# EVIDENCE FOR TRANSLATIONAL REGULATION OF SPECIFIC ENZYME SYNTHESIS BY $N^6$, $O^{2'}$-DIBUTYRYL CYCLIC AMP IN HEPATOMA CELL CULTURES*

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Summary: $N^6$,$O^{2'}$-dibutyryl cyclic 3':5' AMP (dibutyryl cAMP) produces a rapid increase in the activity of phosphoenolpyruvate carboxykinase (PEPCK) in Reuber H35 hepatoma cell cultures. This effect is shown to result from a stimulation of enzyme synthesis. Low concentrations of actinomycin D completely block the response of PEPCK to dexamethasone but not that at early intervals after addition of dibutyryl cAMP. These results suggest that dibutyryl cAMP affects a post-transcriptional step in PEPCK synthesis. Superinduction of tyrosine transaminase after addition of high concentrations of actinomycin D is observed with both dexamethasone and dibutyryl cAMP; PEPCK does not exhibit this phenomenon with either inducer.

The activities of both phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine transaminase (TT) have been shown previously to be elevated by exposure of Reuber hepatoma (H35) cell cultures to dibutyryl cAMP and glucocorticoids (1,2,3). The effects of both agents on TT activity have been shown to result from a stimulation of *de novo* enzyme synthesis in H35 cells (1,2), in adult rat liver (2,4) and fetal rat liver organ cultures (5,6). The present report provides evidence that dibutyryl cAMP also increases the rate of PEPCK synthesis in H35 cells. The early response of both enzymes to dibutyryl cAMP is not prevented by actinomycin D at the same time that the response to dexamethasone ($\Delta^{1,2}$, 9α-fluoro, 16α-methyl cortisol) is blocked. Superinduction of TT is observed after preinduction with dibutyryl cAMP or dexamethasone but does not exhibit this phenomenon with either inducer.

*This is paper II in a series entitled: Regulation of Phosphoenolpyruvate Carboxykinase and Tyrosine Transaminase in Hepatoma Cell Cultures. See Ref. 1 for paper I.

## MATERIALS AND METHODS

H35 cells were grown in monolayer culture as previously described (1). Growth medium was replaced by serum-free medium 12-18 hours before harvest with cells near confluence and assays performed as before (1). RNA was monitored by incorporation of [$^3$H]-uridine into acid-insoluble material by the filter paper disc method (7). Dibutyryl cAMP was obtained from Sigma Chemical Co. or Boehringer Mannheim, [$^3$H]-leucine and [$^3$H]-uridine were purchased from New England Nuclear Corp. Actinomycin D was a generous gift from Merck, Sharpe and Dohme Laboratories. Purification of PEPCK, elicitation of antibodies against it and the details of immunoprecipitation have been described previously (8).

## RESULTS AND DISCUSSION

A 2-3 fold increase in the incorporation of [$^3$H]-leucine into PEPCK occurs at least as early as 1-1/2 hours after addition of dibutyryl cAMP to H35 cells and which is not appreciably greater 2 1/2 hours later (Table I). In Experiment 2 incorporation of [$^3$H]-leucine into TT was measured and also found to be increased 2-3 fold. The relative specific radioactivity of TT was only 5-10% of PEPCK as would be expected on the basis of the relative amounts of these two enzymes in H35 cells (TT ~50-100 ng/culture flask (25 cm$^2$ surface area); PEPCK ~1000-2000 ng/culture flask) assuming roughly comparable rates of synthesis. These results demonstrate that the effects of dibutyryl cAMP on PEPCK activity also are due to stimulation of de novo synthesis in H35 cells as has been reported recently in neonatal (9) and adult rat liver (8).

It has been reported by other laboratories (10,11) and our own (1) that the early effects of dibutyryl cAMP on TT are not inhibited by actinomycin D. The results illustrated in Figure 1 demonstrate that early effects of dibutyryl cAMP on PEPCK activity are also not inhibited by the antibiotic. As time progresses, however, inhibition of increasing intensity is observed. In contrast, the effects of dexamethasone were essentially completely blocked by actinomycin D. Similar results were obtained with TT in the same and other experiments.

TABLE I

EFFECTS OF DIBUTYRYL cAMP ON THE SYNTHESIS OF PEP CARBOXYKINASE IN H35 CELL CULTURES

| Additions | PEP Carboxykinase Activity (units/mg) | Radioactivity In: PEP Carboxykinase (cpm) A | Radioactivity In: Total Soluble Protein (cpm x $10^{-3}$) B | Relative Radioactivity A/B |
|---|---|---|---|---|
| Experiment 1 | | | | |
| None | 43 | 157 | 148 | 1.1 |
| | 48 | 361 | 231 | 1.6 |
| Dibutyryl cAMP (1-1/2 hours) exposure | 70 | 682 | 288 | 2.4 |
| | 88 | 1425 | 300 | 4.8 |
| % change | +75% | +306% | +55% | +170% |
| Experiment 2 | | | | |
| None | 24 | 525 | 315 | 1.7 |
| | 26 | 565 | 296 | 1.8 |
| | 39 | 617 | 314 | 2.0 |
| Dibutyryl cAMP (4 hours) exposure | 70 | 2384 | 406 | 5.9 |
| | 66 | 1857 | 329 | 5.6 |
| | 56 | 1340 | 322 | 4.2 |
| % change | +117% | +227% | +14% | +182% |

Dibutyryl cAMP (0.5 mM) was added to cultures 12-18 hours after transfer to serum-free medium. In Experiment 1, 50 μCi of [$^3$H]-leucine were added 1 hour after dibutyryl cAMP and 30 minutes later the cells were harvested into 4 groups (7 flasks each). PEPCK was partially purified by heating lysates to 58° after addition of PEP (10 mM), $MnCl_2$ (3 mM), dithiothreitol (3 mM) and glycerol (8%). Recovery of enzyme activity averaged 90-95%. Immunoprecipitation and counting were carried out as previously described (8). The antibody used in both experiments had been subjected to preabsorption and, after partial purification, exhibited a single precipitin line against crude, partially purified and highly purified PEPCK after immuno diffusion.

In Experiment 2, 75 μCi of [$^3$H]-leucine were added 3-1/2 hours after dibutyryl cAMP and 30 minutes later the cells were harvested into 6 groups (7 flasks each). PEPCK was partially purified by chromatography on DEAE cellulose and $(NH_4)_2SO_4$ precipitation (8). Recovery of enzyme activity averaged 50-60% in both groups. The radioactivity data have been corrected for losses in both experiments. Radioactivity in control precipitates (performed after 85-95% of initial PEPCK had already been precipitated and counted) ranged from 5-15% of that in the initial precipitate in both experiments.

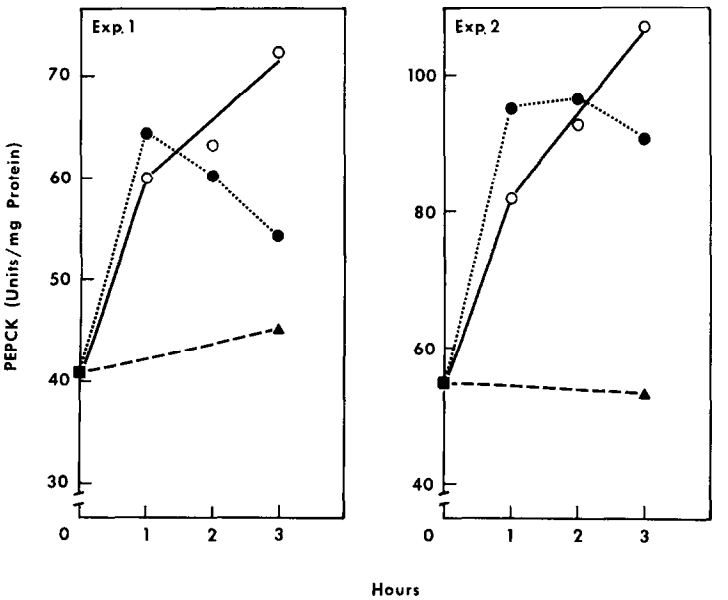


Figure 1. Effects of actinomycin D on early response of PEP carboxykinase activity to dexamethasone and dibutyryl cAMP. Actinomycin D was added 20 minutes prior to inducers at 0.4 µg/ml. Dexamethasone was added at 0.1 µM and dibutyryl cAMP at 0.5 mM. Each point represents the average of 3-5 observations with a standard error of 10-15%. In Experiment 1 the value for enzyme activity 3 hours after dexamethasone alone was 87 units per mg protein. Incorporation of [$^3$H]-uridine into RNA was inhibited by actinomycin D to 85-95% throughout the experimental period. (■) untreated cultures; (●) dibutyryl cAMP + actinomycin D; (○) dibutyryl cAMP; (▲) dexamethasone + actinomycin D.

These results are consistent with the conclusion that the response of PEPCK to dexamethasone requires new RNA synthesis whereas that to dibutyryl cAMP does not. Foster et al (12) reported inhibition by actinomycin D of the response of PEPCK to cortisol in adult rat liver and induction of TT by glucocorticoids is prevented by this antibiotic in all systems examined thus far (2,3). Yeung and Oliver, on the other hand, observed inhibition by actinomycin D of the response of PEPCK to cAMP in neonatal rat liver (13). This result is not unexpected, however, in view of the fact that enzyme activity was measured only 3-1/2 hours after administration of both agents (see Figure 1).

Chuah and Oliver have reported that washed microsomes from neonatal rat

TABLE II

EFFECTS OF HIGH CONCENTRATIONS OF ACTINOMYCIN D ON TRANSAMINASE AND CARBOXYKINASE ACTIVITIES IN PREINDUCED H35 CELLS

| Additions | Final Concentration | Tyrosine Transaminase (units/mg protein) | PEP Carboxykinase (units/mg protein) |
|---|---|---|---|
| None | | 35.5 (7) | 21.3 (7) |
| Dexamethasone | 0.1 μM | 330.3 (9) | 67.0 (9) |
| Dibutyryl cAMP | 0.5 mM | 96.0 (8) | 67.0 (8) |
| Actinomycin D | 5 μg/ml | 47.6 (3) | 30.4 (3) |
| Dexamethasone + Actinomycin D | 0.1 μM<br>5 μg/ml | 443.0 (8) | 55.0 (8) |
| Dibutyryl cAMP + Actinomycin D | 0.5 mM<br>5 μg/ml | 159.4 (8) | 65.6 (8) |

H35 cells were either untreated or incubated overnight with dexamethasone or dibutyryl cAMP; actinomycin D was added in the morning to some of these flasks and two hours later the cells were harvested and enzyme activities measured. The data are averages, with the number of flasks in parenthesis, which were obtained in two separate experiments. The standard errors ranged from 5-10%.

liver will release TT upon addition of cAMP and have suggested that the cyclic nucleotide controls the termination step in TT synthesis (14). PEPCK was not released under the same conditions, however.

The appearance of inhibition of the induction of PEPCK and TT by dibutyryl cAMP after 1-2 hours of exposure to actinomycin D can be accounted for if it is postulated that the template RNA's for these enzymes are relatively labile ($t_{1/2} \tilde{=} 2^{1/2}$-5 hours). Under these conditions eventual inhibition of induction would be anticipated regardless of the site at which the cyclic nucleotide acted. This explanation appears to be valid for TT (15) and experiments are planned to test its validity for PEPCK.

A post-transcriptional site of action for cAMP has also been suggested from studies of its effects on steroidogenesis in adrenal cortex (16) and alanine-

glyoxylate transaminase induction in adult rat liver (17).

High concentrations of actinomycin D have been found to produce a further increase in TT activity above that achieved by prior induction with glucocorticoids or insulin (15,3,20,21). This phenomenon has been referred to as "superinduction and is explained either on the basis of inhibition of the synthesis of an RNA species which inhibits TT synthesis (3), or inhibition of the degradation of TT (20,21). Superinduction of TT can be observed in H35 cells after preinduction with dibutyryl cAMP as well as with dexamethasone (Table II). Butcher et al (9) also reported superinduction of TT with dibutyryl cAMP in H35 cells but only experiments in which cortisol had also been added were reported.

In contrast to TT, high concentrations of actinomycin D did not cause an additional increase in PEPCK activity in the present experiments above that produced by either inducer alone. Studies at later intervals after addition of the antibiotic also did not reveal any superinduction of PEPCK. These results suggest that either a separate regulatory gene for PEPCK does not exist or that if it does, transcription of this gene is not suppressed by very high concentrations of actinomycin D, or that the putative inhibitory RNA species has the same half-life as the template RNA for PEPCK. At present it is not possible to decide which, if any, of these alternatives is correct. However, even the significance of superinduction of TT is in some doubt as a result of a recent report by Butcher et al (22) that cordycepin, (3-deoxyadenosine), at concentrations that inhibit RNA synthesis by more than 90%, does not lead to superinduction of TT in H35 cells after preinduction with cortisol.

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REFERENCES

1. Barnett, C. A., and Wicks, W. D., J. Biol. Chem. 247, 7201 (1971).
2. Kenney, F. T., in H. N. Munro (Ed.), Mammalian Protein Metabolism,

Vol. IV, Academic Press, New York (1970), p. 131.
3. Tomkins, G. R., Gelehrter, T. D., Granner, D., Martin, D., Jr., Samuels, H. H., and Thompson, E. B., Science 166, 1474 (1969).
4. Wicks, W. D., Kenney, F. T., and Lee, K.-L., J. Biol. Chem. 244, 6008 (1969).
5. Wicks, W. D., J. Biol. Chem. 243, 900 (1968).
6. Wicks, W. D., J. Biol. Chem. 246, 217 (1971).
7. Mans, R. J., and Novelli, G. D., Arch. Biochem. Biophys. 94, 48 (1961).
8. Wicks, W. D., Lewis, and McKibbin, J. B., Biochim. Biophys. Acta 264 177 (1972).
9. Hanson, R., Philippidis, H., Reshef, L., and Ballard, F. J., Fed. Proc. 30, 1073 abs. (1971).
10. Butcher, F. R., Becker, J. E., and Potter, V. R., Exp. Cell Res. 66, 231 (1971).
11. Holt, P. G and Oliver, I. T., Biochemistry 8, 1429 (1969).
12. Foster, D. O., Ray, P. D. and Lardy, H. A., Biochemistry 5, 555 (1966).
13. Yeung, D., and Oliver, I. T., Biochemistry 7, 3231 (1968).
14. Chuah, C.-C., and Oliver, I. T., Biochemistry 10, 2990 (1971).
15. Lee, K.-L., Reel, J. R., and Kenney, F. T., J. Biol. Chem. 246, 5806 (1970).
16. Garren, L. D., Vitamins and Hormones 26, 119 (1968).
17. Snell, K., Biochem. J. 123, 657 (1971).
18. Jost, J.-P., Hsie, A., Hughes, S. D. and Ryan L., J. Biol. Chem. 245, 351, (1970).
19. Shiu, S., and Sato, G. H., Biochem. Biophys. Res. commun. 45, 501 (1971).
20. Reel, J. R., Lee, K.-L., and Kenney, F. T., J. Biol. Chem. 246, 5800 (1970).
21. Reel, J. R. and Kenney, F. T., Proc. Nat. Acad. Sci. (USA) 61, 200 (1968).
22. Butcher, F. R., Bushnell, D. E., Becker, J. E., and Potter, V. R., Exp. Cell Res., in press.