PROBLEMS ASSOCIATED WITH THE DETERMINATION OF THE INTRACELLULAR DISTRIBUTION OF ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE IN RAT LIVER

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1. Introduction

The hypothesis that cyclic AMP (adenosine 3′,5′-cyclic monophosphate) is the intracellular effector of catecholamines and pancreatic hormones in the liver [1] has received considerable support from the demonstration that changes in hepatic cyclic AMP concentration occur after exposure to these hormones [1–5,7]. In some cases however, the changes in total cyclic AMP concentration have been shown to be small in relation to the metabolic effects of the hormones [2–4]. It has been suggested that most of the cyclic AMP in the unstimulated cell is sequestered and that only the free cyclic AMP is physiologically active [3]. If the hormones affect only the concentration of free cyclic AMP, these changes may be small compared to the total cell concentration and may not be reflected in measurements of the total cyclic AMP.

The cyclic AMP concentration in rat liver increases after birth [6,7] at a time when there is an increase in activity of several liver enzymes which are thought to be induced by cyclic AMP [8,9]. The largest increase in cyclic AMP however, was only about 2-fold during the first hour after delivery [6,7]. Only the total cyclic AMP in the tissue was measured in these studies. Thus the changes in concentration of the free cyclic AMP could have been greater than that reflected by the measurements of total cyclic AMP. Accordingly, the present study was designed in an attempt to resolve this problem.

Most of the cyclic AMP in liver homogenates from postnatal rats was found to be sequestered. However, there was a significant decrease in the total cyclic AMP concentration during the preparation of the cell fractions. Further experiments revealed marked changes in the cyclic AMP concentration in liver homogenates during the short time required to prepare the subcellular fractions. These results suggest that the distribution of cyclic AMP in the subcellular fractions as prepared does not represent the distribution of cyclic AMP in either the homogenate or the intact liver cell.

2. Materials and methods

2.1. *Reagents and buffers*

Cyclic [8-^{3}H]AMP (6.5 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, Bucks. Cyclic AMP, Norit A activated charcoal, crystalline bovine serum albumin, β-mercaptoethanol and theophylline were products of the Sigma Chemical Co., St. Louis MO. Dextran T500 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Unless otherwise indicated the buffer used was 50 mM Tris–HCl (pH 7.4) containing 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 10 mM theophylline and 6 mM β-mercaptoethanol.

2.2. *Preparation of homogenates and subcellular fractions*

Rats of the Wistar albino strain of *Rattus norwegicus* were used. Foetal rats were delivered by surgical delivery without anaesthesia as previously described [9]. Animals were killed by cervical fracture, the livers quickly excised and kept in ice-cold buffer until the entire litter had been killed. The livers were washed with buffer, blotted dry, weighed and homog-

enized in 2–3 vol. buffer. Control animals were killed by immersion in liquid freon which had been precooled in liquid nitrogen. The livers were dissected while the animals were still frozen and kept in liquid nitrogen until all the livers had been collected, then they were homogenized in 3 vol. buffer.

The homogenate from the unfrozen livers was centrifuged at 10 000 × *g* (max) for 10 min in an International PR-6 refrigerated centrifuge. The pellet (nuclear-mitochondrial fraction) was washed in 1 vol. buffer and the centrifugation step repeated. The supernatants were combined (post-mitochondrial fraction) and centrifuged at 226 600 × *g* (max) for 40 min in a Spinco L2-65 ultracentrifuge (50 Ti rotor). The pellet (microsomal fraction) was washed once with buffer and the supernatants were combined (cytosol fraction). For the determination of free and protein bound cyclic AMP in the cytosol a portion of the cytosol was treated with a charcoal suspension (100 mg charcoal/ml) in Tris–HCl (pH 7.4) containing 10 mg/ml Dextran T500. Sufficient charcoal suspension was added so that the final concentration of charcoal was 33 mg/ml. After standing for 10 min at 0°C, the charcoal was separated by centrifuging at 1600 × *g* for 6 min. The supernatant (bound fraction) was retained.

2.3. *Assays*

Cyclic AMP was measured by the saturation assay of Brown et al. [10] as modified by Di Marco and Oliver (in preparation). Cyclic AMP was extracted from homogenates and subcellular fractions by adding 2 M $HClO_4$ to a final concentration of 0.5 M and the acid extracts were neutralized to pH 7.4 with 5 M KOH.

Protein concentration was determined by the method of Lowry et al. [11] using crystalline bovine serum albumin as a standard.

3. Results and discussion

The apparent subcellular distribution of cyclic AMP in liver homogenates from 2-day-old rats is illustrated in table 1. Most of the cyclic AMP in the homogenate was found in the cytosol fraction. However, 82% of the cyclic AMP in this fraction was sequestered, probably bound to cyclic AMP binding proteins. Thus in the 2-day-old rat as in the adult [4] only a small portion of the cyclic AMP in the liver homogenate was free.

During the preparation of the fractions (3 h at 4°C) the total cyclic AMP concentration had decreased from 2.99–1.24 pmol/mg liver (table 1, sum of b,d,e). Because of this change liver homogenates from foetal and 2-day-old rats were incubated at 4°C and the cyclic AMP concentration monitored (fig.1). The cyclic AMP concentration in the homogenates had doubled after 5 min incubation. In the case of 2-day-old rats, the concentration reached a maximum at 20 min then decreased to initial levels by 90 min. By contrast, the concentration of cyclic AMP remained

Table 1
Subcellular distribution of cyclic AMP in liver homogenates of 2-day-old rats

Subcellular fraction	Cyclic AMP concentration	
	(pmol/mg liver)	(pmol/mg protein)
a. Homogenate	2.99	22.5
b. Nuclear-mitochondrial	0.25	3.84
c. Post-mitochondrial	1.73	23.4
d. Microsomal	0.13	9.58
e. Cytosol	0.86	10.13
1. Bound	0.70	8.25
2. Free	0.16	1.88

Rats were killed by cervical fracture and the livers excised and homogenized in 2 vol. buffer. Subcellular fractions were prepared and cyclic AMP assayed as described in Materials and methods. Each value is the mean from duplicate samples

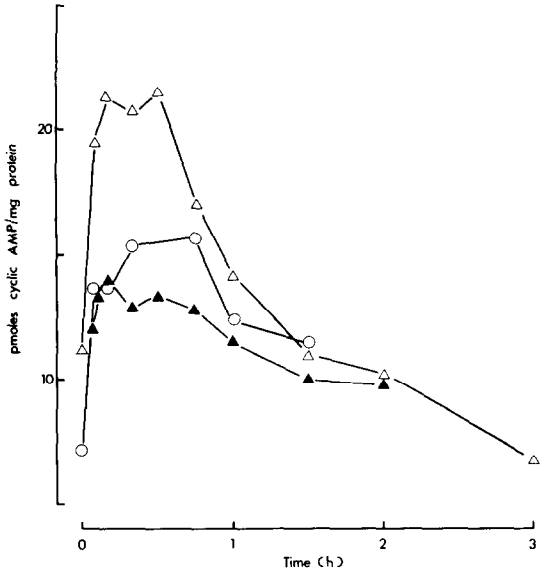

Fig.1. Changes in the cyclic AMP concentration in liver homogenates. Fresh livers (△—△) and frozen livers (▲—▲) from 2-day-old rats and fresh livers from foetal rats (o—o) were homogenized in buffer and incubated at 4°C. At the indicated times samples of the homogenates (0.5 ml) were taken, acidified with 0.2 ml of 2 M PCA and the cyclic AMP extracted and assayed as described in the Materials and methods. All samples were assayed in duplicate. Each point is the mean of duplicate samples for fresh liver homogenates and of one sample for the frozen liver homogenates. Zero time was taken from the time the livers were homogenized. The excision of livers required 8 min in the case of 2-day-old rats and 10 min in the case of day 21 foetal rats.

elevated for at least 90 min in the liver homogenate from foetal rats. Since total concentration in the acid extracts was measured the changes observed were probably due to both synthesis and hydrolysis of cyclic AMP. The results thus suggest that homogenization of the livers activates adenyl cyclase.

Because of the marked changes in the cyclic AMP concentration in the homogenate the results presented in table 1, do not necessarily reflect the distribution of cyclic AMP in the homogenate. Although only the changes in total cyclic AMP concentration in the homogenate were measured (fig.1) it is likely that the distribution of cyclic AMP was also altered because changes in any of the fractions would alter the equilibrium of cyclic AMP between all fractions. These observations suggest that it is not feasible to determine the distribution of cyclic AMP in homogenates from fresh livers. Freezing of the tissues prevented the changes in cyclic AMP concentration (fig.1). However, although freezing of the tissues appeared to inactivate the adenyl cyclase and phosphodiesterase it would probably also disrupt the intracellular organization and thus result in alterations in the distribution of cyclic AMP.

Thus the results reported show that there are rapid changes in the concentration of cyclic AMP in liver homogenates from foetal and postnatal rats which make the interpretation of results from subcellular distribution experiments difficult. This observation emphasizes the need for cautious interpretation of such results.

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