH 6·2
Cyclic GMP	<5	>95
GTP	<1	<1
GDP	<1	$<1{\cdot}3$
GMP	<1	$<1{\cdot}3$
Guanosine	<5	>95
Guanine	<5	<55
P_i	<5	<5

TABLE II

The Identification of Cyclic GMP by Thin Layer Chromatography and Degradation by Phosphodiesterase

Exogenously added cyclic GMP-[^{3}H] and ^{32}P product of guanylate cyclase reaction in the presence of 10 mM-$MnCl_2$ were passed through a short column of AG 50 W resin (0·5 . 2·0 cm) in order to remove theophylline. One half was processed directly and the another was exposed 60 min to brain phosphodiesterase. Then the samples were purified by standard procedure. One half of radioactive cyclic GMP fraction was counted in dioxane scintillation solvent and the other half was evaporated and chromatographed on tetraborate impregnated silica gel thin layers. Areas corresponding to cyclic GMP were scraped off and counted in toluene scintillation solvent.

Conditions	No treatment		Phosphodiesterase	
	^{3}H (c.p.m.)	^{32}P (c.p.m.)	^{3}H (c.p.m.)	^{32}P (c.p.m.)
Standard procedure	1 920	391	473	101
Standard procedure + chromatography	803	263	246	67

and gaunine did not appear in the eluate up to 25 ml (Fig. 1). Inorganic phosphate-[^{32}P] was eluted with GTP and GDP in the first 3·5 ml fraction.

Cyclic GMP eluted from the column of AG 50 W exchange resin can be further purified by negative absorption on the precipitate formed by zinc sulfate and barium hydroxide at pH 6·2–6·4. Table I compares the percentage recovery of various guanine derivatives and inorganic phosphate after one precipitation at pH 6·2 and at pH 7·5 which is used by Krishna and coworkers[11] for the separation of cyclic

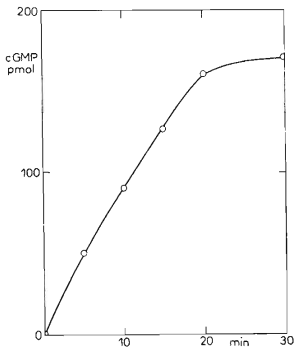

FIG. 3

Time Course of Guanylate Cyclase Activity in Rat Lung Supernatant in the Presence of 5mM-$MnCl_2$

Incubation conditions were the same as in Fig. 2.

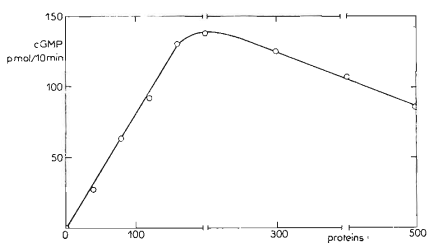

FIG. 4

Guanylate Cyclase Activity in Rat Lung Supernatant in the Presence of 5mM-$MnCl_2$ as a Function of Enzyme Concentration

Conditions of incubation using varying protein content (0–500 μg) were the same as in Fig. 2.

AMP. Decrease in pH during precipitation increased final recovery of cyclic GMP in the supernatant from less than 5% to more than 95%. Reduction of pH did not have any significant effect on the absorption of GTP, GDP, GMP and inorganic phosphate, 95% of guanosine and about 55% of guanine remained in the supernatant. Combination of column chromatography on AG 50 W and purification by barium sulfate precipitation at pH 6·2 can be used for the separation of radioactive cyclic GMP produced by guanylate cyclase from GTP-[^{3}H], GTP-[^{14}C] or GTP-[α-^{32}P]. The purification of radioactive cyclic GMP leaves in samples 0·003 – 0·01% of added radioactivity in GTP-[α-^{32}P]. The technique is rapid enough to detect 50 or more samples in a few hours and sensitive enough to detect 0·005% conversion of labelled GTP to cyclic GMP.

Identification of Cyclic GMP

The proof of the purity of cyclic GMP fraction isolated by the above procedure was made by thin layer chromatography and destruction by phosphodiesterase. Thin layer chromatography on tetraborate impregnated silica gel layers separates cyclic cyclic GMP from other guanine derivatives. The spot of cyclic GMP does not overlap with any spots of its derivatives and metabolites[13]. The product of guanylate cyclase

TABLE III

Comparison of Two Guanylate Cyclase Assays

Guanylate cyclase activity in 100 µg of rat lung supernatant protein was estimated after 10 min incubation period at 37°C by measuring cyclic GMP-[^{32}P] in the medium. Two methods were compared: *A* a procedure using the separation on AG 50W resin and double precipitation with barium sulfate at pH 6·2; and *B* the method of White and Aurbach[8].

$MnCl_2$ mM	Cyclic GMP, pmol/mg protein per 10 min	
	Procedure *A*	Procedure *B*
0	4·7	3·5
	3·8	2·0
0·1	115·3	127·5
	120·6	130·8
0·5	293·7	361·0
	273·9	439·6
1·0	532·9	546·3
	523·9	540·0
5·0	805·9	815·6
	843·4	886·5

reaction travels in thin layer chromatography system together with added cyclic GMP-[^{3}H]. Table II compares radioactivity of tritiated and ^{32}P cyclic GMP before and after the treatment with phosphodiesterase in samples isolated by standard procedure and after additional thin layer chromatgraphy on tetraborate impregnated layers. Percentage of phosphodiesterase digestion of added cyclic GMP-[^{3}H] and of cyclic GMP-[^{32}P] formed by guanylate cyclase in the presence of 10 mM Mn^{2+}-ions was nearly identical in both separation procedures (69–75%).

Activation of Guanylate Cyclase by Mn^{2+}-Ions

Suitability of the above procedure for the separation of radioactive cyclic GMP formed by guanylate cyclase was demostrated by the experiments in which the activation of enzyme by Mn^{2+}-ions under various experimental conditions was tested. Fig. 2 shows the activation of guanylate cyclase in 25000 *g* rat lung supernatant fraction by Mn^{2+}-ions. Under maximal stimulation of guanylate cyclase by 5mM-$MnCl_2$ the accumulation of cyclic GMP by 120 μg of enzyme protein preparation was linear up to 20 min (Fig. 3). By using a 10 min incubation period at 37°C and in the presence of 5mM-$MnCl_2$ there was a linear release of cyclic GMP up to 160 μg of protein per sample. Further increase in protein concentration led to a decreased accumulation of the nucleotide (Fig. 4).

The developed procedure for the separtion of radioactive cyclic GMP was further compared with the method of White and Aurbach[8] which uses combination of column chromatography on AG 50 W, evaporation and chromatography on AR sheets. Data given in Table III show that both methods gave comparable values.

In analogy to the procedures for estimating adenylate cyclase activity in intact cells[14,15], a similar procedure for estimation of guanylate cyclase can be performed by prelabelling the intracellular GTP by the labelled guanine. Above described procedure can also be used for the assessment of the guanylate cyclase activity in intact celles. The simple method for the determination of guanylate cyclase activity might help to study the role of cyclic GMP in biological systems.

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Note added in proof: In experiments in which only ^{32}P samples are measured we now estimate the radioactivity by the use of Cerenkov radiation. Radioactive samples are added to 10 ml of water solution of 1-naphthylamine at concentration 100 mg/ml. All of the counting is done with a Packard Tricarb liquid scintillation spectrometer which operates at maximum gain with a lower discrimination of 50 mV and no upper discrimination. The efficiency of this counting is about 60–70% of that in dioxane scintillation solvent. In these experiments the recovery of cyclic GMP must be assessed by the measurement of optical density at 260 nm.

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