PURIFICATION AND SUCCINYLATION OF CYCLIC GMP FROM LARGE VOLUME SAMPLES AND RADIOIMMUNOASSAY OF SUCCINYL CYCLIC GMP

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Abstract: Improved procedures for isolation of cyclic GMP and cyclic AMP and radioimmunoassay of cyclic GMP with succinylation are described. Procedures involved include modified chromatography on alumina and succinylation of cyclic GMP followed by purification of succinyl cyclic GMP on a Dowex AG 1x8 column. These procedures are convenient and applicable to any volume up to 50 ml of tissue extracts and especially for isotonic incubation mixtures. This assay system is sensitive to 6 femtomoles of cyclic GMP/tube. On radioimmunoassay, free and antibody bound [^{125}I]-labeled cyclic GMP are separated by Millipore filtration. Cyclic GMP levels in several tissue samples were determined in order to show the applicability of the procedures.

Introduction: The development of a radioimmunoassay for cGMP by Steiner et al. (1) has remarkably accelerated the progress of establishing the role of cGMP in physiological processes. In some experimental samples, for example isolated liver and fat cells or plasma, cGMP content is extremely low and in practice some difficulties prevent accurate determination of changes in cGMP levels. From the limitation of sensitivity of the cGMP assay, it is crucial to have a simple and reliable purification method for cGMP from a large volume of experimental sample. Recently, succinylation and acetylation were applied to cyclic nucleotides at the 2'-O-position to increase the sensitivity of radioimmunoassay of cGMP (2-4) and cAMP (4,5). Succinylated cGMP, subjected directly to radioimmunoassay without purification can not always be accurately determined because of interference by reagents used for the succinylation procedure (4) and also, the yield of succinylated product is not calculated. Here we describe a convenient reliable and sensitive assay for cGMP in a large volume experimental sample where cGMP content is extremely low.

Abbreviations used are: cGMP (cyclic GMP), guanosine 3',5'-monophosphate; cAMP (cyclic AMP), adenosine 3',5'-monophosphate; ScGMP, 2'-O-succinyl guanosine 3',5'-monophosphate; [^{125}I] -ScGMP-TME, [^{125}I]-labeled tyrosine methyl ester of 2'-O-succinyl cyclic GMP.

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Materials and Methods

Materials: The cGMP antiserum (IgG fraction) was prepared in our laboratories from immunized rabbits as described by Steiner et al. (1). [^{125}I] labeled tyrosine methyl ester of succinyl cGMP ([^{125}I] ScGMP-TME, 600 Ci/mmole, 1.0 μCi/0.5 ml, used with a dilution of 1:20) was obtained from Collaborative Research, Inc. ScGMP was obtained from Sigma. Alumina (neutral) was purchased from Fisher Scientific Co., Dowex AG 1x8 (100-200 mesh) from Bio-Rad Labs. Other chemicals were commercially available products.

Isolation procedures for cGMP and cAMP: One gram of neutral alumina wetted with AL-W* was poured into a column (0.7 x 4.0 cm, Bio-Rad Labs.). The column was washed with 5 to 7 ml of AL-W. Samples, deproteinized with 10 volumes of 5% TCA containing 1,000 cpm of [^{3}H] cGMP, were loaded on the column. For incubation mixtures, TCA (50% or 10%) was added to a final concentration of 5% together with 1,000 cpm of [^{3}H] cGMP. When the application volume had drained, the column was washed twice with 1 ml of AL-E*.

cGMP and cAMP were eluted from the column with 3 ml of AL-E. This fraction, designated as the cyclic nucleotide fraction, was subjected to succinylation. When cGMP and cAMP were to be assayed, the eluate was applied to Dowex AG 1x8 (0.62 x 10 cm column, 100-200 mesh, formate form) for separation of cGMP and cAMP. Conditions for the chromatography are described in another paper.

Succinylation procedures: 250 μl of triethylamine and 600 μl of succinic anhydride in acetone (200 mg succinic anhydride dissolved in 1.0 ml acetone) were added to the cyclic nucleotide fraction from the alumina column with vigorous mixing after each addition. In the case of cGMP samples from Dowex AG 1x8, 50 μl of triethylamine and 150 μl of succinic anhydride in acetone (200 mg/ml) were added to each tube containing cGMP samples dissolved in 1.0 ml of 50 mM Na-acetate buffer, pH 6.0. After standing at 20-25° for 30 minutes, 0.1 ml of 3.0 M formic acid was added to each tube (with samples from Dowex AG 1x8, 2.0 ml of 0.1 M formic acid was used). The reaction mixtures were then loaded on a Dowex AG 1x8 column (0.62 x 5 cm, 100-200 mesh, formate form). The column was rinsed with 2-3 ml of 0.1 M formic acid and washed with 30 ml of 3.0 M formic acid. ScGMP was eluted from the column with 25 ml of 4.5 M formic acid. After lyophilization, ScGMP was dissolved in 1.0 ml of 50 mM Na-acetate buffer, pH 6.0 and 100-200 μl aliquots were used for radioimmunoassay.

Radioimmunoassay of ScGMP: The radioimmunoassay was carried out in 0.5 ml of 50 mM Na-acetate buffer, pH 6.0, containing 100 μl of ScGMP sample, 100 μl of antibody (diluted 1:6,000), 100 μg of crystallized human albumin and 75 μl of [^{125}I] ScGMP-TME (diluted 1:20, 9,000-10,000 cpm). After incubation for 18 hours at 4°, [^{125}I] ScGMP-TME bound to antibody was separated by passing the incubation mixtures through a Millipore filter (HAWP 02500), followed by washing with 7 ml of 50 mM Na-acetate buffer, pH 6.0. The Millipore filter was dissolved in 1.0 ml of cellosolve and the radioactivity was measured by a liquid scintillation counter in 10 ml of scintillation fluid (2,880 ml toluene, 960 ml cellosolve, 16 g PPO, 0.4 g POPOP (6)).

The values of ScGMP obtained by radioimmunoassay were corrected for the recovery of [^{3}H] labeled ScGMP** converted from [^{3}H] cGMP (about 1,000 cpm) which was added to samples on homogenization with TCA. A ScGMP value equivalent to [^{3}H] cGMP, added for recovery estimation, was subtracted from the ScGMP value obtained after radioimmunoassay.

Tissue samples: For determination of cGMP in tissues, male Sprague-Dawley rats, 150-200 grams, were sacrificed by decapitation. Tissues were removed and crushed by Wolenberger clamps, pre-cooled in liquid nitrogen, within 20 seconds after decapitation. Rat epididymal fat cells and liver cells were prepared according to Rodbell (8) and Berry and Friend (9) as modified by Garrison and Haynes (10) respectively.

* AL-W and AL-E: Tris formate buffer, pH 7.30 at 22°, composed of 0.05 M formate (AL-W) or 0.2 M formate (AL-E).

** 0.5 ml aliquots were counted in 10 ml of scintillation fluid "tT21" (7).

Results and Discussion

Isolation of cGMP and cAMP from large volumes of biological sample: Alumina column chromatography for the isolation of cyclic nucelotides was originally reported by White and Zenser (11). In that procedure, the sample applied to the alumina column is limited to a 1 ml volume and must have neutral pH. TCA or PCA, when used for deproteinization, must be removed from the sample before application to the column. We now report chromatographic conditions with which to purify cGMP and cAMP from samples with comfortable flexability regarding its source or volume. Biological samples which were deproteinized with 5% TCA were applied directly to a neutral alumina column without extraction of TCA. Figure 1 shows the elution patterns of cGMP and cAMP from the alumina column to which 1-50 ml of 5% TCA, containing [^{3}H] cGMP and [^{3}H] cAMP were applied as simulated samples. [^{3}H] GTP and [γ-^{32}P] ATP were also applied and the recoveries of [^{3}H] GTP and [^{32}P] ATP are also shown in Table 1. The elution pattern of cGMP and cAMP was independent of the volume applied (1.5 to 50 ml) and both cyclic nuc-

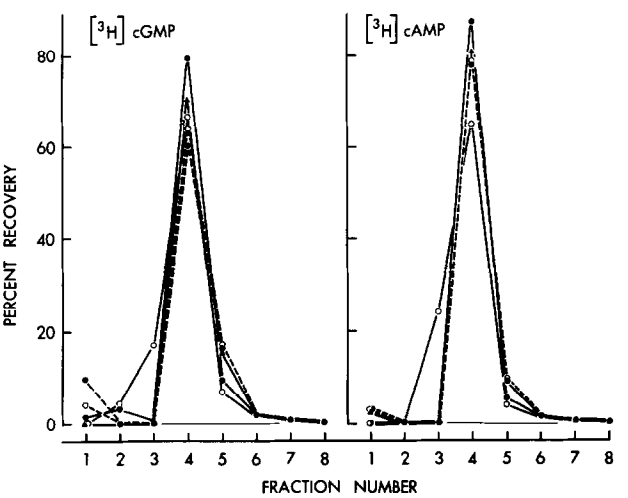

Figure 1. Elution pattern of cGMP and cAMP from alumina column.
[^{3}H] cGMP or [^{3}H] cAMP in 1-50 ml of 5% TCA was loaded on the alumina column. After the applied volume had drained into the first fraction, each 1.0 ml fraction was collected by repeated applications of AL-E to the column. Each point shows percent of applied radioactivity recovered in each fraction Applied volume: o———o, 1.0 ml; ●———●, 1.5 ml; ▲———▲, 10 ml; o- - -o, 30 ml; ●- - -●, 50 ml.

Table 1. Chromatography of [^{3}H] cGMP, [^{3}H] cAMP, [^{3}H] GTP and [γ-^{32}P] ATP on alumina

Samples applied to the column	Recovery in the cyclic nucleotide fraction			
	[^{3}H] cGMP	[^{3}H] cAMP	[^{3}H] GTP*	[γ-^{32}P] ATP*
5% TCA 10 ml	86.9 %	90.6 %	0.16 %	0.022 %
30 ml	84.1	90.2	0.18	0.021
50 ml	83.1	90.3	0.23	0.019
TCA supernatant (30 ml)				
of heart	81.4	84.9	0.12	0.017
brain	77.0	82.7	0.08	0.019
serum	64.8	72.3	0.10	0.020
HBSS**	72.3	81.2	0.15	0.050
KRBB**	71.3	81.2	0.17	0.025

* [^{3}H] GTP and [γ-^{32}P] ATP were used without pre-purification.
** HBSS, Hanks balanced salt solution; KRBB, Krebs-Ringer bicarbonate buffer. The tissue samples applied were prepared as described in the methods section. Conditions for chromatography were the same as those for Fig. 1. The 4th to 6th fraction were combined as the cyclic nucleotide fraction.

leotides were eluted out from the column together resulting in an appreciable concentration of both. Moreover, the presence of up to 150 mM NaCl in the sample had no effect on the elution patterns (Fig. 2). The recoveries of simulated samples of these two nucleotides were excellent, approximately 70-90% and 89-91% for cGMP and cAMP respectively, but were, however, dependent on the concentration of NaCl in the sample. When large volumes of TCA tissue extracts were used as samples, the elution patterns of [^{3}H] cGMP and [^{3}H] cAMP were the same as those of the simulated samples shown in Figures 1 and 2. The recoveries were approximately 65-81 and 72-85% for cGMP and cAMP respectively (Table 1). GTP, GDP, GMP, ATP, ADP and AMP (2 μmoles each) were not recovered from the column. Guanosine and adenosine, however, were eluted in the first to third fraction preceding the elution of both cyclic nucleotides.

Succinylation of cGMP: The procedure, devised by Cailla et al. (5) for succinylation of cAMP in aqueous solution, was adapted for succinylation of the cGMP fraction eluted from the alumina column. With conditions described in the methods section, 80% of cGMP was converted to ScGMP. Acetone was used for solvent

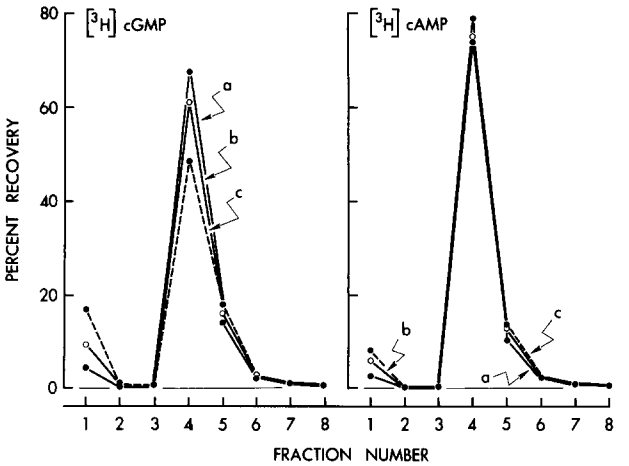

Figure 2. Effect of NaCl content in sample on elution pattern of cGMP and cAMP from alumina column.

Samples of [^{3}H] cGMP and [^{3}H] cAMP applied separately to the column contained 0-150 mM NaCl (a, none; b, 90 mM; c, 150 mM). Conditions for chromatography were the same as those for Figure 1. The volume of sample applied was 30 ml of a 5% TCA solution.

for succinic anhydride (2). Since reagents used in the succinylation reaction have a marked effect on the radioimmunoassay, it is necessary to remove cAMP and its succinyl derivatives and minor contaminations as well as succinylation reagents by chromatography on Dowex AG 1x8. Figure 3 shows the elution pattern of ScGMP from the Dowex AG 1x8 column. About 83% of the applied ScGMP was recovered in the fraction with 4.5 M formic acid (30 ml). With this chromatographic procedure, the separation of ScGMP from cGMP and cAMP with its derivatives, was satisfactory. As the yield of the succinylation reaction is approximately 80%, ScGMP samples, which were obtained by succinylation of cGMP followed by chromatography on Dowex AG 1x8, were contaminated with only 1% of the initial cGMP used. The reaction product of [^{3}H] cGMP, [^{3}H] ScGMP, co-chromatographed with authentic ScGMP on Dowex AG 1x8 (Fig. 3) with elution patterns coinciding. The fraction eluted from Dowex AG 1x8 with 4.5 M formic acid was identified as ScGMP by TLC on cellulose (butanol-glacial acetic acid-water 12:3:5 v/v) and by treatment with 0.1 N NaOH (1). Cailla and Delaage (12) have reported that 2'-O-

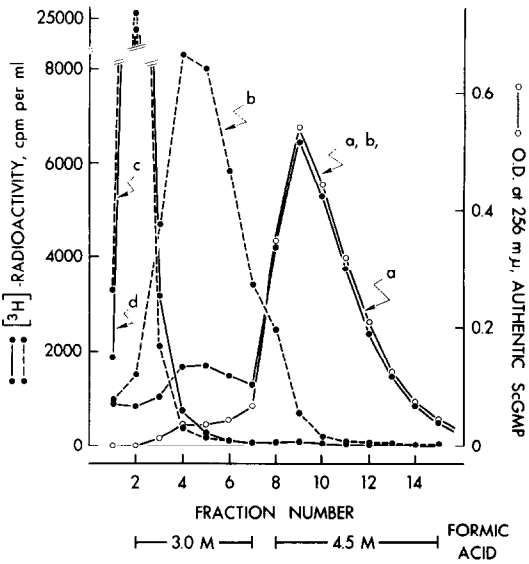

Figure 3. Elution patterns of ScGMP and related compounds on Dowex AG 1x8 (0.62 x 5 cm, formate form).

The cyclic nucleotide fraction from the alumina column was subjected to the succinylation reaction. The reaction mixtures were loaded on Dowex AG 1x8. The column was developed by repeated applications of 5.0 ml of 3.0 M and 4.5 M formic acid. Each 5.0 ml fraction was collected. [^{3}H] cGMP or [3H] cAMP was added to the cyclic nucleotide fraction before (●———● a, [^{3}H] cGMP and d, [^{3}H] cAMP) or after (●- - -● b, [^{3}H] cGMP and c, [^{3}H] cAMP) the succinylation reaction. Before chromatography, authentic ScGMP (o———o) was added to samples a and b. Other conditions are described in the methods section.

succinyl cAMP was hydrolyzed 50% to cAMP upon lyophilization in 0.01 N HCl. ScGMP, however, according to our studies on the stability of ScGMP under conditions used for purification, was stable when lyophilized in 4.5 M formic acid and 1.0 N HCl, and on standing at room temperature in 4.5 M formic acid. Approximately 25% hydrolysis was observed on standing for 12 hours in 1.0 N HCl. Although 1.0 N HCl can be used for elution of ScGMP from Dowex AG 1x8, formic acid appeared to be a better solvent for chromatography of ScGMP.

Radioimmunoassay of ScGMP: The conditions for ScGMP assay were adapted from those used for the assay of cGMP, varying only by the amount of [^{125}I] ScGMP-TME added to the incubation mixtures. For cGMP assay, 100 μl of [^{125}I] ScGMP-TME (diluted 1:20) gave a linear standard curve, whereas for ScGMP assay, high

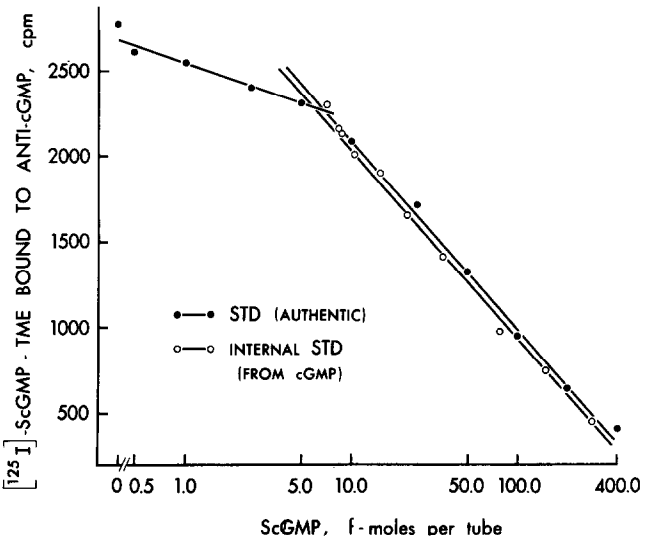

Figure 4. Comparison of external and internal standard curves of ScGMP assay. For the external standard curve, authentic ScGMP was added directly to the incubation mixtures for radioimmunoassay. For the internal standard curve, various known amounts of cGMP in 2 ml of 5% TCA containing 1,000 cpm of [^{3}H] cGMP (S.A. 10.2 Ci/mmole) for recovery estimation, were applied to the entire procedure for purification and succinylation described in the methods section. Final samples of ScGMP were applied to radioimmunoassay for ScGMP. Each value in the standard curve was corrected with its corresponding total recovery (49-52%) of [^{3}H] labeled ScGMP converted from [^{3}H] cGMP. The value of ScGMP equivalent to added [^{3}H] cGMP was added to each plotted value of ScGMP.

sensitivity and linearity on the standard curve was achieved with 75 µl. Figure 4 shows the linearity of the standard curves of ScGMP ranging from 6 to 250 femtomoles/tube. The external and internal standard curves of ScGMP coincide well. For separation of free and antibody bound [^{125}I] ScGMP-TME, the Millipore filtration technique was adopted. [^{125}I] ScGMP-TME bound to antibody was found to be absorbed quantitatively on Millipore filters and not washed away. The results so obtained were comparable to the "second antibody precipitation procedure" with goat anti-rabbit IgG reported by Steiner et al. (1).

Determination of cGMP content: This modified assay system, the combination of a practical and simple isolation procedure with a sensitive radioimmunoassay which can accomodate large volumes of experimental sample (up to 50 ml), can measure as little as a few femtomoles of cGMP, with correction for recov-

Table 2. Concentration of cGMP in rat tissues.

The assay procedures were carried out on 3-3.5 g wet weight of tissue except isolated fat cells (450 mg lipid) and liver cells (30 mg protein) in which cGMP levels were measured from a cell suspension in 10 ml of Krebs-Ringer bicarbonate buffer. For isolated fat cell samples, lipids were extracted with chloroform after the addition of TCA. The values are the average ± ½ range of the values for duplicate samples

	cGMP (p-moles/g wet tissue)	
Pancreas	8.7 ± 0.1	(880.5)*
Tongue		
whole	37.6 ± 2.5	(969.2)*
dorsal one third layer	36.9 ± 1.4	(827.3)*
Isolated fat cells**	0.91 ± 0.03	
Isolated liver cells**	7.08 ± 0.05	

* cGMP and cAMP were separated on Dowex AG 1x8 and measured as described in methods section. cAMP values, obtained by the binding assay (6) are shown in parenthesis (p-moles/g wet tissue).

** Values are expressed as p-moles per g lipid for isolated fat cells and p-moles per g protein for isolated liver cells.

ery of the entire procedure. These procedures were employed for the determination of cGMP in several tissue samples of large volume (30 ml of 5% TCA supernatants). Values are shown in Table 2. cGMP content of rat pancreas and rat tongue has not been previously documented. From the range of linearity of the standard curve, the sensitivity of the assay is limited only by the affinity of antibody to ScGMP, and is not decreased by basal values of [^{3}H] cGMP (6.5-7.5 femtomoles/assay tube) added for recovery estimation. [^{32}P] cGMP with high specific activity as recovery tracer, may increase the accuracy of assay of low values of cGMP.

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References:

1. Steiner, A.L., Parker, C.W., and Kipnis, D.M. (1972) J. Biol. Chem., 247, 1106-1113.

2. Frandsen, E.K., and Krishna, G. (1975) Fed. Proc., 34, 231
3. Zimmerman, T.P., Chu, L-C., and Winston, M.S. (1975) Fed. Proc., 34, 231.
4. Harper, J.F., and Brooker, G. (1975) J. Cyc. Nuc. Res., 1, 207-218.
5. Cailla, H.L., Racine-Weisbuch, M.S., Delaage, M.A. (1973) Anal. Biochem., 56, 394-407.
6. Gilman, A.G. (1970) Proc. Nat. Acad. Sci. USA, 67, 305-312.
7. Patterson, M.S., and Greene, R.C. (1965) Anal. Chem., 37, 854-857.
8. Rodbell, M. (1964) J. Biol. Chem., 239, 375-380.
9. Berry, M.N., and Friend, D.S. (1969) J. Cell Biol., 43, 506-520.
10. Garrison, J.C., and Haynes, R.C. (1973) J. Biol. Chem., 248, 5333-5343.
11. White, A.A., and Zenser, T.V. (1971) Anal. Biochem., 41, 372-396.
12. Cailla, H., and Delaage, M. (1972) Anal. Biochem., 48, 62-72.