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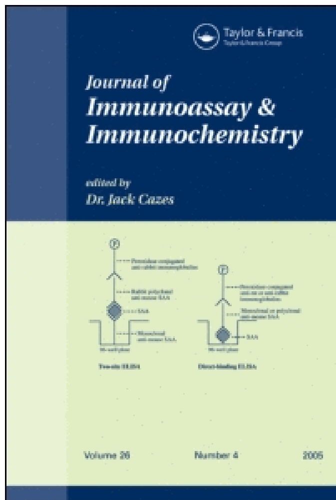

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Double Antibody Enzyme Immunoassay for the Quantitation of Adenosine 3',5'-Cyclic Monophosphate (Cyclic AMP) and Guanosine 3',5'-Cyclic Monophosphate (Cyclic GMP) in Tissue and Plasma

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To cite this Article Yamamoto, I. , Tsuji, J. , Takai, T. and Fujimoto, M.(1982) 'Double Antibody Enzyme Immunoassay for the Quantitation of Adenosine 3',5'-Cyclic Monophosphate (Cyclic AMP) and Guanosine 3',5'-Cyclic Monophosphate (Cyclic GMP) in Tissue and Plasma', Journal of Immunoassay and Immunochemistry, 3: 2, 173 — 196
To link to this Article: DOI: 10.1080/15321818208056994
URL: http://dx.doi.org/10.1080/15321818208056994

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JOURNAL OF IMMUNOASSAY, 3(2), 173-196 (1982)

DOUBLE ANTIBODY ENZYME IMMUNOASSAY FOR THE QUANTITATION OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (CYCLIC AMP) AND GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE (CYCLIC GMP) IN TISSUE AND PLASMA

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ABSTRACT

A sensitive double antibody enzyme immunoassay for the quantitation of cyclic AMP and cyclic GMP is presented. Specific antisera to each nucleotide were raised in rabbits by immunization with succinyl cyclic nucleotide-human serum albumin conjugates. For competitive reaction, antibodies were incubated with a mixture of succinyl cyclic nucleotide labelled with β-D-galactosidase and unlabelled succinylated standard or sample cyclic nucleotides. The antibody-bound enzyme-hapten was separated from free hapten by anti-rabbit IgG immobilized to a polystyrene ball. Activity of the enzyme on the solid phase was fluorometrically determined. The assay system made it possible to ascertain values as low as 5 fmole of cyclic AMP or cyclic GMP. Cyclic nucleotides in plasma could be accurately determined by this method without requiring a deproteinizing reagent as the first step of assay.

KEY WORDS: Enzyme immunoassay, Cyclic AMP, Cyclic GMP, β-D-galactosidase, Polystyrene ball

INTRODUCTION

Immunoassay systems using an enzyme label instead of an isotope have been increasingly employed for measuring the concent-

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rations of antigens, haptens and antibodies in biological fluids. Enzyme immunoassay takes full advantage of the specificity and sensitivity of antibodies while avoiding the use of radionuclides (1-4).

A previous report from our laboratory demonstrated that cyclic AMP in human plasma could be quantitatively determined by enzyme immunoassay (5) which was performed by the competitive binding method with a solid phase antibody. Subsequently we have tried to improve this assay system in terms of precision. In the present paper, we describe a sensitive and reliable enzyme immunoassay for the quantitation of both cyclic AMP and cyclic GMP, which is based upon the principles of competitive reaction and a solid phase double antibody method.

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By use of this immunoassay technique, the concentration of cyclic AMP and cyclic GMP has been determined in various tissues of rats and mice, and in human plasma. Prior separation and deproteinization of human plasma has not been necessary. The values obtained by this assay correlated well with those by radioimmunoassay.

MATERIALS AND METHODS

Materials

β-D-galactosidase (from *Escherichia coli*, Grade IV, 470 units /mg protein) (β-Gal), human serum albumin (crystallized and lyophilized) (HSA), bovine serum albumin (fraction V) (BSA) and 4-

methylumbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma Chemical Co. (St. Louis. Mo.); 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), from E. Merck AG. (Darmstadt); IgG fraction of anti-rabbit IgG (prepared in goat, lyophilized), from Miles Lab., Inc. (Elkhart, Ind.); Succinic anhydride and triethyl-amine, from Tokyo Kasei (Tokyo); Dioxane and acetone, from Ishizu Pharmaceutical Co., Ltd. (Osaka); Freund's complete and incomplete adjuvants, from Difco Lab. (Detroit, Mich.); ACS II scintillation cocktail, from RCC Amersham (London). 8-[^{3}H]-Cyclic AMP ammonium salt (20-30 Ci/mmole) and 8-[^{3}H]-cyclic GMP ammonium salt (10-30 Ci/mmole) were purchased from Radiochemical Centre, Amersham. Polystyrene balls (diameter, 1/4 inch) were obtained from Ichiko Co. Ltd. (Nagoya). All other chemicals and solvents from Commer-cial sources were of reagent grade quality.

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Preparation of Immunogens

To obtain the conjugate of cyclic nucleotide and HSA, 2'-*O*-succinyl cyclic nucleotide was synthesized, purified by chromato-graphy, and coupled to HSA through the carboxyl group using EDC essentially as described by Steiner et al. (6). Briefly, 50 mg of cyclic nucleotide (Na salt) was dissolved in 2.5 ml of re-distilled water followed by addition of 5 ml of dioxane-triethyl-amine mixture containing succinic anhydride (which was prepared by mixing 9 ml of dioxane containing 400 mg of succinic anhydride with 1 ml of triethylamine). After 10 min at room temperature, unreacted succinic anhydride was hydrolyzed by addition of 40 ml of redistilled water, and the reaction mixture was concentrated to 3 ml by rotary evaporator under reduced pressure. Succinyl cyclic

nucleotide was purified by paper chromatography with butanol-glacial acetic acid-water (12:3:5, v/v). Succinyl cyclic nucleotide migrated ahead of cyclic nucleotide. Ten mg of succinyl cyclic nucleotide was dissolved in 2 ml of redistilled water followed by addition of 20 mg of HSA and 10 mg of EDC, the pH being adjusted to 5.5 after each addition. The reaction mixture was incubated in the dark at room temperature for 18 hr and then dialyzed against 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl at 4°C for 48 hr with frequent changes of the dialyzing solution. Spectrophotometric determination indicated a conjugate of about 10 succinyl cyclic nucleotide residues per HSA molecule.

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Immunization

Antibodies were produced in rabbits by repeated immunizations. Succinyl cyclic nucleotide-HSA conjugate was dissolved in sterile redistilled water and emulsified with an equal amount of complete Freund's adjuvant. An emulsion (0.25 ml) containing 0.5 mg of immunogen was injected intracutaneously on the animal's back. The rabbits were boosted once every 4 weeks for 2 months with 0.2 mg of immunogen in incomplete Freund's adjuvant, followed 11 or 15 weeks thereafter by 2-4 weeks intervals of booster injection with the same dose of antigen. Bleedings were taken from the carotid artery 7-10 days after the last injection. The separated sera were lyophilized and stored at -20°C.

Ligand Binding Affinity Test

In order to obtain high quality anti-succinyl cyclic nucleotide sera in terms of sensitivity and specificity, antisera were screened by a binding affinity test using ^{3}H-succinyl cyclic

nucleotide as ligand essentially as described by Honma et al. (7). One volume of ^{3}H-cyclic nucleotide (100 nM) was mixed with the same volume of the succinylation reagent which was prepared by mixing 9 ml of dioxane containing 400 mg of succinic anhydride with 1 ml of triethylamine. After 10 min at room temperature, the mixture was added to eight volumes of 50 mM sodium acetate buffer, pH 6.5 containing 0.1 % BSA. To 100 µl of this mixture was added 200 µl of antisera diluted 1 : 500 in the above buffer. After the mixture had stood at 4°C for 18 hr, 0.5 ml dextran coated charcoal suspension (0.075 % dextran, 0.5 % Norit extra and 0.5 % BSA) was added and the mixture cooled in an ice bath. The charcoal was spun down at 3000 rpm for 10 min, and 0.5 ml of the supernatant was mixed with 8 ml of ACS II scintillation cocktail and counted in a scintillation counter. A "Blank" was set up containing buffer instead of antiserum. Bound cpm/total cpm (B/T) (%) was calculated as $\frac{\text{cpm (bound)} - \text{cpm (blank)}}{\text{cpm (total)} - \text{cpm (blank)}} \times 100$. The B/T (%) is a function of the succinyl cyclic nucleotide titer in rabbit sera.

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Preparation of Hapten-Enzyme Conjugate

Succinyl cyclic nucleotide was conjugated with β-D-galactosidase using EDC as described by Yamamoto and Tsuji (5); 100 µl of β-Gal (0.5 mg protein, 10^{-9} mole) and 50 µl of 2 % EDC solution (1 mg) were mixed with 1 ml of 10 mM phosphate buffer, pH 6.3, containing 0.15 M NaCl, 1 mM $MgCl_2$ and 20 mM succinyl cyclic nucleotide. The mixture was incubated at 4°C for 3 hr in the dark. The mixture was dialyzed for 4 days against a total volume of 2 liters of phosphate buffer (as above) and used for enzyme immunoassay without purification. The succinyl cyclic nucleotide-β-Gal

conjugate thus obtained (ca. 1 ml volume) was stable for at least one year at -20°C in a mixture with an equal volume of glycerin. On the basis of the spectrum of succinyl cyclic nucleotide-β-Gal conjugate and unconjugated β-Gal and assuming molar absorbance coefficients of 14,650 (258 nm) and 12,950 (254 nm) for the cyclic AMP and cyclic GMP, respectively, the number of residues of succinyl cyclic nucleotide bound to β-Gal was estimated to be about 10 per molecule. The enzyme activity was not significantly reduced by conjugation of the enzyme with the hapten.

Preparation of Second Antibody-Bound Polystyrene Balls

Second antibody was immobilized on polystyrene balls by physical adsorption according to the following procedures: balls were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (50-fold diluted with 50 mM phosphate buffered saline (PBS), pH 7.5, containing 0.1 % NaN_3) at 4°C for 24 hr and washed in A_1 buffer (10 mM phosphate buffer, pH 6.8, containing 0.1 M NaCl, 1 mM $MgCl_2$ and 0.1 % BSA). They were then kept in the same buffer at 4°C for at least 18 hr until use.

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Preparation of Samples

Whole blood withdrawn from healthy persons was immediately mixed with a 1 % by volume of 0.5 M EDTA sodium salt (an isotonic neutral solution) and plasma was obtained by centrifugation at 2000 rpm for 10 min at 4°C. Various tissues of mice and rats were excised and rapidly frozen between stainless blocks which had been previously cooled under liquid nitrogen. Frozen tissues were weighed and ground to a fine powder under liquid nitrogen in a stainless mortar. The frozen powders were homogenized in 10-50

volumes of cold 6 % TCA in a glass-Teflon tissue grinder. The homogenates were clarified by centrifugation, and the resultant supernatants were extracted three times with 3 volumes of water-saturated ether to remove TCA. The remaining ether in samples were evaporated by immersing them in the hot water at 70°C for 10 min. These samples were kept at -20°C until assayed.

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Assay Procedure

Cyclic nucleotides in test samples were succinylated prior to assay as described by Yamamoto and Tsuji (5). Briefly, 100 µl of succinylating reagent (4 mg of succinic anhydride, 10 µl of triethylamine and 90 µl of acetone) was added to equal volumes of samples or standard solution (80 nM). After vortexing, the mixture was allowed to stand at room temperature for 10 min. The sample was treated with 800 µl of A_2 buffer (0.1 M phosphate buffer, pH 6.6, containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.1 % BSA and 0.5 % gelatin) to hydrolyze the untreated succinic anhydride, and was cooled to 0°C. The extent of succinylation of [^{3}H]-cyclic nucleotide added to the sample solution was checked by cellulose-TLC in butanol:glacial acetic acid:water (12:3:5), and was shown to be approximately 98 %. For the competitive reaction, assay tubes containing 100 µl of β-Gal-succinyl cyclic nucleotide (diluted 1:40000 for cyclic AMP and 1:10000 for cyclic GMP with A_2 buffer), 100 µl of antiserum (diluted with the same buffer) and 200 µl of the succinylated sample or standard solution (diluted with a mixture of water, succinyl reagent and A_2 buffer (1:1:8)) were incubated at 4°C for 18 hr. Antibody-bound β-Gal-succinyl cyclic nucleotide was bound to second antibody immobilized to a polystyrene

ball by rocking the ball in the mixture for 4 hr at room temperature. The ball was then washed with A_1 buffer and transferred to a new tube containing 200 µl of A_1 buffer. The activity of enzyme bound to the solid phase was determined by incubating the ball with 200 µl of 0.3 mM 4-MUG at 37°C for 1 hr, and terminating the reaction by addition of 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3. The amount of the 4-MU liberated was determined by fluorescence spectrophotometry with an excitation wave-length at 360 nm and emission wave-length at 450 nm.

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RESULTS

EIAs by the Solid Phase Method and Double Antibody Solid Phase Method

In EIA the double antibody solid phase method is used next to the solid phase procedure which was the assay system that we adopted in previous experiment (5). Then, we tried to compare these two methods in terms of precision. As is shown in Table 1, the coefficient of variation was lower in the double antibody solid phase method than in the solid phase method.

Standard Curve and Cross Reactivities for EIAs for cyclic AMP and cyclic GMP

Typical calibration curves for the enzyme immunoassays of cyclic AMP and cyclic GMP show a linear displacement of enzyme labelled hapten by unlabelled succinyl cyclic nucleotide, when plotted as a semilogarithmic function from 6.25 to 1600 fmol/tube (Figure 1).

TABLE 1

Comparison of Two Assay Methods in terms of Precision

Assay System	Coefficient of Variation[b] (C.V.) %	
	cAMP Assay	cGMP Assay
Solid Phase Method[a]	4.7	5.0
Double Antibody Solid Phase Method	3.5	3.9

[a]The procedure for the solid phase method is essentially the same as described previously. Briefly, a polystyrene ball physically bound with the anti-succinyl cyclic AMP or cyclic GMP serum was incubated in a test tube with 200 μl of zero-standard solution and the same volume of enzyme-succinyl cyclic AMP or cyclic GMP conjugate. After 24 hr incubation at 4°C, the reaction mixture was removed and the ball was washed. The fluorescence intensity of 4-MU liberated from 4-MUG as substrate by the enzyme bound to the ball was then determined as described in the section of Materials and Methods.

[b]Fluorescence intensity at zero-standard (n=20) were measured by the both methods and the variance of the measured value were indicated by coefficient of variation.

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This sensitivity allows the cyclic nucleotides to be measured in triplicate on 5-10 μl of human plasma. The sensitivity has been increased by several hundred fold by the preliminary succinylation of the samples as previously described by Cailla et al. for the RIA of cyclic AMP (8).

Specificity is shown in Figure 2. The most important competitors are cyclic GMP and cyclic AMP for the assays of cyclic AMP and cyclic GMP, respectively, but these cross-reactivities are negligible. Other nucleotides and nucleosides including cyclic CMP, cyclic UMP, ATP, GTP, ADP, GDP, adenosine, guanosine, adenine and guanine show even less reactivity to these assays.

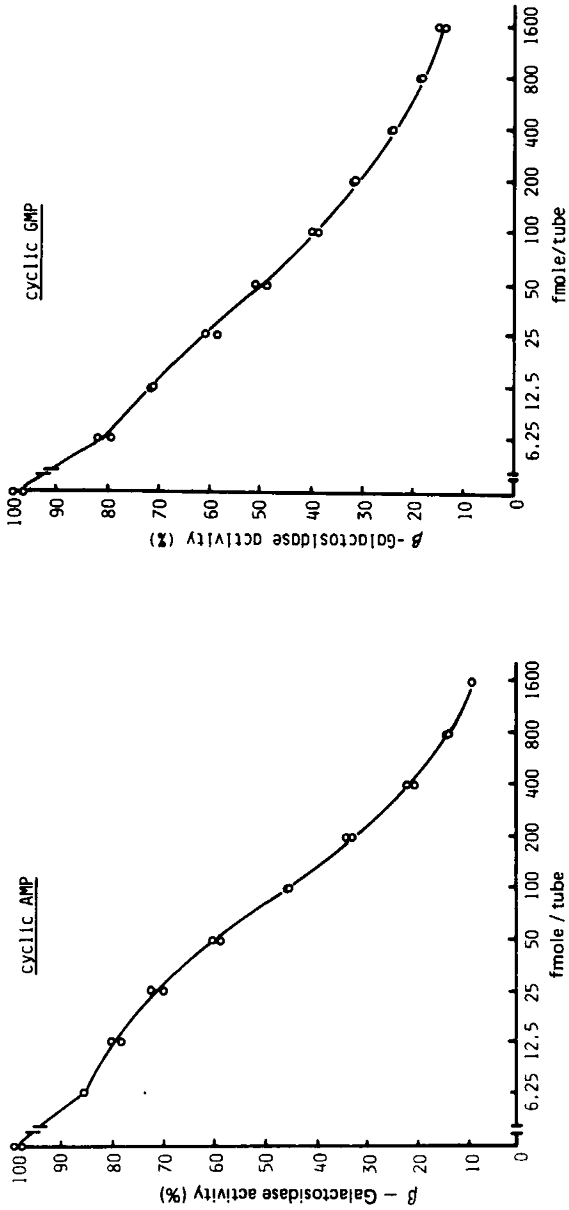

FIGURE 1. Calibration curves for cyclic AMP and cyclic GMP obtained by double antibody solid phase method.

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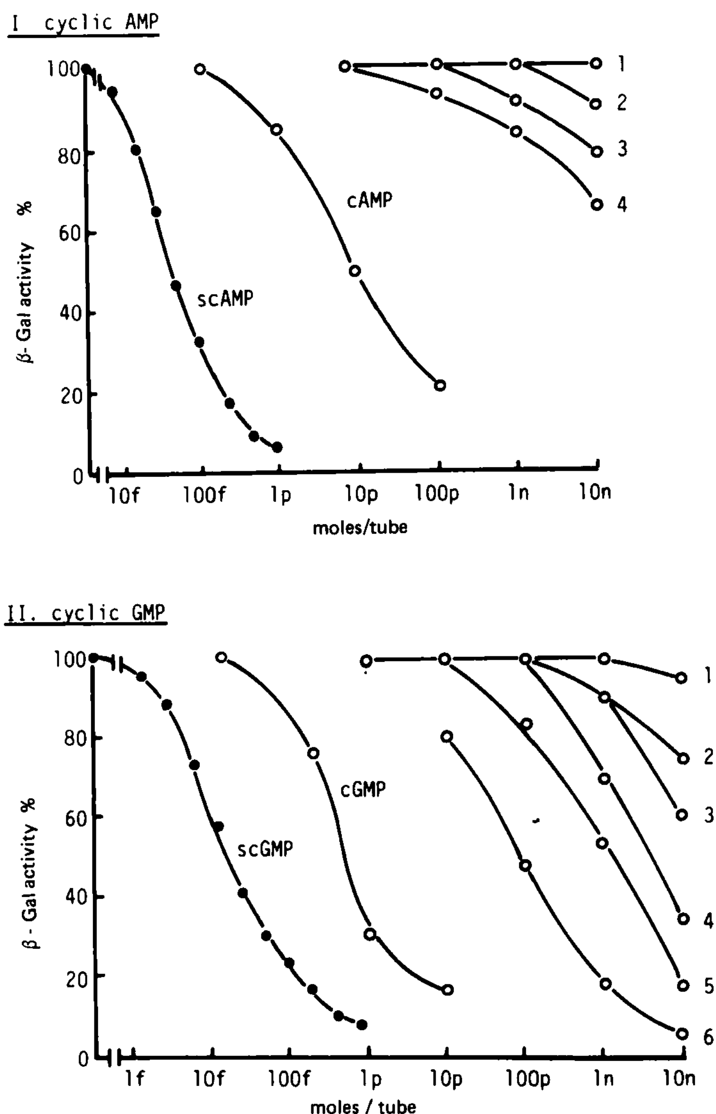

FIGURE 2. Specificity of anti-succinyl cyclic AMP and anti-succinyl cyclic GMP sera.

I. cyclic AMP
1: ATP, ADP, GTP, GDP, GMP, guanosine. 2: adenosine, adenine, guanine. 3: AMP. 4: 3',5'-cGMP.

II. cyclic GMP
1: AMP, 3',5'-cCMP, 3',5'-cUMP. 2: ATP, ADP, adenosine. 3: adenine, guanine. 4: guanosine, GMP. 5: GTP, GDP. 6: 3',5'-cAMP.

Choice of Buffer

As many authors described in their articles (7,9) about RIA for cyclic AMP and cyclic GMP, free succinate present in succinylated samples interferes with the subsequent binding reaction. To overcome this obstacle, M. Honma et al. (7) chose an imidazole buffer as a diluent for the succinylated samples in their RIA for cyclic nucleotides. In this experiment, we settled this problem by diluting the succinylated samples with a phosphate buffer five-fold. As is shown in Table 2, comparison of various kinds of buffers as a diluent revealed that a phosphate buffer reduced the succinate interference so effectively as to make the dilution of succinylated samples minimal in the present assay.

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Recovery and Dilution Tests

Plasma and TCA-extracts of tissues may contain substances which interfere with the binding reaction or the enzyme activity. Matrix problems were minimized by incubating the assay in A_2 buffer (0.1 M phosphate buffer, pH 6.8, containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.1 % BSA and 0.5 % gelatin) as shown in Table 2,3 and 4. Dilution experiments (Table 4) resulted in amounts of measured cyclic AMP and cyclic GMP which were directly proportional to those of the undiluted samples.

Recovery studies with mean recoveries of 103-109 % are shown in Table 2 and 3.

These results demonstrate that there are no inhibitory or interfering substances in the samples (TCA-extracts and plasma) and that the measured values may be taken as accurate representations of the true cyclic nucleotide concentrations in the samples.

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TABLE 2

Recovery of Cyclic AMP added to Human Plasma and Correlation between the Values obtained by Radioimmunoassay and Enzyme Immunoassay with different Buffer

Human Plasma		Phosphate Buffer[a]			Imidazole Buffer			Acetate Buffer		
			cAMP 50 pmole	Recovery (%)		cAMP 50 pmole	Recovery (%)		cAMP 50 pmole	Recovery (%)
1	(21.1)[b]	20.5[c]	73.0	105	21.0	91.0	140	28.0	107	158
2	(17.6)	16.9	67.5	101	17.1	83.5	133	28.0	97.5	139
3	(23.6)	25.5	70.0	89	19.7	105	171	30.4	98.0	135
4	(20.1)	23.0	79.0	112	21.5	97.5	152	28.5	108	159
5	(15.4)	14.7	81.0	133	16.3	70.5	108	22.5	97.5	150
6	(27.9)	33.9	82.0	96	29.0	91.0	124	34.5	112	155
7	(21.0)	21.1	78.5	115	22.0	80.0	116	34.5	103	137
8	(19.5)	14.7	75.5	122	22.3	80.5	116	20.3	116	191
9	(14.5)	18.5	63.5	90	18.4	82.0	127	22.4	97.5	150
10	(21.8)	23.5	74.5	102	22.0	77.0	110	24.2	111	174
11	(25.0)	25.9	76.5	101	25.0	86.0	122	30.0	112	164
12	(23.5)	24.0	68.0	88	26.0	95.0	138	28.7	100	143
13	(30.6)	35.0	79.0	88	31.5	88.5	114	41.0	126	170
14	(23.9)	24.2	76.0	104	33.9	91.0	114	40.5	105	129
Mean	(21.8)	23.0		103	23.3		128	29.5		154
Regression Equation (Y: EIA, X: RIA)		Y = 1.26X - 4.47			Y = 0.96X - 2.37			Y = 1.07X - 6.17		
Correlation Coefficient		0.92			0.81			0.76		

[a] A_2 buffer (0.1 M phosphate buffer, pH 6.8, containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.1 % BSA and 0.5 % gelatin)

[b] measured by RIA (YAMASA cyclic AMP assay kit)   [c] units of pmole per milliliter of plasma

TABLE 3

Recovery of Cyclic AMP and Cyclic GMP Added to Plasma and Tissues

I. Cyclic AMP

No.	Specimen		Intrinsic cAMP	Added cAMP	Measured Value	Recovery (%)
1	Human Plasma	(35.2)[a]	34.5[b]		85.5	102
2		(25.1)	26.0	50	89.5	127
3		(21.9)	22.5		78.5	112
4	Mouse Plasma	(142.0)	104		206	102
5		(97.5)	91.0	100	206	115
6		(52.1)	50.0		141	91
7	Rat Tissue Extract	(269.0)	250		375	125
8		(167.0)	139	100	245	106
9		(77.8)	68.5		167	99
	Mean					109

II. Cyclic GMP

No.	Specimen		Intrinsic cGMP	Added cGMP	Measured Value	Recovery (%)
1	Human Plasma	(4.7)[a]	4.1[b]		15.0	109
2		(7.6)	5.6	10	15.4	98
3		(5.4)	3.7		15.4	117
4	Mouse Plasma	(n.d.)	34.0		95.0	122
5		(n.d.)	31.0	50	80.0	98
6		(n.d.)	14.0		58.5	89
7	Rat Tissue Extract	(n.d.)	6.6		19.0	124
8		(n.d.)	1.9	10	12.0	101
9		(n.d.)	0.9		10.0	91
	Mean					105

[a] measured by RIA (YAMASA cyclic AMP or cyclic GMP assay kit)

[b] units of pmole per milliliter of plasma or tissue extract

The reaction was performed in 0.1 M sodium phosphate buffer, pH 6.8 containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.5 % gelatin and 0.1 % BSA and then applyed to the different assays.

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TABLE 4

Dilution Study

I. Cyclic AMP

Sample	Dilution[a]	Human Plasma		Mouse Plasma		Mouse Tissue Extract	
		fmole/tube as read from Standard Curve	cAMP pmole/ml of Plasma	fmole/tube as read from Standard Curve	cAMP pmole/ml of Plasma	fmole/tube as read from Standard Curve	cAMP pmole/ml of Extract
1	1:1	478	23.9	1270	63.5	550	27.5
	1:2	228	22.8	638	63.8	288	28.8
	1:5	90.0	22.5	237	59.3	122	30.5
	1:10	50.0	25.0	113	56.5	47.7	23.9
	1:20	23.5	23.5	56.0	56.0	34.3	34.3
2	1:1	308	15.4	1480	74.0	900	45.0
	1:2	154	15.4	620	62.0	470	47.0
	1:5	63.5	15.9	245	61.3	187	46.8
	1:10	33.8	16.9	128	64.0	119	59.5
	1:20	16.8	16.8	72.0	72.0	57.5	57.5

II. Cyclic GMP

Sample	Dilution[a]	Human Plasma		Mouse Plasma		Mouse Tissue Extract	
		fmole/tube as read from Standard Curve	cGMP pmole/ml of Plasma	fmole/tube as read from Standard Curve	cGMP pmole/ml of Plasma	fmole/tube as read from Standard Curve	cGMP pmole/ml of Extract
1	1:1	98.0	4.90	80.5	4.03	188	9.40
	1:2	47.0	4.70	n.d.[b]		99.0	9.90
	1:5	20.4	5.10	14.9	3.73	42.0	10.5
	1:10	11.0	5.50	8.00	4.00	19.5	9.75
2	1:1	96.0	4.80	388	19.4	60.0	3.00
	1:2	46.5	4.65	n.d.[b]		28.5	2.85
	1:5	15.1	3.78	70.0	17.5	11.2	2.80
	1:10	9.1	4.55	35.6	17.8	7.9	3.95

[a]Diluted with distilled water and succinylated by mixing with the succinic anhydride-triethylamine reagent.

[b]n.d. : not done

The reaction was performed in 0.1 M sodium phosphate buffer, pH 6.8 containing 0.1 % BSA, 0.5 % gelatin, 0.3 M NaCl and 1 mM $MgCl_2$.

TABLE 5

Intraassay Variance Study

I. Cyclic AMP

Sample	1	2	3	4
cyclic AMP assay (fmole/tube)	390	438	116	82.0
	406	440	102	87.0
	390	444	99.5	82.0
	430	448	104	81.5
	430	463	101	78.5
	445	467	106	81.0
	438	450	88.5	78.5
	521	455	93.5	99.9
	385	460	100	90.0
	465	405	108	74.5
Mean	430	447	102	83.5
C.V. (%)	9.7	3.9	7.4	8.6

II. Cyclic GMP

Sample	1'	2'	3'	4'	5'
cyclic GMP assay (fmole/tube)	980	530	107	91.0	40.0
	1060	540	110	89.9	35.7
	1070	578	109	93.0	42.0
	1010	520	113	83.0	36.0
	1050	560	118	88.0	36.5
	940	530	107	100	40.3
	1030	600	123	90.0	38.8
	900	530	100	96.0	39.0
	920	560	110	85.0	43.0
	920	578	124	86.0	40.3
Mean	988	553	112	90.2	39.3
C.V. (%)	6.5	4.8	6.7	5.7	6.3

Intra-assay coefficient of variation was determined by testing mouse tissue extract 10 times in an assay.

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TABLE 6

Interassay Variance Study

I. Cyclic AMP

Sample	1	2	3	4
cyclic AMP assay (fmole/tube)	400	241	142	61.0
	420	258	170	64.0
	452	252	176	70.0
	470	262	156	69.0
	419	247	173	71.0
Mean	432	252	163	67.0
C.V. (%)	6.5	3.3	8.7	6.4

II. Cyclic GMP

Sample	1'	2'	3'	4'	5'
cyclic GMP assay (fmole/tube)	358	208	53.0	50.0	14.3
	382	210	52.0	52.0	12.2
	355	191	56.0	52.0	11.0
	315	190	58.0	50.0	11.9
	342	216	56.0	54.5	13.2
Mean	350	203	55.0	51.7	12.5
C.V. (%)	7.0	5.8	4.5	3.6	10.1

Inter-assay coefficient of variation was evaluated by testing mouse tissue extract in duplicate each assay for 5 assays.

In the assay of plasma cyclic AMP and cyclic GMP, all the values corresponded closely with results by RIA.

Intra- and Inter-assay Coefficient of Variation for the EIAs

The intra-assay coefficient of variation with six samples of mouse tissue extract was 3.9-9.7 % for cyclic AMP, and 4.8-6.7 % for cyclic GMP (Table 5).

The inter-assay coefficient of variation with five samples of mouse tissue extract was 3.3-8.7 % for cyclic AMP, and 3.6-10.0 % for cyclic GMP (Table 6).

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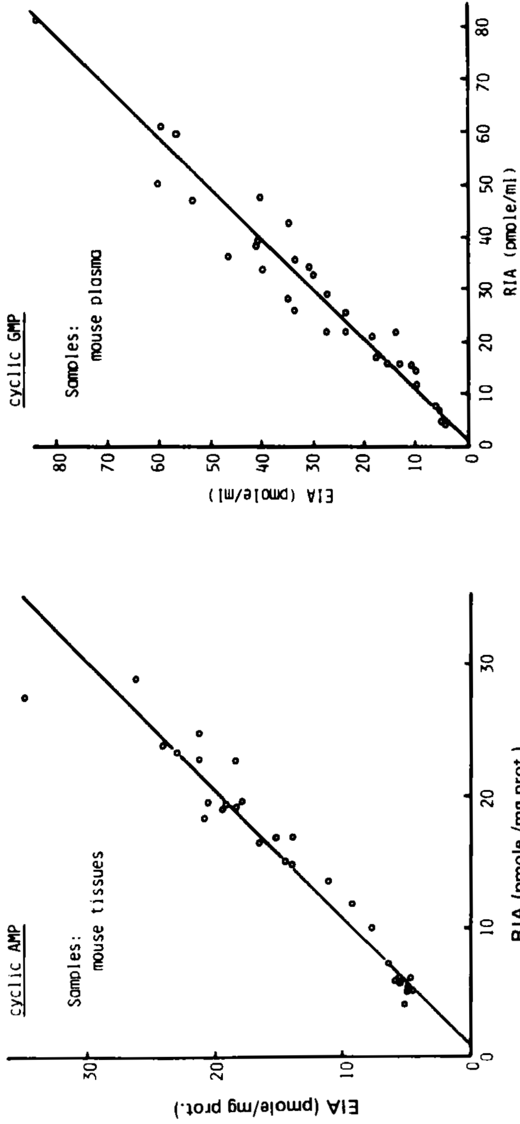

FIGURE 3. Correlation between cyclic nucleotide concentrations measured by the proposed EIA and RIA methods. The ddY strain mice (8 weeks old) were sacrificed and plasma and TCA-tissue extract were prepared as described in Methods. Samples were assayed by the proposed EIAs and commercially available RIA kits.

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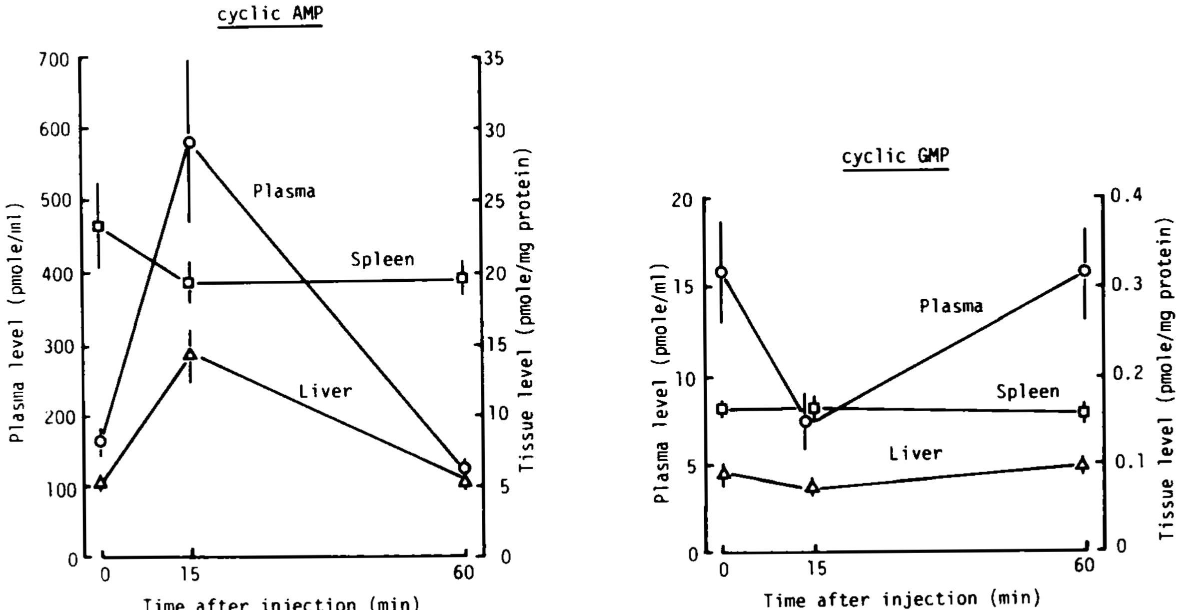

FIGURE 4. Changes in the content of cyclic AMP and cyclic GMP in the tissue and plasma in mice following glucagon administration. Male mice of ddY strain were sacrificed either 15 or 60 min after subcutaneous injection of glucagon at the dose of 10 µg/100 g body weight. Samples were prepared and cyclic nucleotides were assayed as described in Methods. Each point represents the average of duplicate determinations on 5 samples each group. The bars delineate standard errors.

Levels of Cyclic AMP and Cyclic GMP in Plasma and Tissues

The levels of cyclic AMP from various tissues of mice (brain, kidney, liver and spleen) and plasma levels of cyclic GMP in mice are shown in Figure 3. There was a good correlation between the values obtained by this EIA and by RIA kits (Yamasa Shoyu Co., Ltd.). (cyclic AMP: $Y=1.02X-0.97$, $r=0.96$, $n=30$) (cyclic GMP: $Y=1.05X-1.19$, $r=0.97$, $n=32$)

Increased levels of cyclic AMP in animals have been reported after glucagon administration (10,11). We found that the levels of cyclic AMP, although not of cyclic GMP, were increased in liver and plasma after subcutaneous injection of glucagon (Figure 4). The control levels of these nucleotides are in the same range as those previously reported by RIA (7,8,12).

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DISCUSSION

The levels of both cyclic AMP and cyclic GMP are extremely low; those of cyclic GMP are about one order of magnitude lower than those of cyclic AMP (7,8,12-14). A very sensitive assay is required for these nucleotides and a variety of assay procedures have been described to overcome experimental problems. These include enzymatic displacement (15-17), high-pressure liquid chromatography (18), protein kinase activation (19), luminescence (20), enzyme recycling (21), protein binding (22) and radioimmunoassay (6-8,12-14,23). Of these, radioimmunoassay is one of the most sensitive and has the practical advantages that all of the reagents are commercially available. However, radioimmunoassays

have certain practical limitations such as the relatively short-life of the isotope and the special regulations that surround transport and use of isotope.

Recent studies have indicated that enzyme immunoassay may be advantageous (1-5). This is the first description of enzyme immunoassays for cyclic AMP and for cyclic GMP using a double antibody solid phase separation. Their limit of detection is comparable to that of RIAs for cyclic AMP and cyclic GMP (6-8,12). Tissue and plasma concentrations of cyclic AMP and cyclic GMP are reported using these enzyme immunoassays.

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Development of these highly sensitive immunoassays was attributable to (a) obtaining an anti-succinyl cyclic nucleotide serum with excellent specificity and affinity, (b) the use as a label of β-D-galactosidase which is very stable and has a large turnover number, (c) utilization of 4-MUG, a fluorescent substrate, for sensitive analysis, (d) employment of a double antibody solid phase using polystyrene balls, (e) elimination of interferences with the binding reaction or enzyme activity by use of an incubation buffer containing BSA, gelatin and NaCl and (f) using succinylation of samples prior to assay.

Application of this method to cyclic CMP has been accomplished and will be published (24).

ACKNOWLEDGEMENTS

This work was supported in part by Sanyo Hoso Grant (1980), Okayama. The skilled technical assistance of Miss Mariko Owaki is gratefully acknowledged.

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The abbreviations used are: A_1 buffer, 0.01 M phosphate buffer, pH 6.8, containing 0.1 % BSA, 0.1 M NaCl and 1 mM $MgCl_2$; A_2 buffer, 0.1 M phosphate buffer, pH 6.6, supplemented with 0.3 M NaCl, 1 mM $MgCl_2$, 0.1 % BSA and 0.5 % gelatin; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; B/F, bound/free; BSA, bovine serum albumin; cyclic AMP, adenosine 3',5'-cyclic monophosphate; cyclic CMP, cytidine 3',5'-cyclic monophosphate; cyclic GMP, guanosine 3',5'-cyclic monophosphate; cyclic UMP, uridine 3',5'-cyclic monophosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediamine tetraacetic acid; EIA, enzyme immunoassay; β-Gal, β-D-galactosidase; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HSA, human serum albumin; 4-MU, 4-methylumbelliferone; 4-MUG, 4-methylumbelliferyl-β-D-galactoside; PBS, phosphate buffered saline; RIA, radioimmunoassay; TCA, trichloroacetic acid.

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