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A Rapid Method for Measuring Guanylate Cyclase Activity in Mammary Tissue[1] (39120)

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Several laboratories have recently published methods for quantitating guanylate cyclase activity in broken cell preparations from biological tissues (1–8). Each of these methods employs an incubation period with the substrate, GTP, followed by a variety of techniques for isolating and quantitating the guanosine-3′:5′-cyclic monophosphoric acid (cGMP) formed during the incubation. The isolation techniques used are generally laborious and time-consuming. Here we report a rapid, simplified technique for measuring guanylate cyclase activity in mammary tissue using ion exchange chromatography followed by a $ZnSO_4$–$Ba(OH)_2$ precipitation for the isolation of cyclic-GMP. The analysis of 30–50 tissue samples can be carried out within a 5–6 hr period when this method is employed.

Materials. The following products were purchased from the Sigma Chemical Company: 5′-guanosine monophosphate (GMP), 5′-guanosine diphosphate (GDP), 5′-guanosine triphosphate (GTP), 3′:5′-cyclic guanosine monophosphate (cyclic-GMP), 5′-xanthosine triphosphate (XTP), 5′-xanthine diphosphate (XDP), 5′-xanthosine monophosphate (XMP), 3′:5′-cyclic xanthosine monophosphate (cyclic-XMP), 5′-inosine monophosphate (IMP), 3′:5′-cyclic inosine monophosphate (cyclic-IMP), 3′:5′-cyclic-adenosine monophosphate (cyclic-AMP), phosphocreatine, creatine phosphokinase (rabbit skeletal muscle), and cyclic nucleotide phosphodiesterase (beef heart). Dowex 50W-X8 (200–400 mesh) in the hydrogen form was purchased from Bio Rad Laboratories. (α-^{32}P)-GTP (20.7 Ci/mmole) and ^{3}H-cyclic-GMP (G, 2.1 Ci/mmole) were purchased from the New England Nuclear Corp. and were used without further purification. The mammary gland tissues used in this study were obtained from Swiss Webster mice in the twelfth to fourteenth day of their first pregnancy; these animals were purchased from Spartan Research Animals, Inc., Haslett, Mich. Prolactin (NIH-P-S-10) was a gift from the National Institute of Arthritis and Metabolic Diseases.

Methods, results, and discussion. The experimental protocol for these studies was as follows. Mice were killed by cervical dislocation and the inguinal and abdominal mammary glands were excised. The glands were then either used immediately or they were frozen by immersion in liquid nitrogen and stored at −80°C for future use. In preparation for the guanylate cyclase assay, the tissues were blotted on filter paper, weighed, and homogenized at 0–4°C in a ground glass homogenizer; the tissues were homogenized 1:10 (wt/vol) in medium containing 0.04 *M* Tris–HCl buffer (pH 7.4, 30°C), 0.25 *M* sucrose, 0.01 *M* $MnCl_2$, and 0.01 *M* theophylline. Enzyme activity was then determined within a 1-hr period. Tissues stored at −80°C for up to 2 weeks showed no loss of enzyme activity.

The following methods were used to carry out the guanylate cyclase assay. Mammary gland homogenate (0.1 ml) or boiled homogenate (the blank) was added to 0.05 ml of incubation medium at 0–4°C. The final concentrations of components in the reaction mixture were 0.04 *M* Tris–HCl buffer (pH 7.4, 30°C), 0.01 *M* $MnCl_2$, 0.01 *M* theophylline, 0.003 *M* cyclic-GMP, 0.5–1.0 μCi (α^{32}P)-GTP (5 mmoles), and a GTP regenerating system consisting of 0.02 *M* creatine phosphate and 1 mg/ml creatine phosphokinase. The enzyme assay was initiated by placing the reaction tubes (12 × 75 mm) in a water bath maintained at 30°C; unless otherwise specified, incubation was carried out for 5 min with shaking. The enzyme reaction was terminated by adding 0.025 ml of 1.0 *M* HCl

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to the tubes and then placing the tubes in a boiling water bath for 2 min. Then, 10,000 cpm ^{3}H-cyclic-GMP in 0.1 ml of 1 m*M* cyclic-GMP were added to each reaction tube; the ^{3}H-cyclic-GMP was added to monitor for procedural losses during the subsequent isolation steps.

Cyclic-GMP was next isolated from the reaction mixture by a two-step procedure. The first step entails ion exchange chromatography on a 0.4 × 3.5 cm Dowex 50W-X8 column which had been equilibrated with 0.1 *N* HCl. After centrifugation of the reaction tubes at 3000 rpm for 5 min, the resulting supernatants were applied to the Dowex columns and eluted with 0.1 *N* HCl. The first 0.4 ml of the eluent was discarded and the next 1 ml containing the cyclic-GMP was collected. Fig. 1 shows the elution pattern of GTP, GDP, GMP, and cyclic-GMP from the columns. Cyclic-GMP is clearly separated from the other guanosine derivatives via this method. The second step for the isolation of cyclic-GMP from the reaction mixture involves a precipitation with $ZnSO_4$ and $Ba(OH)_2$. 0.090–0.095 ml of 1 *N* NaOH was first added to each tube. Then 0.25 ml of 5% $ZnSO_4$ was added, followed by the addition of 0.1 ml of a saturated aqueous solution of $Ba(OH)_2$; the pH of this final mixture was about 5.7. The tubes were then centrifuged for 5 min at 3000 rpm. The resulting supernatant was poured into new tubes and the $ZnSO_4$–$Ba(OH)_2$ procedure was repeated one additional time. The data in Table I show

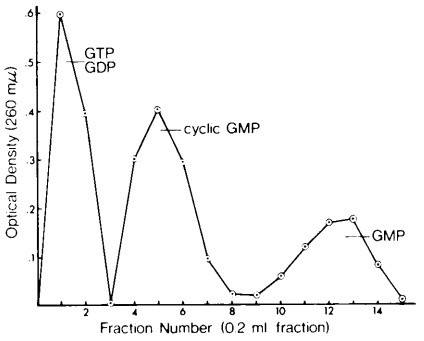

FIG. 1. Elution of guanine nucleotides from Dowex-50 columns. About 50 μg of each nucleotide was dissolved in 0.25 ml of the incubation medium; this mixture was acidified and eluted from the columns with 0.1 *N* HCl as described in the text.

TABLE I. RECOVERY OF GUANINE NUCLEOTIDES IN THE SUPERNATANT FRACTION FOLLOWING A $ZnSO_4$–$Ba(OH)_2$ PRECIPITATION[a]

Nucleotide	Optical density (260 mμm)	
	Before precipitation	After precipitation
Cyclic-GMP	0.41	0.41
GMP	0.30	0.00
GDP	0.30	0.00
GTP	0.40	0.00

[a] 0.2 micromoles of nucleotides were dissolved in 1 ml of 0.1 *N* HCl; 0.09 ml of 1 *N* NaOH was then added to each tube. The $ZnSO_4$–$Ba(OH)_2$ procedure as described in the text was then carried out.

the effectiveness of the $ZnSO_4$–$Ba(OH)_2$ procedure for the isolation of cyclic-GMP from the other guanosine derivatives. The fact that White and Zenser (2) were previously unsuccessful in their attempt to isolate cyclic-GMP by a $ZnSO_4$–$Ba(OH)_2$ precipitation is due to the pH at which they carried out the precipitation. Following the $ZnSO_4$–$Ba(OH)_2$ precipitations, the ^{3}H and ^{32}P in the resulting supernatants were measured by liquid scintillation spectrometry. The quantity of cyclic GMP represented by the ^{32}P was then calculated. An appropriate adjustment for procedural losses of ^{32}P was made from the relative amount of ^{3}H-cyclic-GMP present in the final supernatant; we could normally recover 60–90% of the ^{32}P. Also the presence of ^{32}P in the final supernatant which did not represent cyclic-GMP formed by guanylate cyclase in the tissue homogenates was accounted for by subtraction of values obtained with incubation blanks (see above); these blanks for most experiments were about 25% of the values obtained after incubations.

Following the isolation procedures, two methods were used to identify the ^{32}P-labeled product as cyclic-GMP. First, descending paper chromatography was carried out for 20 hr on Whatman 3 MM paper using a solvent system consisting of 1 *M* ammonium acetate (pH 5.0):95% ethanol (30:70). Table II shows the results of this study. Of the nucleotides tested, only the area on the chromatogram corresponding to cyclic-GMP contained ^{32}P counts that were not also present

TABLE II. PAPER CHROMATOGRAPHY OF PRODUCT FROM GUANYLATE CYCLASE ASSAY[a]

Nucleotide	Migration distance (cm)	^{32}P activity (cpm)	
		Blank	Sample[b]
GTP	3.8	0	0
GDP	6.4	0	0
GMP	11.4	0	3
Cyclic-GMP	21.6	80	902
XTP	0	0	0
XDP	3.2	0	0
XMP	8.3	0	1
Cyclic-XMP	14.0	0	0
IMP	15.9	0	0
Cyclic-IMP	23.5	0	7
Cyclic-AMP	24.3	40	38

[a] Guanylate cyclase assay was carried out as described in the text with the exception that ^{3}H-cyclic-GMP was not added during the isolation procedure. Descending paper chromatography was carried out on 1 ml of the solution containing the reaction product. Chromatography was carried out for 20 hr using 1 *M* ammonium acetate:95% ethanol (30:70). Areas on the paper corresponding to the appropriate nucleotide were cut out and radioactivity was measured.

[b] Numbers in the table represent the average of three samples.

in the incubation blanks. Similar results were obtained when descending chromatography was carried out on Whatman 3 MM paper using a solvent system consisting of isobutyric acid:water:ammonium hydroxide (63:35:2). The reaction product was further identified as cyclic-GMP by conducting the enzyme assay in the presence of added cyclic nucleotide phosphodiesterase (2, 9). For this experiment, 0.08 units of cyclic nucleotide phosphodiesterase were added to the reaction medium and theophylline was omitted; the reaction was allowed to proceed for 10 min. This incubation procedure reduced by 95% the amount of ^{32}P present in the cyclic-GMP fraction, thus further suggesting that the ^{32}P-labeled product isolated by the above procedures is cyclic-GMP.

Figure 2 shows the time-course of product formation in the guanylate cyclase assay. The amount of cyclic-GMP formed is linear with time for up to 15 min. Fig. 2 also shows that enzyme activity decreases in direct proportion to the dilution of the tissue homogenate. Further characterization of the enzyme showed that the enzyme is activated by manganese and calcium ions (Table III), whereas fluoride had no effect. Similar ionic characteristics were reported earlier for guanylate cyclase found in other tissues (1, 4, 5, 10–12). Finally, although previous studies indicated that cyclic-GMP may mediate certain of the metabolic effects of prolactin in the mammary gland (13, 14), this hormone was found to have no effect on guanylate cyclase activity

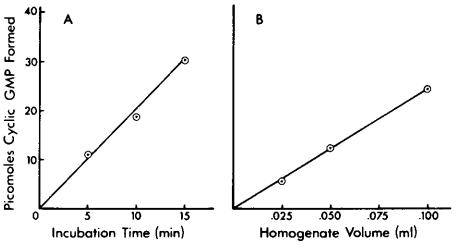

FIG. 2. (A) Time-course for guanylate cyclse assay. Homogenates of mammary glands were prepared and assayed by methods described in the text except that the incubation time was varied. (B) Guanylate cyclase activity in dilutions of homogenates of mammary glands. Incubation volumes were maintained constant by the addition of an appropriate volume of incubation medium. Incubations were carried out for 10 min by methods described in the text.

TABLE III. EFFECT OF IONS AND PROLACTIN ON GUANYLATE CYCLASE ACTIVITY[a]

Experiment	Agent added	Guanylate cyclase activity (pmoles cyclic-GMP/min/mg tissue)
I	—	0[b]
	1 m*M* Mn^{2+}	0.100
	5 m*M* Mn^{2+}	0.286
	10 m*M* Mn^{2+}	0.407
II	—	0.375
	5 m*M* Ca^{2+}	0.473
III	—	0.304
	10 m*M* NaF	0.310
IV	—	0.399
	10 μg prolactin/tube	0.390
	25 μg prolactin/tube	0.399

[a] Guanylate cyclase activity was measured by methods described in the text except in Experiment I where the Mn^{2+} concentration was varied.

[b] Results are means of duplicate determinations. These data are representative of those obtained in several experiments.

in broken cell preparations of the mammary gland.

In experiments not presented the K_m for guanylate cyclase in mammary tissue was found to be about 0.2 mM; this value is similar to that observed in other tissues (see references above). In further studies, guanylate cyclase activity was measured in 50,000g pellet and supernatant fractions of mammary gland homogenates. About 30% of the activity was found in the pellet and the remainder in the supernatant; addition of the activities found in these two fractions provided a value corresponding to the activity found in the homogenate prior to centrifugation.

Summary. A simple and rapid method for measuring guanylate cyclase activity in broken cell preparations of biological tissues is described. This method employs the rate of conversion of [^{32}P]GTP to [^{32}P]cyclic-GMP. The product of this reaction is isolated by ion-exchange chromatography and by a $ZnSO_4$–$Ba(OH)_2$ precipitation at pH 5.7. Using this method, about 30–50 samples can be assayed for guanylate cyclase activity during a 5–6 hr period. The characteristics of this enzyme in the mammary gland were found to be similar to those described for other tissues using different methods for measuring guanylate cyclase activity.

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