Improvement on the competitive binding assay for the measurement of cyclic AMP by using ammonium sulphate precipitation

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The protein-binding assay developed by Brown, Albano, Ekins, Sgherzi & Tampion [(1971) *Biochem. J.* **121**, 561–562] and Brown, Ekins & Albano [(1972) *Adv. Cyclic Nucleotide Res.* **2**, 25–40] was modified by using precipitation with $(\text{NH}_4)_2\text{SO}_4$ of the protein–cyclic AMP complex instead of adsorption of the free nucleotide on charcoal. The half-life of the protein–cyclic AMP complex obtained in the presence of charcoal was lower than that of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated complex. In consequence, owing to the great stability of the precipitated protein–cyclic AMP complex, this method allows more accurate and reproducible determinations.

INTRODUCTION

The method initially described by Brown *et al.* [1,2] for the determination of cyclic AMP is very simple, specific and sensitive. This method is based on the separation of the protein-bound cyclic AMP from the unbound nucleotide by adsorption of the free nucleotide on charcoal. However, we found that the half-life of the protein–cyclic AMP complex is very short in the presence of charcoal. The amount of radioactivity bound to the charcoal increases with time, leading to overestimation of cyclic AMP values when a considerable number of samples are handled. Another disadvantage is the low firmness of the charcoal/bovine serum albumin pellet, which is easily disaggregates with time.

In order to circumvent this problem, we have modified the technique of separation between the protein–cyclic AMP complex and the free nucleotide. The assay was performed as described by Brown *et al.* [1,2], except that the charcoal step was replaced by precipitation with $(\text{NH}_4)_2\text{SO}_4$ of the protein–nucleotide complex.

MATERIALS AND METHODS

Cyclic [^3H]AMP (37 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). All other reagents were analytical grade. For the calibration curve, half-life and cyclic AMP determinations, the incubations were performed as described by Brown *et al.* [1]. Briefly, the tubes (all in duplicate) contained the sample (50 μl), 20 000 d.p.m. of cyclic [^3H]AMP in 100 μl of buffer A (50 mM-Tris/HCl, 8 mM-theophylline and 6 mM-mercaptoethanol, pH 7.4) and the crude binding protein (diluted 1:10 in buffer A, 50 μl). The samples were incubated for 1.5 h at 0–4 °C. Then 300 μl of a saturated $(\text{NH}_4)_2\text{SO}_4$ solution (53.3 g/100 ml satd. soln. at 20 °C) or 200 μl of a charcoal slurry [5% (w/v)

charcoal and 1% (w/v) bovine serum albumin, in buffer A] were added, both at 0–4 °C. For determinations of the half-life and cyclic AMP, at the indicated times (see Figs. 1 and 2b) the tubes were centrifuged (7000 g, 20 min, at 4 °C). The ‘zero time’ was taken immediately after addition of charcoal or $(\text{NH}_4)_2\text{SO}_4$. For the calibration curve, the tubes were maintained for 15 min in the presence of charcoal or $(\text{NH}_4)_2\text{SO}_4$. After centrifugation, the pellets from the $(\text{NH}_4)_2\text{SO}_4$ assay were resuspended in buffer A (0.2 ml), and 0.15 ml samples from all tubes were finally taken for radioactivity counting in scintil-

Fig. 1. Half-life of the cyclic AMP–protein complex measured by the charcoal method (●) and by the $(\text{NH}_4)_2\text{SO}_4$ precipitation method (○)

The half-lives obtained after fitting the curves by an exponential model (see the text) were $t_1 = 16 \pm 4$ min and $t_2 = 115 \pm 35$ min in the presence of charcoal and $t > 1000$ min with $(\text{NH}_4)_2\text{SO}_4$. These results clearly indicate that the cyclic AMP–protein complex is more stable in the presence of $(\text{NH}_4)_2\text{SO}_4$ than in the presence of charcoal.

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Fig. 2. (a) Calibration curves corresponding to the charcoal (●) and to the $(\text{NH}_4)_2\text{SO}_4$ precipitation (○) methods, and (b) values of cyclic AMP obtained by interpolation in the curves shown above, with a constant amount of cyclic AMP (7.5 pmol)

(a) The measurements were conducted as described in the Materials and methods section by using different amounts of cyclic AMP (0.02–30 pmol). B_0 represents values determined in the absence of unlabelled cyclic AMP minus non-specific binding, and B represents binding values at each point of the standard dilutions minus non-specific binding; non-specific binding was determined in the presence of 100-fold excess unlabelled cyclic AMP. Both curves were similar in slope and accuracy (slope = 2.3 and $r^2 = 0.98$). (b) The samples were maintained in the presence of charcoal or $(\text{NH}_4)_2\text{SO}_4$ at different times. Whereas a constant value of cyclic AMP was obtained with the precipitation method, clearly different values were obtained with charcoal, in agreement with the half-life studies (Fig. 1).

lation solution [Omnifluor/toluene with 30% (v/v) Triton X-100].

RESULTS AND DISCUSSION

Fig. 1 shows the results of half-life determination. The curves were fitted by a least-squares iterative method [3], by using the following function:

$$Y = ae^{-k_1x} + be^{-k_2x}$$

taking $b = 0$ for the $(\text{NH}_4)_2\text{SO}_4$ precipitation assay. The half-lives obtained with the charcoal method were clearly lower than those obtained with the $(\text{NH}_4)_2\text{SO}_4$ precipitation assay (16 ± 4 and 115 ± 35 min versus > 1000 min). These results indicate that the complex in the presence of $(\text{NH}_4)_2\text{SO}_4$ is more stable than that in the presence of charcoal.

The calibration curves (Fig. 2a) indicate that the methods are similar in slope and accuracy (slope = 2.3 and $r^2 = 0.98$ in both cases). However, as shown in Fig. 2(b), the cyclic AMP values obtained with the $(\text{NH}_4)_2\text{SO}_4$ precipitation assay were constant with time, whereas those obtained with the charcoal method were clearly different with time, in agreement with the half-life values. Thus the results confirm that the charcoal method can lead to serious errors, particularly when a considerable number of samples are handled. In consequence, owing to the stability of the precipitated complex, the modification of the separation technique allows both a more accurate determination of cyclic AMP and handling of a considerable number of samples. In addition, the assay maintains the simplicity, specificity and sensitivity of the original method [1,2], since no modification was made at the binding step.

REFERENCES

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