3',5'-CYCLIC AMP AND THE RELEASE OF POLYSOME-BOUND PROTEINS *IN VITRO*[1]

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Previous studies from this laboratory have indicated that during glucose repression of serine dehydratase in rat liver the rate of *de novo* synthesis of the enzyme is greatly depressed (Pitot and Jost, 1967), while there is up to a 25-fold increase in the incorporation of amino acids into the ribosomal-bound enzyme antigen which may be released by puromycin *in vitro* (Khairallah *et al.*, 1967). This evidence suggested that glucose or a metabolite associated with glucose metabolism may be involved in the release of newly synthesized proteins from polysomes.

Since glucagon and epinephrine can both reverse the glucose effect on these enzymes (Peraino and Pitot, 1964), and since both these hormones can increase the levels of cyclic AMP in tissues from 3-50-fold (Robison *et al.*, 1967), we investigated the effect of the addition of 3',5'-cyclic AMP to a cell free system prepared from rat liver designed to test the release of newly synthesized proteins. This communication presents evidence suggesting that cyclic AMP may be involved in the enzymatic release of nascent proteins from the polysomes of rat liver.

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MATERIALS AND METHODS

Preparation of prelabeled polysomes. Male Holtzman rats (180-220 g), which had been previously fed a protein-free diet for 5 days, were fasted overnight and then intubated with 1 gram of enzymatic casein hydrolysate in 4 ml of water. Six hours later the rats were intubated again with one gram of glucose together with 100 mg of a complete mixture of essential amino acids lacking only valine (Peraino et al., 1965). Thirty minutes after the second intubation the animals were injected with 12 μc of L-valine-1-C^{14} (22 μc/μM). They were sacrificed 40 minutes later and their livers were homogenized in 3 volumes of 0.33 M sucrose buffered in TKM (0.05 M Tris-HCl; 0.025 M KCl; and 0.005 M $MgCl_2$, pH 7.4). The post-mitochondrial supernatant (Webb et al., 1966) was treated with deoxycholate to a final concentration of 1.0% and 3.5 ml of this solution was layered over a discontinuous sucrose gradient consisting of 4 ml of 1.3 M sucrose-TKM over 3 ml of 2.0 M sucrose-TKM. After centrifugation at 105,000 x g for 8-10 hours, the polysome pellet was again layered over 3 ml of 2.0 M sucrose-TKM and spun for 2.5 hours at 165,000 x g. This procedure selectively sedimented the highly aggregated polysomes which are generally regarded as the most active protein synthesizing units (Fleck et al., 1965; Webb et al., 1966). The polysomal pellet was then washed in TKM, centrifuged for 60 minutes at 105,000 x g and resuspended in TKM. After centrifugation at 600 x g for 1 minute to remove clumped particulates, the supernatant suspension of polysomes was used in the system outlined below.

Assay for release of soluble labeled proteins. The in vitro release of the nascent proteins prelabeled in vivo was assayed by incubating the polysomal suspension (0.33 mg RNA and 3400 dpm.) for 10 minutes at 37°C in the presence of 50 mM Tris-HCl, pH 8.0; 275 mM KCl; 5 mM $MgCl_2$; 1 mM ATP; 0.25 mM GTP; 5 mM phosphoenolpyruvate; 10 units of pyruvate kinase; 5 mM GSH; and 200 γ of a "releasing enzyme" prepared as described by Pitot and Cho (1961). This "releasing enzyme" appears to be a high

molecular weight, thermolabile component prepared by passing a 35-55% ammonium sulfate cut of the pH 5.2 supernatant of rat liver through Sephadex G-50. It should be noted that this enzyme fraction still contains the transferase I and II described by Moldave and his associates (Fessenden and Moldave, 1963; Gasior and Moldave, 1965).

The reaction was stopped by diluting the incubation mixture with 8 volumes of pH 6.4 potassium-free buffer and then centrifuging at 78,000 x g for 2 hours to remove the polysomes. The released proteins in the supernatant were then precipitated with TCA, washed twice, redissolved in 98% formic acid and their radioactivity determined.

RESULTS

Table I presents the results of a typical experiment showing the requirements of the complete releasing system. It can be seen that ATP,

Table I

THE RELEASE OF C^{14}-PROTEINS FROM POLYSOMES PREPARED FROM RATS PRELABELED IN VIVO WITH C^{14}-VALINE. (COMPLETE SYSTEM)

System	dpm released/mg RNA
Complete	722
- ATP (10^{-3} M)	111
- GTP (2.5×10^{-4} M)	382
- PK 60 γ	128
- PEP (5.0×10^{-3} M)	268
- GSH (5.0×10^{-3} M)	708
- K (0.275 M)	250
- "Releasing Enzyme" (200γ)	55

GTP, as well as an energy generating system are required for the release. GSH, which seems to be required for the transferase II (Sutter and Moldave, 1966) does not appear to be essential in our system. The requirement for a high potassium concentration is consistent with the

findings of Traut and Monro (1964) and of Hultin (1966). NH_4^+ may replace the potassium ion. Approximately 50% greater release occurred at pH 8.0 than at pH 7.4. Controls run in the presence of TKM alone or stopped at zero time released 10-16% of the total counts incubated. The figures presented in the tables are corrected for this background activity.

Table II shows the results of replacing the triphosphate nucleotides by

Table 2

THE EFFECT OF 3'5' CYCLIC AMP (CAMP) ON THE RELEASE OF C^{14}-PROTEINS FROM POLYSOMES OF RATS PRELABELED IN VIVO WITH C^{14}-VALINE.

System	dpm released/mg RNA
A) Complete	722
B) Complete - ATP - GTP	15
(1) + CAMP (10^{-3}M)	524
(2) + AMP (10^{-3}M)	48
(3) + CAMP + DMAP (10^{-3}M)	1320
(4) + DMAP	312
(5) + CAMP - Releasing Enzyme	18

All incubations in B (1-5) contained the complete system minus ATP and GTP.

3',5'-cyclic AMP in the above system. It can be seen that cyclic AMP without the addition of triphosphates can release as much as 75% of the counts liberated by the complete system. In the results presented, 10^{-3}M cyclic AMP was used; however, in other experiments we have been able to demonstrate enhanced release of nascent proteins with as little as 10^{-5}M cyclic AMP. AMP at the same concentrations was used as a negative control. The addition of 10^{-3}M dimethylaminopurine (DMAP) or theophylline, both known inhibitors of the phosphodiesterase specific for cyclic nucleotides, usually resulted in a 1-3-fold increase in the release of labeled protein. The fact that DMAP alone can release some nascent proteins may be due to

the presence of some adenylate cyclase in our system. However, it should be noted that the effect of the addition of both cyclic AMP and DMAP was more than additive in this experiment, suggesting that this phenomenon may be of physiological importance. The "releasing enzyme" appears to be essential for the cyclic AMP to have an effect.

DISCUSSION

The results presented suggest that cyclic AMP can replace ATP and GTP in the in vitro energy dependent release of soluble protein from its polysome-bound form. This release requires the presence of a non-dialyzable, thermolabile factor which has been partially purified from rat liver. Earlier studies by Morris and Schweet (1961) and Morris (1964) demonstrated that the release of polysome-bound "nascent" protein in vitro was an enzymatic process which could, in part, be differentiated from the process of amino acid incorporation into soluble protein. Studies from this laboratory (Pitot and Cho, 1961) suggested that the release of particulate bound tryptophan pyrrolase activity into a soluble form was an energy dependent enzymatic process.

Although the data presented here do not indicate the actual role of cyclic AMP in the release process, several mechanisms are possible. (1) Cyclic AMP may be interacting with the "releasing enzyme"; the fact that cyclic nucleotides can activate many enzymes is well documented. (Sutherland and Robison 1966). (2) The cyclic nucleotide could react with the completed polypeptide chain which is still bound to the terminating transfer RNA and facilitate release by a puromycin-like action, or (3) Cyclic AMP might interact with the terminating transfer RNA itself thus replacing the polypeptide bound to it. Efforts to elucidate which, if any, of the mechanisms are active in this system, as well as the role of this process in the hormonal control of protein synthesis, are now being pursued.

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