A SIMPLE AND SPECIFIC LOCUST PROTEIN BINDING ASSAY FOR CYCLIC 3'5' GMP

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The 10,000 g x 15 min supernatant from homogenised whole fifth instar Schistocerca gregaria hoppers provides a binding protein which may be used for the measurement of cyclic GMP without further purification. Specificity is greater than that reported in previously published protein binding procedures. Urine levels may be measured directly and agree well with results obtained by radioimmunoassay.

The increasing interest in the role of cyclic GMP* as an alternative second messenger to cyclic AMP*[1,2] has led to a search for suitable methods for its measurement.

Radioimmunoassay is well established[3] but the production or purchase of antisera is a drawback. The bovine-adrenal receptor assay[4] has had a major impact on the measurement of cyclic AMP and a protein binding assay for cyclic GMP of similar simplicity and low cost is desirable. Published receptor procedures for the measurement of cyclic GMP[5,6,7,8] require purification of the binding protein and have not achieved a specificity comparable to that shown by cyclic AMP assays[4,9].

Cyclic GMP is used as the abbreviation for guanosine-3'5'-cyclic phosphate, Cyclic AMP as the abbreviation for adenosine-3'5'-cyclic phosphate.

We have developed an assay of high specificity for cyclic GMP using, as a source of binding protein, a supernatant fraction from whole desert locusts (Schistocerca gregaria) at the fifth instar stage.

Members of Sutherland's group reported[10] in 1969 that "Cricket" tissue was markedly different from the wide range of mammalian tissues tested in that it contained more than two nanomoles cyclic GMP per gram, with a two- to three-fold excess of cyclic GMP over cyclic AMP. These findings, which have recently been confirmed[8 11] led us first to investigate crickets and, later, locusts as a source of binding protein for cyclic GMP.

EXPERIMENTAL

Preparation of the binding protein

Fifth instar Schistocerca gregaria, each weighing 1.5-2 g, were rapidly frozen and ground to a powder in liquid nitrogen. The cold powder was extracted with 6.0 ml ice-cold homogenisation medium (250 mM-sucrose; 50 mM-Tris, pH 7.4; 25 mM-KCl; 15 mM-$MgCl_2$) per gram, using two thirty-second periods at maximum speed on a 'Polytron' blender (Northern Media Supply Limited) with cooling in an ice-bath. The homogenate was centrifuged at 10,000 g for 15 minutes, the supernatant decanted through nylon cloth (125 μ pore size) and respun for a further 15 minutes at 10,000 g. The resulting supernatant was again decanted through nylon cloth and 5.0 ml aliquots were frozen and stored at $-20^{0}C$. Aliquots were thawed immediately before use, the frozen preparation maintaining its binding properties for at least three months.

Assay procedure (at $4^{o}C$)

The incubation mixture comprised 100 μl assay buffer (50 mM-HEPES,

pH 7.4; 8 mM-theophylline; 6 mM-2-mercaptoethanol; 5 g/l bovine serum albumin (Armour)) in which is dissolved 200 pmol cyclic AMP; 100 µl of binding protein (3.5-4.0 mg protein); and 100 µl of test or standard (in the range 0-50 pmol cyclic GMP) made up in the assay buffer.

One picomole of [8-^{3}H] cyclic GMP (Radiochemical Centre; 19 Ci/mmol), in 50 µl of assay buffer, was added after a thirty minute pre-incubation at 4°C and the incubation continued for a further two and a half hours.

Bound cyclic GMP was separated by the addition of 1.0 ml of ice-cold 3.8 M-ammonium sulphate solution. The tubes were mixed in a 'Whirlimixer', kept at 4°C for ten minutes, then centrifuged for thirty minutes at 1,800 g (MSE 'Mistral 4L' refrigerated centrifuge).

The supernatant was aspirated; the precipitate taken up in 1.0 ml distilled water and mixed. An aliquot (0.5 ml) from each tube was transferred to a 'Minivial' (G.D. Searle and Company), four ml of toluene - 'Synperonic-NXP' scintillant[12] added and the tritium content measured with a counting efficiency of 35%.

RESULTS AND DISCUSSION

Preliminary experiments, in which cyclic AMP was not included in the assay buffer, gave cyclic GMP concentrations in urine which were two to three times higher than those obtained by radioimmunoassay using antisera raised in this laboratory. The addition of a known amount of cyclic GMP to urine samples gave recovery values with the locust assay in the range 150-300% in comparison with standards in assay buffer. The setting-up of standards in phosphodiesterase pre-treated urine did not alter these recovery values. Addition of cyclic AMP to the cyclic GMP standards increased the sensitivity of the assay. The effect was

TABLE I

Cyclic GMP levels in urine

Nine random urine samples were diluted 1 in 20 and analysed in duplicate by the locust protein binding method and by radioimmunoassay with two different rabbit anti-cyclic GMP antisera of high specificity. The radioimmunoassay is based on the method of Steiner et al (1972).

SAMPLE	LOCUST ASSAY		RADIOIMMUNOASSAY			
			ANTISERUM 1		ANTISERUM 2	
	Cyclic GMP		Cyclic GMP			
	pmol/ml	µmol/g creatinine	pmol/ml	µmol/g creatinine	pmol/ml	µmol/g creatinine
1	240	1.18	200	0.99	240	1.18
2	400	1.22	420	1.28	420	1.28
3	560	0.92	600	0.98	540	0.89
4	320	1.14	300	1.07	340	1.21
5	90	0.50	90	0.50	100	0.56
6	180	0.64	200	0.71	160	0.57
7	680	1.19	700	1.23	640	1.12
8	860	1.26	860	1.26	1000	1.47
9	400	0.80	360	0.72	380	0.76

detectable with the addition of as little as 5 pmol cyclic AMP per tube, and was maximal at a level of 100-200 pmol/tube. Thus, cyclic AMP affects the binding of cyclic GMP and the addition of 200 pmol cyclic AMP per tube to both standards and tests gave results which agreed well, in the urine samples investigated, with those determined by radioimmunoassay (Table 1). Moreover, recovery of cyclic GMP added to ten urine specimens now averaged 98%. Measurements of cyclic GMP were independent of urine dilution in the range 1:2 to 1:32.

The detection limit of the locust assay was calculated[13] to be 0.2-0.3 pmol cyclic GMP per tube. Preincubation of urine samples with excess ox-heart phosphodiesterase at pH 7.4 reduced the cyclic GMP content to below the detection limit.

Cross reactivities of fifteen nucleotides in the assay are illustrated in Figure 1, similar results being obtained with three different binding protein preparations. Cyclic IMP cross-reacted with less than one fiftieth the avidity of cyclic GMP and cyclic AMP with less than one-thousandth. Other cyclic nucleotides cross-reacted to the same extent or less than cyclic AMP. Specificity is, therefore, considerably higher than that of existing protein binding methods for cyclic GMP.

Scatchard plots of the binding data revealed smooth curves which could be interpreted in terms of two populations of binding sites with dissociation constants of the order $K_{D1} = 1.5\text{-}2.5 \times 10^{-9}M$ and $K_{D2} = 2.4\text{-}5.2 \times 10^{-8}M$.

The use of EDTA as an anticoagulant and phsphodiesterase inhibitor in plasma cyclic AMP studies[14] led us to investigate the effect of replacing theophylline by this compound in the assay buffer. This resulted in a 20-30% increase in zero standard binding but also markedly

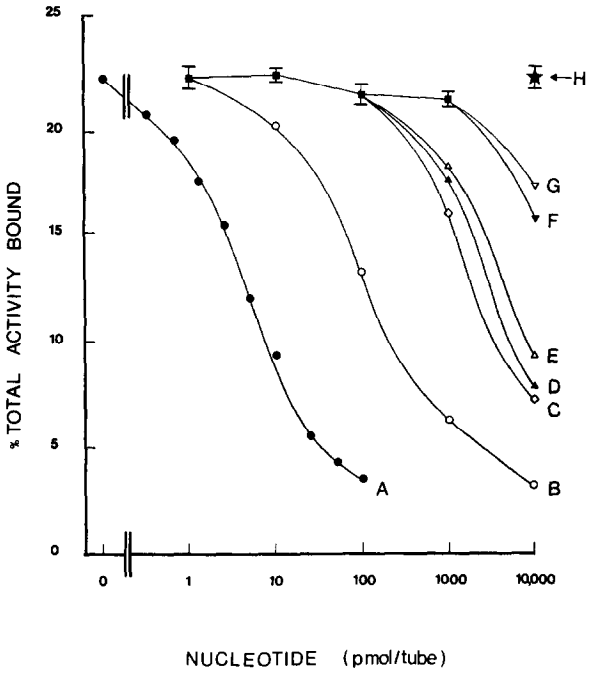

FIGURE 1

Specificity of the Method

The cross reactivities of fifteen nucleotides were determined using the incubation procedure described in the test.

A:- 3'5' cyclic GMP; B:- 3'5' cyclic IMP; C:- 3'5' cyclic CMP; D:- dibutyryl 3'5' cyclic GMP; E:- 3'5' cyclic AMP; F:- 3'5' cyclic UMP; G:- dibutyryl 3'5' cyclic AMP; H:- Mean result (± SEM) for 3'5' cyclic-dTMP, 5' AMP, 5' ATP, 5' GTP, 2'3' cyclic AMP, 2'3' cyclic GMP, AMP-PNP, GMP-PNP, and monobutyryl 3'5' cyclic UMP.

(★) and (■) show the means (±SEM) of results for nucleotides which are not plotted individually.

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increased the cross-reactivity of cyclic AMP. In contrast to this, the use of isobutylmethyl xanthine (8mM) in the assay buffer gave binding and specificity results in good agreement with those found for theophylline.

Binding protein extracts from Schistocerca gregaria showed distinct differences in specificity between the adult and juvenile stages. Tissue preparations from adult males (either whole insects or isolated abdomens) showed much greater cyclic AMP cross-reactivity than fourth or fifth instar hoppers. Further investigation of this phenomenon may prove of interest in studies on insect endocrinology.

Preliminary investigations with fifth instar Locusta migratoria migratorioides indicated that this species will also be suitable as a source of cyclic GMP binding protein.

The locust method combines the advantages of a simple procedure for the preparation of binding protein with high specificity for cyclic GMP. Urine may be analysed without preliminary purification, and it may be possible to carry out measurements on plasma and tissue extracts without prior nucleotide fractionation.

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