A Simple and Sensitive Saturation Assay Method for the Measurement of Adenosine 3':5'-Cyclic Monophosphate

By B. L. BROWN, J. D. M. ALBANO, R. P. EKINS and A. M. SGHERZI

Institute of Nuclear Medicine, The Middlesex Hospital Medical School, London W1N 7RL, U.K.

and W. TAMPION

Department of Biochemistry, Royal Free Hospital Medical School, London W.C.1, U.K.

(Received 13 November 1970)

The role of cyclic AMP (adenosine 3':5'-cyclic monophosphate) in cellular metabolism is a subject of intense interest in the life sciences despite the great difficulties associated with the assay of the nucleotide. A report of a binding protein with a very high specificity for cyclic AMP in adrenal glands (Gill & Garren, 1969) led us to consider the feasibility of a 'saturation assay' for this nucleotide (Brown & Ekins, 1970). We have confirmed the possibility of utilizing this analytical principle in the development of a sensitive and simple method (Brown, Ekins & Tampion, 1970).

Bovine adrenals, collected as soon as possible after slaughter, were transported on ice to a cold-room at 4°C. The cortices were separated, chopped and homogenized in a mixer-emulsifier (Silverson Machines Ltd., London S.E.1, U.K.) with 1.5 vol. of an ice-cold medium comprising 0.25 M-sucrose, 50 mM-tris-HCl buffer, pH 7.4, 25 mM-potassium chloride and 5 mM-magnesium chloride. The supernatant, after centrifugation of the homogenate at 2000g for 5 min, was respun at 5000g for 15 min. The resultant supernatant was stored in 0.5 ml or 1.0 ml portions at -20°C; negligible loss of binding activity has been found after 3 months. This preparation was thawed and diluted as required with 50 mM-tris-HCl buffer, pH 7.4 (containing 8 mM-theophylline and 6 mM-2-mercaptoethanol). This buffer was used for all subsequent procedures.

Each assay tube contained 50 µl of either a known amount (0-15 pmol) of cyclic AMP standard [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] or of an unknown sample, 50 µl of tritiated cyclic AMP (9.1 mCi/mg, approx. 8 nCi; The Radiochemical Centre, Amersham, Bucks., U.K.) or buffer, 100 µl of an appropriate dilution of the binding reagent and buffer to a final volume of 350 µl. A portion (50 µl) was taken into 4 ml of scintillator and its radioactivity counted to give an estimate of the total radioactivity in the tube. Incubation tubes were placed in the cold-room, and after 1.5 h 100 µl of a suspension of 10 mg of charcoal (Norit GSX; Norit Clydesdale, Glasgow, U.K.) in buffer containing 2% (w/v) of bovine serum albumin was added to each and the tubes were briefly agitated. After centrifugation (1200g for 15 min at 4°C) a

100 µl portion of the supernatant (i.e. the bound fraction) was taken for counting of radioactivity. Calibration curves were plotted in terms of the percentage radioactivity bound against amount (or concentration) or standard nucleotide (Fig. 1). Amounts of cyclic AMP in unknown samples were determined by reference to the appropriate calibration curve.

Optimum concentrations of tracer and binding reagent were chosen in accordance with the theoretical precepts of Ekins & Newman (1970). Currently, for high sensitivity, we normally employ a final dilution of binding protein (as initially prepared) of 1:24.

Tissue samples were prepared by one of two methods.

(i) Freeze-clamped tissue was weighed, pulverized under liquid N₂ and then thoroughly mixed with 10 vol. of cold 0.5 M-perchloric acid in 25% (v/v) ethanol. After homogenization the supernatant was neutralized with potassium hydroxide and the precipitate removed by centrifugation. Portions of this supernatant were dried and taken up in buffer for assay.

(ii) Tissue samples were frozen in liquid N₂ immediately on collection. Pieces of frozen tissue (10-20 mg) were weighed and dropped unthawed into ice-cold glass homogenizers containing 0.6 ml of 6% (w/v) trichloroacetic acid to which had been added tritiated cyclic AMP tracer. The amount of tracer was so chosen that the assay incubation tube would contain 8-10 nCi. After homogenization and centrifugation, the supernatant was pipetted off and washed four times with 10 vol. of water-saturated diethyl ether. Residual ether was removed in a stream of N₂ and portions of the washed extract were dried *in vacuo* over phosphorus pentoxide. The dried residues were taken up in buffer for assay. An appropriate correction for extraction recovery (usually 70-90%) was made for each sample. When residues were subjected to paper chromatography (Carminatti & Passeron, 1966) and the chromatogram was subsequently scanned with a windowless gas-flow counter only one peak of ³H radioactivity was detected, the R_F of which corresponded to that of marker cyclic AMP.

Fig. 1. Response curves for cyclic AMP (●), cyclic IMP (▲), cyclic GMP (○), cyclic CMP (■) and cyclic UMP (△). It should be noted that this assay was set up under sub-optimum sensitivity conditions. Adenosine, AMP, ADP, ATP, GMP, thymidine 3':5'-cyclic monophosphate and adenosine 2':3'-cyclic monophosphate did not appear to cross-react in the system at concentrations up to 30 nmol/incubation tube.

The specificity characteristics of the binding reagent were investigated by incubating dilutions of a number of nucleotides and nucleosides [all from Sigma (London) Chemical Co. Ltd. with tritiated cyclic AMP and binding reagent under the normal conditions of assay.

Of the nucleotides examined, slight cross-reaction was observed with cyclic UMP (uridine 3':5'-cyclic monophosphate), cyclic IMP (inosine 3':5'-cyclic monophosphate), cyclic CMP (cytosine 3':5'-cyclic monophosphate) and cyclic GMP (guanosine 3':5'-cyclic monophosphate (Fig. 1).

Serial dilutions of liver extracts were assayed with the intention of distinguishing non-specific effects (Ekins & Newman, 1970). Though such data constitute poor evidence for assay specificity, we observed parallel dilution curves on semilogarithmic plots with respect to standard cyclic AMP preparations for all biological samples thus tested. Likewise standard curves in buffer and in corresponding reagent 'blank' mixtures were superimposable, indicating that both trichloroacetic acid extracts and perchloric acid extracts do not subsequently interfere significantly with the binding-protein reaction. Response curves derived from standards set up in rat liver extract were parallel to both buffer and reagent-blank curves, suggesting that incubation tubes differed only in the amount of endogenous cyclic AMP in the liver sample.

Liver extracts treated with charcoal to remove endogenous cyclic AMP and employed as 'standard diluent' yielded response curves that were superimposable on buffer and reagent-blank curves. Material that was assayed as endogenous nucleotide was destroyed in the same way as was standard cyclic AMP by ox heart cyclic phosphodiesterase [Boehringer Corp. (London) Ltd., London W.5, U.K.].

To check the reproducibility of tissue cyclic AMP determinations rat liver was chosen for reasons of convenience. With the perchloric acid extraction procedure (i) on eight individual rat livers the mean was 0.79 pmol/mg wet wt. (range 0.57–1.01). With extraction procedure (ii) on eight samples from the same liver separately weighed, homogenized, processed and assayed the coefficient of variation was 8.7%; in four samples of this liver individually homogenized in trichloroacetic acid containing both tracer and known amounts of unlabelled cyclic AMP (3–7 pmol/sample) the recovery of the latter was 96–110%.

The lower limit of detection for cyclic AMP in this laboratory is currently about 0.2 pmol/incubation tube with an incubation volume of 350 μ l. With incubation volumes of 50 μ l of the detection limit is lowered to approx. 0.03 pmol, though at the cost of prolonged counting times with the low-specific-radioactivity labelled cyclic AMP available to us during this study.

Assay methods fundamentally similar in principle to that described here relying on specific antibodies (Steiner, Kipnis, Utiger & Parker, 1969) or on a purified binding protein from bovine muscle (Gilman, 1970) have been reported. However, these assay methods, although possessing essentially similar sensitivity and specificity characteristics, appear to be technically more demanding.

Note added in proof. Since submission of this paper, Walton & Garren (1970) have reported an assay method using a more highly purified adrenal protein as specific binding protein. Their technique appears to be considerably less sensitive than that described here, and possesses no compensating advantages.

The financial assistance of the Medical Research Council is gratefully acknowledged. We also thank Dr P. McLean and Dr K. Gumaa of the Courtauld Institute, The Middlesex Hospital, for their valuable assistance and expertise in preparing some of the tissue extracts.

Brown, B. L. & Ekins, R. P. (1970). *Clin. Sci.* **38**, 27 p.

Brown, B. L., Ekins, R. P. & Tampion, W. (1970). *Biochem. J.* **120**, 8 p.

Carminatti, H. & Passeron, S. (1966). In *Methods in Enzymology*, vol. 8, p. 108. Ed. by Neufeld, E. F. & Ginsburg, V. G. New York: Academic Press Inc.

Ekins, R. P. & Newman, B. (1970). *Acta endocr., Copenh.*, **64**, Suppl. no. 147, p. 11.

Gill, G. N. & Garren, L. D. (1969). *Proc. natn. Acad. Sci. U.S.A.* **63**, 512.

Gilman, A. G. (1970). *Proc. natn. Acad. Sci. U.S.A.* **67**, 305.

Steiner, A. L., Kipnis, D. M., Utiger, R. & Parker, C. (1969). *Proc. natn. Acad. Sci. U.S.A.* **64**, 367.

Walton, G. M. & Garren, L. D. (1970). *Biochemistry, Easton*, **9**, 4225.