Anti-Anabolic Effects of Adenosine 3':5'-Cyclic Monophosphate

INHIBITION OF PROTEIN SYNTHESIS

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1. Evidence is presented that cyclic AMP inhibits the incorporation of L-[4,5-³H]leucine into protein in a cell-free system from rat liver. This inhibition occurs after aminoacyl-tRNA formation. 2. Microsomal fractions, isolated after the incubation of postmitochondrial supernatant with cyclic AMP and ATP, show a diminished ability to synthesize protein. Both cyclic AMP and ATP are required for this effect. 3. A possible physiological role for the anti-anabolic action of cyclic AMP is discussed in terms of the control of gluconeogenesis.

It is well established that the metabolic effects of several hormones are mediated through the intracellular participation of cyclic AMP (Robison *et al.*, 1971). Our studies on the mechanism by which cyclic AMP stimulates gluconeogenesis in the liver have shown that in rat liver slices cyclic AMP inhibits the incorporation of several radioactive precursors into protein, lipid and cholesterol under conditions when the balance of metabolism favours gluconeogenesis (Akhtar & Bloxham, 1970; Bloxham & Akhtar, 1972). The inhibition of lipid and cholesterol biosynthesis has been confirmed by other workers (Bricker & Levey, 1972*a,b*; Goodridge, 1973). A systematic investigation of the mechanism for the cyclic AMP-dependent decrease in the incorporation of precursors into cholesterol has permitted the identification of a precise enzymic step in the biosynthetic pathway which is sensitive to inhibition by cyclic AMP (Bloxham & Akhtar, 1971; Bloxham *et al.*, 1971).

The present paper extends the observation (Akhtar & Bloxham, 1970; Bloxham & Akhtar, 1972) that cyclic AMP inhibits the incorporation of labelled amino acids into proteins in a cell-free system and reports observations on the mechanism of this inhibition.

Materials and Methods

Chemicals

Cyclic AMP and phosphoenolpyruvate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambridge, U.K. Hyamine hydroxide (1.0M in methanol) was purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. L-[4,5-³H]leucine (19 mCi/ μ mol

or 52 mCi/ μ mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. British Drug Houses Ltd., Poole, Dorset, U.K., or May and Baker Ltd., Dagenham, Essex, U.K., was the source of all other chemicals.

Measurement of protein

Delipidated protein was measured with biuret reagent by the method of Cleland & Slater (1953) with bovine serum albumin (fraction V) as standard.

Experimental animals

Male Wistar Albino rats (150-250g) were used throughout. Animals were allowed food and water *ad libitum* and maintained in a fixed-temperature environment in a room with daylight illumination. Animals were killed by cervical dislocation.

This paper was first prepared in 1972 but not submitted for publication because at that time we noticed a considerable variation in the sensitivity of our cell-free system to cyclic AMP. Our records over the past 2 years show that trouble-free, consistent and reproducible responses were generally obtained during the winter months (October to April). Reproducible responses were again obtained during winter 1972-73. Our difficulties in the summer may be primarily due to the long sunlight hours, which disturb the eating habits of the animals. A recent study has shown that concentrations of cyclic AMP are susceptible to diurnal variation, being high during light periods and low during the dark (Marks & Grimm, 1972). It may be advisable to keep animals under strict dark/light period regimen and kill them before the onset of the light phase.

Measurement of radioactivity

Radioactive samples in organic solvents were counted in butyl-PBD in toluene (10–15 ml; 8 g/litre). ^3H radioactivity was counted at 58% efficiency in an Intertechnique ABAC SL-40 liquid-scintillation counter at 12°C and values were automatically corrected for quenching.

Preparation of cell fractions for cell-free protein synthesis

All operations were carried out at 4°C. Minced liver (16–20 g) from normal fed rats was homogenized in 0.25 M-sucrose containing 70 mM-Tris-HCl buffer, pH 7.8, 25 mM-KCl and 10 mM-MgCl₂ (40 ml) by using a piston-barrel homogenizer (clearance 1 mm, Bucher, 1953). Cell debris was removed by centrifugation at 1000g for 10 min. The supernatant was then centrifuged for 10 min at 10000g to give a postmitochondrial supernatant fraction, which was finally centrifuged at 105000g for 30 min, to give the microsomal pellet and a crude postmicrosomal supernatant.

The pellet derived from 6.0 ml of 10000g supernatant was resuspended by gentle homogenization with a Potter-Elvehjem homogenizer in 1.5 ml of the homogenization buffer, giving the 'unwashed' microsomal preparation.

By submitting the unwashed microsomal preparation to further centrifugation at 105000g for 30 min, a supernatant rich in activating enzymes was produced. This supernatant was used as the source of activating enzymes for the cross-addition experiments. Resuspension of the resulting microsomal fractions in 8–10 ml of homogenization buffer, resedimentation at 105000g and suspension in a further 1.5 ml of homogenization buffer yielded a 'washed' microsomal preparation which incorporated amino acids only when supplemented with the supernatant.

Standard assay for incorporation of radioactive leucine into protein

Samples of 10000g supernatant, 'unwashed' microsomal fraction or 'washed' microsomal fraction plus the supernatant fraction were incubated in the presence of phosphoenolpyruvate (5 mM), ATP (3 mM) and L-[4,5- ^3H]leucine (1 μCi ; 53 pmol, unless otherwise stated) for 15 min at 37°C in a final volume of 1.0 ml. The reaction was stopped by the addition of 3 ml of ice-cold buffer and 2 ml of 30% (w/v) trichloroacetic acid.

Tubes were left for 1 h, and the protein was then harvested by centrifugation with a bench centrifuge. The pellet was washed by resuspension and centrifugation in ice-cold 5% (w/v) trichloroacetic acid (4 $\times$ 5 ml), in hot (90°C) 5% trichloroacetic acid

(2 $\times$ 5 ml) and in acetone (2 $\times$ 5 ml) at room temperature. The resulting pellet was dissolved in 1.0 ml of Hyamine hydroxide for 15–30 min at 50°C and a sample (0.2–0.4 ml) was counted for radioactivity.

For the incorporation of leucine into the aminoacyl-tRNA fraction, the hot-trichloroacetic acid extract was pooled (Schneider, 1945) and a 1.0 ml sample was counted for radioactivity in 10 ml of Instagel (Packard Instrument Co. Inc., Warrenville, Ill. 60515, U.S.A.).

Results

Inhibition of cell-free protein synthesis by cyclic AMP

Fig. 1 shows that cyclic AMP causes a dose-dependent inhibition of the incorporation of L-[4,5- ^3H]leucine into protein by a postmitochondrial supernatant from rat liver. This effect could be the result of an enhanced rate of protein degradation in the presence of cyclic AMP, since it is known that cyclic AMP causes net protein catabolism in perfused livers (Mallette *et al.*, 1969a,b). To study this possibility *in vitro*, the effect of cyclic AMP on the retention of radioactivity by protein labelled *in vitro* was examined. For these experiments, protein was labelled by incubating the postmitochondrial supernatant (0.7 ml) at 37°C in the presence of L-[4,5- ^3H]leucine (4 μCi ; 212 pmol), ATP (3 mM) and phosphoenolpyruvate (5 mM). After 7 min, further protein

Fig. 1. Inhibition of protein synthesis by various concentrations of cyclic AMP in a postmitochondrial supernatant from rat liver

Portions (0.7 ml) of postmitochondrial supernatant were incubated for 15 min at 37°C as described in the Materials and Methods section. The incorporation in the absence of exogenous cyclic AMP was 2600 d.p.m./15 min per mg of protein and is taken as 100%. A similar pattern of inhibition was observed in four separate experiments.

synthesis was stopped completely by the addition of cycloheximide (0.36mM). The samples were then subjected to a second incubation of 10min at 37°C in the presence or absence of cyclic AMP (2mM). In both cases the residual radioactivity after the second incubation was 2860 ± 20 d.p.m./mg of protein (eight observations), showing that even high concentrations of cyclic AMP do not accelerate the rate of protein degradation. Further, since the specific radioactivity at the point of addition of cycloheximide was 2900 d.p.m./mg of protein, it is apparent that under the conditions of these experiments the amount of degradation is insignificant when compared with the rate of incorporation into protein. These results, however, do not exclude the possibility that cycloheximide might inhibit protein degradation.

The possible site of inhibition of protein synthesis was investigated further by examining the distribution of L-[4,5-³H]leucine between protein and aminoacyl-tRNA fractions. Table 1 shows that under conditions where cyclic AMP caused a pronounced inhibition of protein synthesis, there was no detectable inhibition of incorporation into the fraction associated with tRNA; indeed there was a slight increase in the incorporation into this fraction. The fact that incorporation into aminoacyl-tRNA was not inhibited by cyclic AMP eliminates the possibility that a change in specific radioactivity of the leucine pool could have led to the decrease in incorporation into protein.

The cumulative evidence presented above is consistent with the concept that cyclic AMP inhibits the pathway for protein synthesis at the level of aminoacyl-tRNA assimilation into peptide bonds.

Requirement for cyclic AMP and ATP in the inhibition of microsomal protein synthesis

The site of inhibition was studied further by a preliminary incubation of the postmitochondrial system with cyclic AMP and/or ATP followed by isolation of the separate components of protein synthesis, which allowed us to assay their synthetic capability. However, in the presence of ATP, protein synthesis occurs in the postmitochondrial supernatant and ceases after only 15min (Bloxham & Akhtar, 1972). The effect of varying the concentration of ATP in the incubation of the postmitochondrial supernatant on the activity of the subsequently isolated microsomal fraction was studied. [Attention is drawn to the fact that a microsomal fraction sedimented once from a postmitochondrial supernatant incorporates amino acids at an impressive rate without the addition of soluble activating enzymes (Bloxham & Akhtar, 1972). This fraction will be referred to as the 'unwashed' microsomal fraction.] Parallel experiments (Fig. 2) showed that

Table 1. *Effect of cyclic AMP on the incorporation of leucine into leucyl-tRNA and protein*

Portions (0.7ml) of postmitochondrial supernatant were incubated for 15min at 37°C with L-[4,5-³H]leucine (1 μ Ci), ATP (3mM) and phosphoenolpyruvate (5mM) in the presence or absence of cyclic AMP (0.5mM). The incorporation into protein and leucyl-tRNA was measured as described in the text.

Expt. no.	Conditions	Incorporation into leucyl-tRNA (d.p.m.)	Incorporation into protein (d.p.m.)
1	Control	10900	7420
	Cyclic AMP	12800	3800
2	Control	6625	7500
	Cyclic AMP	6880	5075
3	Control	8050	7500
	Cyclic AMP	8750	3900

Fig. 2. *Loss of protein-synthetic capability of 'unwashed' microsomal fraction isolated from postmitochondrial supernatant incubated with various concentrations of ATP*

The postmitochondrial supernatant was incubated with L-[4,5-³H]leucine in the presence of various concentrations of ATP and the incorporation was compared with the standard assay system containing 3mM-ATP and 5mM-phosphoenolpyruvate. Simultaneously, parallel incubations were performed for 10min at 37°C in the absence of labelled leucine. The 'unwashed' microsomal fraction was then isolated and incubated with L-[4,5-³H]leucine in the standard assay system. The loss of activity owing to the presence of ATP in the first incubation was determined by comparison with the control when ATP was omitted.

as the protein-synthetic activity of the postmitochondrial supernatant was enhanced by the addition of ATP, then the ability of the subsequently isolated 'unwashed' microsomal fraction to incorporate radioactivity into proteins decreased. However, provided that the concentration of ATP is maintained below 2mM, it is apparent that there is only a minor loss of protein-synthetic activity in the 'unwashed' microsomal fraction. Therefore only 1.5mM-ATP was used in the preincubation experiments described below.

Initially it was shown (Fig. 3) that incubating the postmitochondrial supernatant with both cyclic AMP and ATP resulted in the time-dependent loss of protein-synthetic activity in the isolated 'unwashed' microsomal fraction. No significant inhibition was observed after preincubation with cyclic AMP alone. The requirement for ATP as well as cyclic AMP in these experiments indicates that the

Fig. 3. Time-course of the modification of the protein-synthesis system by cyclic AMP

Portions (6.0ml) of postmitochondrial supernatant from rat liver were incubated at 37°C for various time-intervals in the presence of nucleotides as indicated below. The microsomal fractions were harvested and their ability to incorporate L-[4,5-³H]leucine into protein was assayed. ○, Preincubation with cyclic AMP (0.5mM) and ATP (1.5mM); Δ, preincubation with cyclic AMP (0.5mM) and ATP (1.5mM); □, preincubation with cyclic AMP (0.5mM). An identical pattern was observed in three separate experiments.

inhibitory effect may be due to the phosphorylation of a component of the protein-synthetic system. That the phosphorylation may be mediated through the participation of an enzyme system (presumably a phosphokinase) present in the postmicrosomal supernatant is suggested by experiments outlined in Fig. 4, which show that the 'unwashed' microsomal preparation itself is not susceptible to cyclic AMP-dependent inhibition of incorporation, but the addition of the crude postmitochondrial supernatant largely restores the inhibitory property of the nucleotide.

Cross-addition experiments

The above experiments established that the inhibition of protein synthesis by cyclic AMP is dependent on the presence of ATP. The next problem was whether the loss of protein-synthetic ability in the 'unwashed' microsomal fraction caused by cyclic AMP and ATP was due to inhibition of the microsomal or the soluble components of protein synthesis. The postmitochondrial supernatant was therefore incubated in the absence of further

Fig. 4. Participation of a postmicrosomal-supernatant enzyme system in the expression of the inhibitory effect of cyclic AMP

Incorporation of radioactivity into protein in the presence of different concentrations of cyclic AMP was studied by using either 'unwashed' microsomal fraction (□), or 'unwashed' microsomal fraction supplemented with crude postmicrosomal supernatant (ratio of the microsomal:supernatant protein 1:2, w/w) (Δ) or postmitochondrial supernatant (○).

additions, with ATP alone, with cyclic AMP alone and with cyclic AMP plus ATP. The 'unwashed' microsomal pellet was then isolated and processed further to yield the 'washed' microsomal pellet and a supernatant containing activating enzymes. These two components must be combined to obtain protein synthesis. This enabled us to cross-match the components in various ways in order to test which component of the protein-synthetic system was inactivated by preincubation with cyclic AMP and ATP (Table 2). Preincubation with ATP alone does not lead to any loss in protein-synthetic activity of the soluble activating enzymes (Expt. *b*) whereas the activity of the 'washed' microsomal fraction is decreased by 10–15% (Expts. *c* and *d*). This loss of activity is in accord with the inhibition noted in earlier preincubation experiments (see Figs. 2 and 3).

The site of action of cyclic AMP and ATP was elucidated by the cross-addition experiments (*e*) and (*f*). When a 'washed' microsomal fraction isolated from preincubation with cyclic AMP was reconstituted with activating enzymes isolated from preincubation with cyclic AMP and ATP, the activity of the system was normal (compare Expt. *e* with Expts. *a* and *b*, Table 2). In comparison, the system was markedly inhibited when a 'washed' microsomal fraction isolated from preincubation with cyclic AMP and ATP was reconstituted with activating enzymes from preincubation with cyclic AMP alone (compare Expt. *f* with Expt. *e*, Table 2). Thus the data indicate that the combined effect of cyclic AMP and ATP is to inhibit the function of the microsomal fraction whereas none of the pretreatments significantly affects the role of the soluble activating enzymes.

Other workers (Eil & Wool, 1971; Loeb & Blat, 1970) have demonstrated a cyclic AMP-dependent phosphorylation of ribosomal proteins but have not linked this to ribosomal function experimentally. However, evidence has been presented suggesting that phosphorylation of liver ribosomes by a phosphokinase from rabbit skeletal muscle inhibits the efficiency with which ribosomes incorporate aminoacyl-tRNA into protein (Monier *et al.*, 1972).

Discussion

The present work shows the direct inhibition of protein biosynthesis by cyclic AMP, which may occur through phosphorylation of a component of the microsomal fraction. The modification results in a 40–50% decrease in the protein-synthetic capability in both cellular and cell-free systems (Bloxham & Akhtar, 1972). Although there is a net decrease in protein synthesis in the presence of the cyclic nucleotide, this does not preclude the enhanced synthesis of specific enzymes involving newly available mRNA (Wicks, 1971). The inhibition of cholesterol biosynthesis has been established in previous studies (Akhtar & Bloxham, 1970; Bloxham & Akhtar, 1971; Bloxham *et al.*, 1971; Bloxham & Akhtar, 1972) and occurs at the level of C-4 demethylation, where the enzyme β -hydroxy steroid dehydrogenase is inhibited (Bloxham & Akhtar, 1971). Recent work has also indicated that cyclic AMP may inhibit the enzyme β -hydroxy- β -methylglutaryl-CoA reductase in rat liver in an ATP-dependent process (Beg *et al.*, 1973). These observations, when considered in conjunction with the evidence for the inhibition of lipid biosynthesis

Table 2. *Protein-synthesizing ability of systems reconstituted from 'washed' microsomal and activating enzyme fractions subjected to various pretreatments*

Portions (6.0ml) of postmitochondrial supernatant from rat liver homogenate were incubated for 7 min at 37°C in the presence of either cyclic AMP (0.5mM) plus ATP (1.5mM), cyclic AMP (0.5mM) alone or ATP (1.5mM) alone and then immediately cooled to 4°C. Control incubations were carried out in a similar manner in the absence of any additions. In each case the 'unwashed' microsomal pellet was harvested and processed to give the supernatant containing the activating enzymes (1.5ml) and the 'washed' microsomal pellets as described in the Materials and Methods section. The 'washed' microsomal fraction (5mg of protein) was then supplemented with supernatant (1.5ml; 1mg of protein) as indicated below. The ability of the reconstituted system to incorporate [³H]leucine into protein was assayed. Each value represents the mean $\pm$ S.E.M. for three observations.

Expt.	'Washed' microsomal fraction from preincubation with:	Supernatant containing activating enzymes from preincubation with:	Incorporation into protein (d.p.m./15 min per mg of protein)
(a)	No addition	No addition	7850 $\pm$ 75
(b)	No addition	ATP	7960 $\pm$ 75
(c)	ATP	No addition	6680 $\pm$ 25
(d)	ATP	ATP	7100 $\pm$ 50
(e)	Cyclic AMP	Cyclic AMP + ATP	8350 $\pm$ 50
(f)	Cyclic AMP + ATP	Cyclic AMP	3580 $\pm$ 50

by cyclic AMP (Akhtar & Bloxham, 1970; Bloxham & Akhtar, 1972; Bricker & Levey, 1972a; Goodridge, 1973), suggest that under conditions of enhanced gluconeogenesis cyclic AMP may act as an anti-anabolic agent by inhibiting the biosynthesis of protein, lipid and cholesterol (Bloxham & Akhtar, 1972). These results *in vitro* may be compared with reported observations *in vivo*: starvation is a major stimulus of gluconeogenesis in the liver (Krebs, 1964) and is accompanied by an increase in concentration of cyclic AMP (Jefferson *et al.*, 1968; Exton *et al.*, 1970) and a decreased synthesis of cholesterol (Bucher, 1959), fatty acids (Stetton & Boxer, 1944; Lowenstein, 1968) and protein (Munro, 1968; Henshaw *et al.*, 1971). Decreasing concentrations of plasma insulin in diabetes leads to an increase in liver cyclic AMP concentrations (Jefferson *et al.*, 1968; Exton *et al.*, 1970), which is accompanied by inhibition of synthesis of protein (Krahl, 1952, 1953) and fatty acid (Brady & Gurin, 1950; Renold *et al.*, 1955). If relevant *in vivo*, then a possible physiological role for an anti-anabolic action of cyclic AMP could involve the inhibition of biosynthetic pathways which would compete for substrates and cofactors during periods of maximum gluconeogenic flux. Thus it would be desirable to decrease the activity of the protein-, cholesterol- and fatty acid-synthesizing pathways in order to divert ATP and reducing equivalents to gluconeogenesis. Also these pathways would remove intermediates such as pyruvate, tri-carboxylic acid-cycle intermediates and amino acids, all of which serve as intermediates for gluconeogenesis.

Finally, if the cyclic AMP-dependent inhibition of protein synthesis by a phosphorylation mechanism operates *in vivo* under conditions of gluconeogenesis, it follows that when an animal is returned to an anabolic state a converse regulatory mechanism must come into play which replenishes or indeed enhances the activity of the protein-synthetic system.

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