REVISTA ESPAÑOLA DE FISIOLOGIA, 29, 233-238. 1973

Protein Binding of 3′, 5′-Adenosine Cyclic Monophosphate Acid

J. M. Mato and M. Serrano-Ríos

Instituto de Investigaciones Médicas
Fundación Jiménez Díaz
Madrid-3 (Spain)

(Received on June 18, 1973)

J. M. MATO and M. SERRANO-RIOS. *Protein Binding of 3′, 5′-Adenosine Cyclic Monophosphate Acid.* Rev. esp. Fisiol., 29, 233-238. 1973.

It is described the adaptation of a protein binding method for cAMP determination using charcoal for separation of bound/free nucleotide. The effect of cyclic receptor protein (CRP) dilution, concentration of charcoal, incubation time and crossreactivities with different nucleotides were studied. It was found a high precision in measurements of replicates of identical samples with a lower detection limit of 0.12 pmol/tube and excellent yield in recovery studies (100 ± 6.9 SD). Also the specificity was rather high as proved by only moderate displacement of H^3-cAMP-CRP binding by dybutiryl cAMP and cGMP at very high concentrations of these nucleotides. The results in normal urine (1.950 ± 1.106 SD nmol/ml) and plasma (11.2 ± 4.2 SD pmol/ml) samples are in agreement with most of the reported in the literature.

In recent years the assay of 3′, 5′-cyclic adenosine monophosphate acid (cAMP) and related nucleotides in biological fluids and tissues has become available by protein-binding (3, 5, 6, 10) and radioimmunoassay methods (9).

The purpose of this paper is to present the experience with a protein-binding technique adapted from that of BROWN *et al.* (3).

Materials and Methods

cAMP receptor protein (CRP) was obtained from adrenal glands supplied by a local abbatoir. Charcoal (Norit A), cAMP, cCMP, cGMP, cIMP, cUMP, dybutiryl cAMP, GMP, AMP and ATP were purchased from Sigma Ltd., Co. H^3-cAMP (specific activity 27.5×10^3 mCi/mmol), PPO and POPOP were obtained from Amershan Radiochemical. Naphtalene and Methylglicol were obtained from Scharlau, and Tris, Sucrose, potasium chloride and magnesium chloride from Merck.

Cyclic Receptor Protein Extraction. — Fresh adrenal glands (200-250 g) obtained from young calves, were immediately transferred in ice to the Laboratory. The glands were carefully stripped off fat and conective tissue, weighed and homogenized in a medium containing: 0.25 M sucrose,

50 mM tris-HCl buffer, *p*H: 7.4, 25 mM potassium chloride and 5 mM magnesium chloride, in an ice bath, using a «Virtix Mixer» for 2-4 minutes. The homogenate suspended in the medium, was centrifuged twice at 4° C for 20 minutes at 5,000 rpm in a refrigerated cetrifuge. The supernatant was collected, conveniently aliquoted and stored undiluted at —20° C until use.

Binding Activity of the CRP. — CRP was conveniently diluted with 50 mM tris-HCl buffer, *p*H: 7.4 (containing 8 mM theophylline and 6 mM 2-mercaptoethanol). 100 μl of this dilution was incubated with 50 μl of H^3-cAMP (at a concentration of 120 pg/tube), 50 μl of «cold» cAMP (concentration ranging from 0.05 to 50 pmol/tube) or of an unknow sample and tris-HCl buffer, to a final volume of 300 μl.

Proper blank (in which H^3-cAMP was substituted for buffer) and controls without added (CRP) were used on identical conditions. After 90 minutes at 4° C 100 μl of charcoal (10 %) in tris-HCl solution containing 2.0 % of bovine serum albumin was added and immediately centrifuged at 3,000 rpm for 20 minutes at 4° C. The supernatant was pippetted off (200 μl), into vials containing 10 ml of the scintillation mixture (composition: 700 ml of toluene, 300 ml of methylglicol, 80 g of naphtalene, 6.0 g of PPO and 0.2 g of POPOP). The vials were counted in a Nuclear Chicago Scintillation Counter with an efficiency around 30-35 %. The d.p.m. were plotted as % bound of the total standard vs. «cold» cAMP concentration.

Samples Preparation. — Urine samples were diluted (1:40) in tris-HCl buffer and used directly in the assay.

Plasma samples (1 ml) were extracted in absolute ethanol (2 ml), centrifuged (10 minutes, 3,000 rpm) and the supernatant pipetted to another tube. The precipitate was washed with 1.0 ml of ethanol (33 %) and centrifuged. After this second centrifugation the precipitate was discarded and the corresponding supernatant pooled with the first one, dessicated at 60° C under a nitrogen stream. The residue dissolved in 0.5 ml of tris-HCl buffer was centrifuged and the supernatant used in the assay.

Results

Separation: Concentration of Charcoal. In figure 1, lower panel, is presented the effect of different concentrations of charcoal (2.5-40 %). It seems, that from 10 to 40 % charcoal concentrations the control

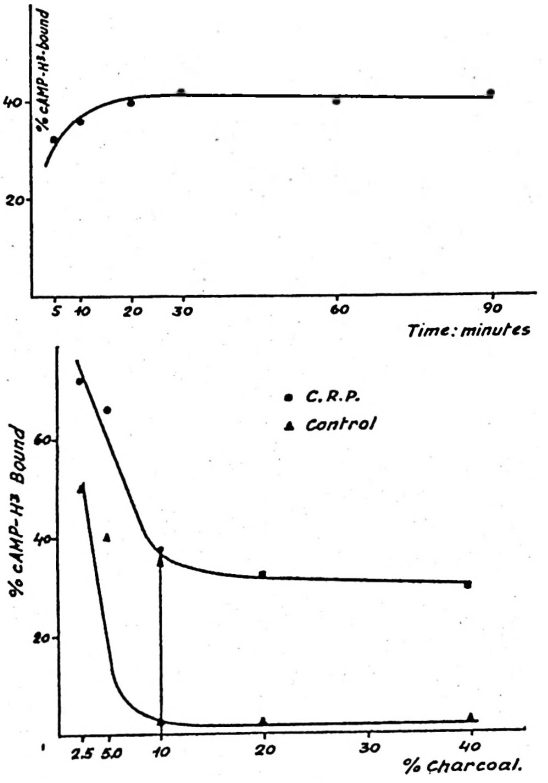

Fig. 1. *Binding properties of cAMP-CRP. I. Lower panel.* Effect of different charcoal concentrations. Optimal concentration indicated by arrow. *Upper panel.* Effect of different incubation times on the equilibrium of the CRP-H^3-cAMP reaction.

binding was negligible (2.5 %), but the maximum of H^3-cAMP binding was 10 % charcoal. Lower charcoal concentration (2.5-5.0 %) gave higher control binding (27.5-52.5 %) interferring with the sensitivity of the method.

Incubation Time. — In figure 1, upper panel, is described the effect of incubation time on % bound H^3-cAMP (240 pg/tube). The equilibrium was reached by 30 minutes (% bound = 42.5) and remained unaltered up to 90 minutes. Shorter incubation times (5, 10 and 20 minutes) gave 32.5, 35.0 and 40 % bound respectively. The effect of incubation time when are in competition 120 pg/tube of H^3-cAMP and «cold» cAMP (concentration ranging from 0.05 to 50 pm/tube) is presented in figure 2, (right panel). As in the previous figure, the equilibrium was reached at 30 minutes and remained unaltered up to 90 minutes. With shorter incubation times (5 and 10 minutes) is apparent a significant decrease in binding.

Cyclic Receptor Protein Dilution. — In figure 2, left panel, are presented standard curves at different dilutions of CRP extract. The H^3-cAMP (120 pg/tube) and «cold» cAMP (0.05 to 50 pm/tube) concentrations remained unaltered at the three CRP dilutions (1:4, 1:8 and 1:14). The CRP concentrations for this binding ranged from one extract to another between 1 to 2 g %. The protein keeps intact its binding activity after 3 months at —20° C.

Specificity. — Figure 3, lower panel, represent the crossreactivity between H^3-cAMP (120 pg/tube) and different nucleotides in concentrations ranging from 10^{-11} to 5×10^{-7} mol/tube. No crossreactivity existed with ATP and GMP. Displace-

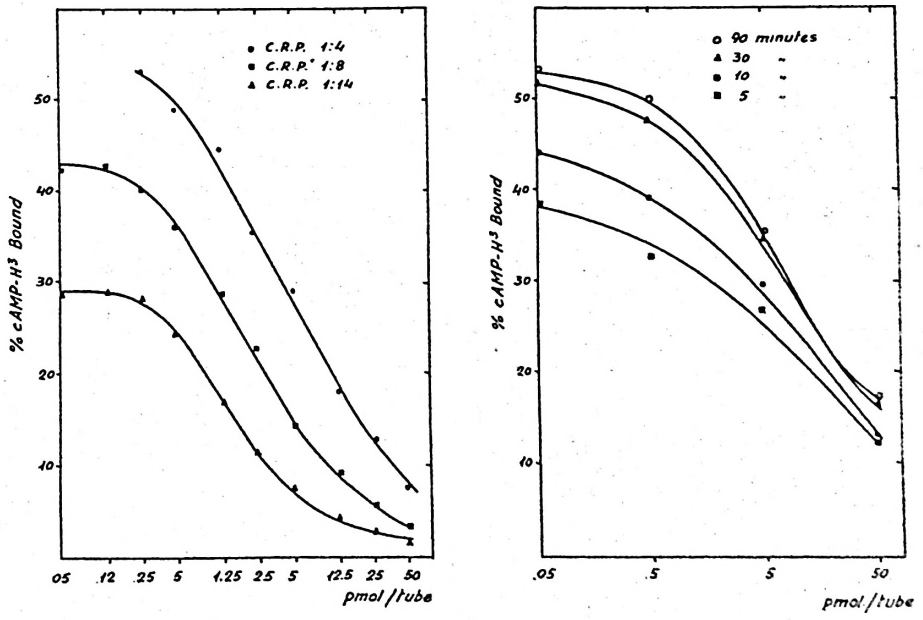

Fig. 2. *Binding properties of cAMP-CRP. II.*
Lower panel. Effect of different incubation times on the equilibrium of CRP with H^3-cAMP and cAMP. *Upper panel.* Effect of different dilutions of CRP on binding of H^3-cAMP versus increasing concentrations of «cold» cAMP.

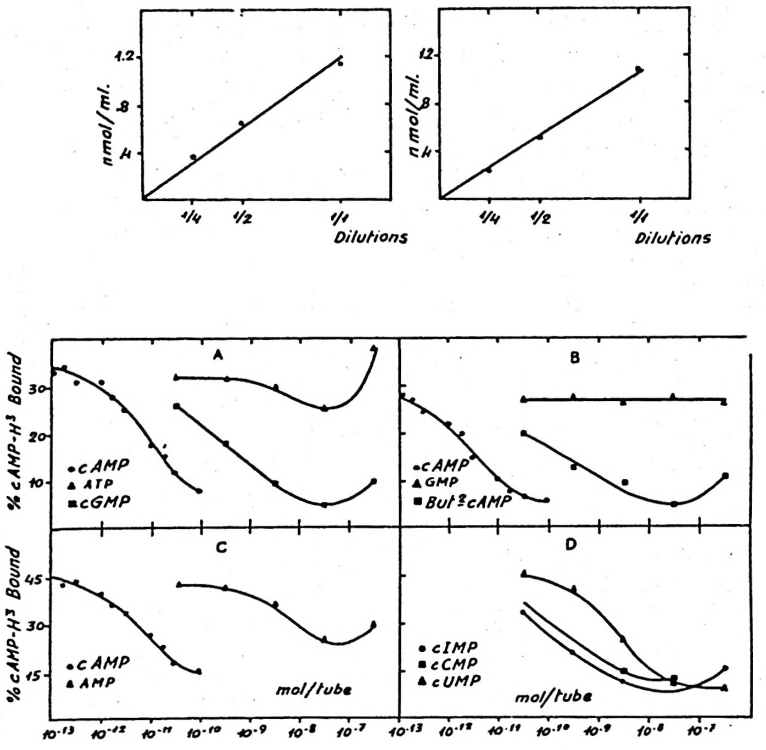

Fig. 3. *Specificity of cAMP-CRP.*
Lower panel. Crossreactivities of H^3-cAMP with different nucleotides. *Upper panel.* Effect of dilution on urine samples of two different normal subjects.

Table I. *Normal values of cAMP levels on plasma and urine samples.*

AUTHORS	METHOD	PLASMA pmol/ml	URINE
BROADUS et al. (2)	Enzymatic	10 - 20	—
TSANG et al. (10)	Protein-binding (Resin)	21.8 ± 8.8 n * = 17	2.9 ± 1.1 μmol/g creat. n = 21
STEINER et al. (9)	Radioimmunoassay	8 - 20	4.41 ± 0.39 μmol/24 h. n = 15
BUTCHER et al. (4)	Enzymatic	—	1.0 nmol/ml.
GILMAN (6)	Protein-binding (Millipore)	—	1.76 nmol/ml. n = 4
BROWN et al. (3)	Protein-binding (Charcoal)	10 - 20	1.08 ± 5.26 nmol/ml. n = 4
Present authors	Protein-binding (Charcoal)	11.2 ± 4.2 n = 13	1.950 ± 1.106 nmol/ml. n = 11

* n = number of individual sample.

ment of H^3-cAMP was observed with cIMP, cCMP, cUMP and AMP (at concentrations thousandfold that of cAMP); dybutiryl cAMP and cGMP exerted a moderate crossreactivity at concentrations hundred times that of cAMP. Figure 3, upper panel, shows the dilution effect on two different urines samples of 2 normal subjects.

Precision and Sensitivity. — Precision of measurements performed on 10 identical samples within the same assay were as follows: ± 0.02 pmol at the level of 0.5 pmol/tube, ± 0.25 pmol at the level of 5.0 pmol/tube and 2.8 pmol at the level of 50.0 pmol/tube. The lower detection limit was 0.12 pmol/tube.

Recovery and Normal Values.—Added cAMP was recovered from plasma and urine samples with an efficiency of 100 ± 6.9 SD ($n = 8$). The results in normal plasma (11.2 ± 4.2 SD pmol/ml; $n = 13$) and urine (1.950 ± 1.106 SD nmol/ml; $n = 11$) are in agreement with most of those reported in the literature (Table I).

Discussion

Many methods for assay of cAMP levels in biological fluids and tissues have been described. Classical enzymatic procedures (1, 2, 4, 7) are time consuming and elaborated, precluding its systematic use for those purposes. More recently a radioimmunoassay (RIA) method has been described with a high degree of precision, sensitivity and specificity but involving great difficulties in raising antibodies to cAMP and labeling the chemically modified molecule of the nucleotide (9). On the other hand, GILMAN *et al.* (6) have described a technically simple and specific technique of protein-binding, exploiting the high affinity properties for cAMP of a protein obtained from muscle (6, 8, 11). In this original method, Millipore filter was used at the final stage of the assay to separate «free» from «protein-bound» cAMP. Lately, modifications of the GILMAN's technique using charcoal or resin as separation method have been introduced (3, 10) using adrenal glands as CRP source (3, 5, 10). It seems apparent by comparing the results of these authors that no substantial differences in the optimal conditions for cAMP assay arise when using different separation methods. The present results are, in overall, in agreement with those reported by BROWN *et al.* (3). Minor differences with the results reported by TSANG *et al.* (10) using resin and by GILMAN *et al.* (6) with Millipore were observed. GILMAN (6) gave a sensitivity of 0.05-0.10 pmol/tube, while it was of 0.2 pmol/tube in Brown's method and 0.12 pmol/tube in the present work. Recovery Brown's values were 96-110 %, highly comparable with the present results (100 ± 6.9 SD) and better than those of Tsang (96 ± 8.5 SE, $n = 21$). Displacement of cAMP by other nucleotides was only apparent with dybutiryl cAMP and cGMP at essentially the same concentrations reported by BROWN *et al.* and TSANG *et al.* (3, 10). Also the displacement effected by other nucleotides was superimposible to the results of these authors and those of STEINER *et al.* using RIA (9). As shown in table I, the present results in normal urine samples are well in agreement with the range reported by several others authors (3, 4, 6). As for plasma cAMP concentrations we obtained similar results to most authors (2, 3, 9) but slightly lower than those of TSANG (10).

Conclusions

According to the present experience this method for cAMP assay is technically simple, sensitive and highly reproducible. As shown by crossreactivity experiments the method has also a high degree of specificity. In fact, the crossreactivities observed with dybutiryl cAMP and cGMP are at so high levels that will not significantly alter the measurement of cAMP in

tissues or in biological fluids. Finally, the results in normal urine and plasma samples are in agreement with most of those reported in the literature.

Resumen

Se describe la adaptación de un método de «receptores proteicos» para la determinación de AMPc utilizando «charcoal» para separar el nucleótido ligado del libre. Se ha estudiado el efecto de la dilución de la proteína receptora de AMPc (CRP), de la concentración de «charcoal», del tiempo de incubación y de la reactividad cruzada con otros nucleótidos diferentes. Se ha encontrado una alta precisión en la medida de muestras idénticas con una sensibilidad de 0,12 pmol/tubo y excelentes rendimientos en los estudios de recuperación (100 ± 6,9 SD). Del mismo modo, la especificidad es elevada como lo demuestra el hecho de encontrar un moderado desplazamiento de la unión H^3-AMPc-CRP por: dibutiril AMPc y GMPc a concentraciones muy altas de estos nucleótidos. Los resultados en muestras de orina (1.950 ± 1.106 SD nmol/ml) y plasma (11,2 ± 4,2 SD pmol/ml) de sujetos normales están de acuerdo con la mayoría de los dados en la bibliografía.

References

1. BALL, T. W. and SUTHERLAND, E. W.: *J. Biol. Chem.*, **232**, 1065, 1958.
2. BROADUS, A. E., KAMINSKY, N. I., HARDMAN, J. G., SUTHERLAND, E. W. and LITTLE, G. W.: *J. Clin. Invest.*, **49**, 222, 1970.
3. BROWN, B. L., ALBANO, J. D. M., EKINS, R. P., SGHERZI, A. M. and TAMPION, W.: *Biochem. J.*, **121**, 561, 1971.
4. BUTCHER, R. W., HO, R. J., MENG, H. C. and SUTHERLAND, E. W.: *J. Biol. Chem.*, **240**, 4515, 1965.
5. GILL, G. N. and GARREN, L. D.: *Biochem. Biophys. Res. Commun.*, **39**, 335, 1970.
6. GILMAN, A. G.: *Proc. Natl. Acad. Sci. USA.*, **67**, 305, 1970.
7. GOLDBERG, N. D. and O'TOOLE, A. G.: *Methods Biochemi. Anal.*, **20**, 1, 1971.
8. LANGAN, T. A.: *Science*, **162**, 579, 1968.
9. STEINER, A. L., KIPNIS, D. M., UTIGER, R. and PARKER, C. W.: *Proc. Natl. Acad. Sci. USA.*, **64**, 367, 1969.
10. TSANG, C. P. W., LEHOTAY, D. C. and MURPHY, B. E. P.: *J. Clin. Endocrinol. Metab.*, **35**, 809, 1972.
11. WALSH, D. A., PERKINS, J. P. and KREBS, E. G.: *J. Biol. Chem.*, **243**, 3763, 1968.