# Effects of Cyclic Adenosine Monophosphate, Dexamethasone and Insulin on Phosphoenolpyruvate Carboxykinase Synthesis in Reuber H-35 Hepatoma Cells†

J. Martyn Gunn,* Shirley M. Tilghman, Richard W. Hanson, Lea Reshef,‡ and F. John Ballard§

ABSTRACT: Antiserum prepared against rat liver cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is shown to specifically precipitate the enzyme from Reuber H-35 cells. Synthesis of phosphoenolpyruvate carboxykinase, as measured immunochemically, is increased by dibutyryl cAMP and dexamethasone, the nucleotide maximally producing a sixfold and the glucocorticoid a threefold change in rate. Studies with actinomycin D, cordycepin, and cycloheximide suggest dibutyryl cAMP acts at a translational or post-transcriptional site. Insulin prevents the increase in synthesis of phosphoenolpyruvate carboxykinase produced by either dibutyryl cAMP or dexamethasone. This antagonism is concentration dependent and does not require the simultaneous presence of glucose, pointing to a direct effect of the hormone on liver enzyme induction. It is suggested that hepatic phosphoenolpyruvate carboxykinase activity is regulated predominantly by the antagonistic interaction of cAMP (glucagon) and insulin on enzyme synthesis.

The activity of hepatic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32)[1] is regulated by glucagon, acting via cAMP, by insulin, and by glucocorticoids (Shrago et al., 1963; Foster et al., 1966; Reshef et al., 1969; Reshef and Hanson, 1972). Dibutyryl cAMP has been shown to increase the synthesis rate of this enzyme in fetal (Hanson et al., 1973) and adult (Wicks et al., 1972) rat liver, as well as in hepatoma cells in culture (Wicks and McKibbin, 1972). Insulin, on the other hand, causes a rapid decline in hepatic P-enolpyruvate carboxykinase synthesis when injected into diabetic rats (Tilghman et al., 1974). Glucocorticoids increase the rate of enzyme synthesis in the livers of diabetic rats, but actually decrease P-enolpyruvate carboxykinase synthesis in normal animals, probably due to a glucocorticoid-mediated increase in insulin release.[2] The regulation of this enzyme is, therefore, a potentially useful model for understanding the mechanism of hormone action. It is important, however, to delineate the effects of these various hormones directly in the absence of secondary effects caused by hormone interactions in vivo. The Reuber H-35 hepatoma cell in culture contains P-enolpyruvate carboxykinase so that these cells may be used to measure directly the effects of a variety of hormones on enzyme activity and synthesis. In this paper we have tested the effect of dibutyryl cAMP, dexamethasone, and insulin on P-enolpyruvate carboxykinase synthesis in H-35 cells.

## Experimental Procedures

*Materials.* Dibutyryl cAMP (sodium salt), dexamethasone, theophylline, actinomycin D, and cordycepin (grade III) were obtained from Sigma Chemical Co., St. Louis, Mo.; inosine 5′-diphosphate (trisodium salt), phosphoenolpyruvate (potassium salt), NADH (disodium salt, grade II), and malate dehydrogenase used in the assay of P-enolpyruvate carboxykinase were from the Boehringer Mannheim Corp., New York, N.Y.; L-leucine-*4,5*-$^3H$ (30–50 Ci/mmol), orotic acid-*5*-$^3H$ (10–20 Ci/mmol) and $NaH^{14}CO_3$ (2–10 Ci/mol) were purchased from New England Nuclear, Boston, Mass., and NCS solubilizer was from Amersham/Searle Corp., Arlington Heights, Ill. Disposable, plastic, sterile, tissue culture flasks (25 $cm^2$) and Petri dishes (80 $cm^2$) were obtained from Falcon, Oxnaval, Calif., and all media constituents used for cell culture from Flow Laboratories, Inc., Bethesda, Md. Glucagon-free insulin was the gift of Eli Lilly & Co., Indianapolis, Ind.

*Cell Culture Conditions.* Reuber H-35 cells (strain H-4-II-E) (Reuber, 1961; Pitot et al., 1964) adapted to tissue culture were obtained from Drs. Joyce Becker and Van R. Potter. Stock cultures were grown in glass roller bottles at 37° in Williams' Medium E (Williams and Gunn, 1974) containing 10% fetal bovine serum, 2 m*M* glutamine, penicillin G, and streptomycin. Stock cultures were passaged by trypsinization every 2 weeks and either plated into fresh roller bottles to maintain the stock or into flasks and Petri dishes for experimental purposes. Experimental cell cultures were grown in Eagle's Minimal Essential Medium (Eagle, 1959), containing Hanks' salts and supplemented with 10% fetal bovine serum, 15 m*M* (final concentration) glucose, penicillin G, and streptomycin, and with vitamins and amino acids, including nonessential amino acids, at twice the original formulation. These cultures were grown

† From the Fels Research Institute and Department of Biochemistry, Temple University Medical School, Philadelphia, Pennsylvania 19140 (J.M.G., S.M.T., and R.W.H.), Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel (L.R.), and C.S.I.R.O. Division of Human Nutrition, Adelaide, SA5000, Australia (F.J.B.). *Received November 15, 1974.* This work was supported in part by Grants AM-16009, CA-10916, and CA-12227 from the U.S. Public Health Service, by an American Cancer Society Institutional Grant, IN-88G, and by Research Grant Agreement 206, U.S.-Israel Binational Science Foundation. R.W.H. is the recipient of Career Development Award AM-15365 from the National Institute of Health.

[1] Abbreviations used are: P-enolpyruvate carboxykinase, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); dibutyryl cAMP, $N^6$, $O^2$-dibutyryladenosine 3′,5′-cyclic monophosphoric acid; cAMP, adenosine 3′,5′-cyclic monophosphoric acid; dexamethasone, 9*a*-fluoro-16*a*-methylprednisolone.

[2] J. M. Gunn, unpublished observations.

in a humidified incubator at 37° in an atmosphere of 5% $CO_2$–95% air. The medium was changed twice weekly and the cells were used for experiments at confluency, i.e., 10–14 days after subculture.

*Experimental Conditions.* For experimental purposes, Eagle's medium was replaced with a serum-free medium of exactly the same formulation and the cells left overnight (ca. 18 hr). The various additions of inducers and inhibitors were then made from solutions in the same serum-free medium, the final volume of medium being adjusted to 5 ml/flask and 10 ml/Petri dish. At various time intervals thereafter the cultures were pulsed for 15 min with [$^3$H]leucine (20 $\mu$Ci/flask or 50 $\mu$Ci/dish). Throughout the experimental period the cultures were maintained under sterile conditions at 37° in a moist atmosphere of 5% $CO_2$–95% air. Stock solutions of dexamethasone, insulin, and actinomycin D were prepared in ethanol, 0.006 *N* HCl, and acetone, respectively, these being diluted at least 100-fold with serum-free medium before addition to the cultures.

*Preparation of Extracts.* At the end of the 15-min pulse-labeling period the flasks or dishes were placed on ice, washed twice with cold 0.15 *M* NaCl, and detached into 1 ml of cold 0.25 *M* sucrose. The cells were then homogenized with a Virtis Model 45 microhomogenizer and centrifuged at 100,000*g* for 30 min at 0°. The supernatants were used for the determination of protein content, radioactivity incorporated into cytosol proteins and for the assay, and immunoprecipitation of P-enolpyruvate carboxykinase.

*Isolation and Quantitation of Radioactive P-enolpyruvate Carboxykinase.* Enzyme activity was measured in cytosol fractions as described previously (Ballard and Hanson, 1969). One unit of enzyme activity catalyzes the fixation of 1 $\mu$mol of $NaH^{14}CO_3$/min at 37°.

To 0.3–0.4 ml of supernatant, prepared from Reuber H-35 cells previously labeled with [$^3$H]leucine and assayed for P-enolpyruvate carboxykinase, was added sufficient, unlabeled, purified rat liver cytosol enzyme (Ballard and Hanson, 1969) to bring the total activity to 180 mU, Triton X-405 to a final concentration of 0.6%, and 250 mU of specific antibody (Hopgood et al., 1973) raised in goats against purified rat liver cytosol P-enolpyruvate carboxykinase. All subsequent procedures, including a second, control antigen–antibody precipitation, were exactly as reported by Hopgood et al. (1973). The radioactivity in cytosol proteins was determined following precipitation with 10% trichloroacetic acid and the protein concentration measured according to Lowry et al. (1951) using bovine serum albumin as standard.

In order to verify the specificity of the antibody, the antigen–antibody complex was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Cell cultures were placed in a serum-free medium containing 0.5 m*M* dibutyryl cAMP, 1 m*M* theophylline, and 2 $\mu M$ dexamethasone for 18 hr to induce P-enolpyruvate carboxykinase activity. Each flask was then pulsed-labeled with [$^3$H]leucine for 30 min, the cell contents of five similarly treated flasks were combined in 1 ml of sucrose, P-enolpyruvate carboxykinase activity was assayed, and 360 mU of radioactive enzyme was precipitated with 500 mU of antibody as described. The procedure adopted for gel electrophoresis of the first and second precipitates was as previously reported (Hopgood et al., 1973). A sample of purified, $^{14}$C-labeled rat liver cytosol enzyme (Ballard et al., 1974) was added as a marker before electrophoresis. Following electrophoresis the gels were scanned at 280 nm in 7% acetic acid on a Gilford Model 2400 spectrophotometer fitted with a linear transport attachment, then sliced into 1-mm fractions and the radioactivity in each fraction was counted in an Intertechnique SL30 liquid scintillation spectrometer (Hopgood et al., 1973).

The synthesis of P-enolpyruvate carboxykinase is reported both as a relative rate (percent incorporation of label into enzyme compared to cytosol protein in a 15-min pulse) and as cpm incorporated into enzyme per mg of cytosol protein per 15 min. The former expression corrects for differences in the labeling of the free amino acid pools.

*Measurement of Radioactivity in RNA.* The experiment was performed as described in the legend to Figure 4. At the end of the labeling period, the cells were washed thoroughly in 0.15 *M* NaCl and homogenized in 0.1 *M* sodium acetate, 5 m*M* NaEDTA, and 0.5% sodium dodecyl sulfate (pH 5). A portion of the homogenate was adjusted to 10% trichloroacetic acid, left at 0° for at least 1 hr, and centrifuged at 2000*g* for 10 min. The pellet was washed three times with 10% trichloroacetic acid, dissolved in NCS solubilizer, and the incorporation of radioactivity into total RNA determined.

The incorporation of label into poly(A) containing mRNA was determined as follows. An equal volume of buffer-saturated phenol was added to the homogenate and the mixture shaken for 2 min. An equal volume, with respect to phenol, of chloroform was then added and the mixture shaken again for a further 2 min. After centrifugation at 30,000*g* for 5 min the aqueous phase was removed and the organic phase reextracted with a half-volume of homogenization buffer. The aqueous phases were combined and extracted twice with 2 volumes of chloroform. After adjusting to 0.2 *M* NaCl, the aqueous solution was precipitated overnight with 2 volumes of ethanol. The nucleic acid precipitate was centrifuged at 30,000*g* for 10 min, dissolved in 0.1 *M* sodium acetate and 5 m*M* NaEDTA (pH 7), and reprecipitated with 2 volumes of ethanol at −20° for 1 hr. The precipitate was washed two times with ethanol and dissolved at a concentration of 10 $A_{260}$ units/ml in cellulose column buffer (10 m*M* Tris-HCl (pH 7.6), 0.5 *M* KCl, and 0.2 m*M* $MgCl_2$). One milliliter of the solution was passed through a cellulose column (0.25 g dry weight) previously equilibrated with cellulose column buffer (Schutz et al., 1972). The RNA retained by the column was eluted by washing with 10 m*M* Tris-HCl (pH 7.6) and a portion of the eluted RNA was counted for radioactivity and its $A_{260}$ determined. Using this procedure, poly(A) containing mRNA (i.e., that RNA bound to the cellulose column) was found to comprise approximately 5% of the isolated RNA applied to the column.

## Results and Discussion

We have determined the synthesis rate of P-enolpyruvate carboxykinase in Reuber H-35 cells as the rate of incorporation of [$^3$H]leucine into enzyme protein precipitated as an antigen–antibody complex. Since, by definition, one unit of antibody activity is that volume of antiserum needed to precipitate one unit of rat liver cytosol enzyme, the titration curve for this enzyme will have unit slope. Titration curves for cytosol preparations of control, cAMP, and glucocorticoid-induced Reuber H-35 cells satisfied the same condition,[2] indicating that P-enolpyruvate carboxykinase from the hepatoma cells and rat liver are antigenically similar. This conclusion is supported (Figure 1) by the sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of a

Table I: Effects of Dibutyryl cAMP and Dexamethasone on the Relative Synthesis Rate of P-enolpyruvate Carboxykinase.[a]

| Addition | Concn ($M$) | P-enolpyruvate Carboxykinase (mu/mg) | Radioactivity Incorporated into: Protein (cpm/mg × $10^{-3}$) | Radioactivity Incorporated into: P-enolpyruvate carboxykinase (% of total) |
|---|---|---|---|---|
| None | | 46 ± 3.2 | 28.27 ± 1.39 | 0.53 ± 0.08 |
| Dibutyryl cAMP | $5 \times 10^{-4}$ | 72 ± 4.2 | 27.20 ± 2.05 | 3.57 ± 0.53 |
| Dexamethasone | $2 \times 10^{-7}$ | 57 ± 6.0 | 36.62 ± 3.66 | 1.38 ± 0.08 |
| Theophylline | $10^{-3}$ | 51 ± 3.5 | 25.12 ± 0.75 | 0.94 ± 0.10 |
| Isobutyrate | $5 \times 10^{-4}$ | 53 ± 12.0 | 31.37 ± 2.12 | 0.83 ± 0.18 |
| Theophylline plus isobutyrate | $10^{-3}$ + $5 \times 10^{-4}$ | 44 ± 8.5 | 35.28 ± 9.71 | 1.25 ± 0.02 |

[a] The experimental procedure is described in the text. All additions were made at time zero, and the cells were pulsed with [$^3$H]leucine 3 hr later. Each value is the mean ±SEM of an average of four observations.

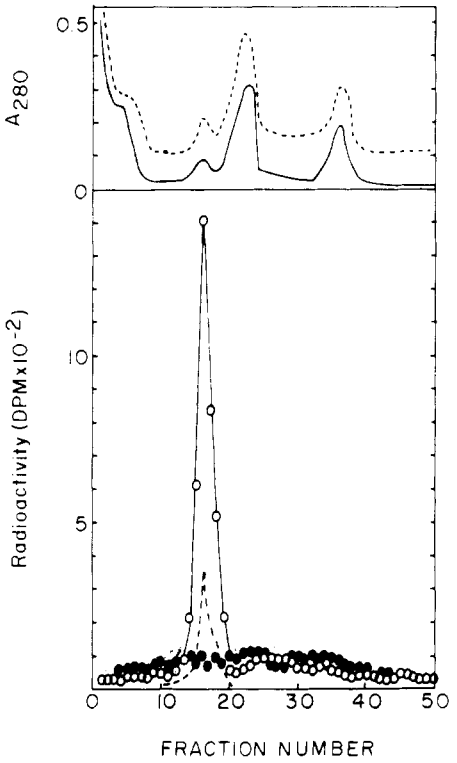


FIGURE 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of P-enolpyruvate carboxykinase from Reuber H-35 hepatoma cells. For details of the methodology, see Experimental Procedures. Fraction 1 is the top of the gel. Upper figure: $A_{280}$ scan of the gels for the first (- - - -) and the second (—) antigen-antibody precipitates. The ordinate for the first precipitate is displaced by 0.1 $A_{280}$ unit for convenience. Lower figure: radioactivity associated with 1-mm fractions of the same gels for the $^3$H-labeled hepatoma enzyme in the first antigen-antibody precipitate (○), the $^3$H-radioactivity associated with the second, control precipitate (●), and the $^{14}$C-labeled rat liver marker enzyme added prior to electrophoresis (- - - -).

$^3$H-labeled antigen-antibody precipitate (see Experimental Procedures). The absorption profiles at 280 nm for the gels of the first precipitate (hepatoma enzyme) and the second, control precipitate (purified rat liver enzyme) show that both enzymes are of similar molecular weight. The other two peaks correspond to the heavy and light chains of the antibody, respectively. Similarly, the $^3$H-labeled hepatoma enzyme comigrates with the $^{14}$C-labeled purified rat liver enzyme added as a marker. Specificity of the antibody is shown by the fact that most of the labeled protein precipitated comigrates with marker enzyme. The radioactivity associated with the second, control precipitate is due to nonspecific, low-affinity binding of labeled proteins. This represents 0.5–0.6% of the label incorporated into total cytosol proteins and, depending on the experimental condition, from 10 to 50% of the label in the first precipitate. Thus in preinduced cultures (Figure 1) the radioactivity in the second precipitate is approximately 10%, while in uninduced cultures it is about 50% of the labeling in the first precipitate.

We have used the specific antibody to P-enolpyruvate carboxykinase to establish that the increase in enzyme activity after the addition of dibutyryl cAMP or dexamethasone to Reuber H-35 cells (Barnett and Wicks, 1971) represents new antigenic material rather than an activation of preexisting enzyme (Table I). Since 1 m$M$ theophylline was present at all concentrations of dibutyryl cAMP used, the effect of theophylline alone is reported for comparison. Similarly, the effect of 0.5 m$M$ isobutyrate as well as 1 m$M$ theophylline plus 0.5 m$M$ isobutyrate is also included. Theophylline, which is capable of increasing P-enolpyruvate carboxykinase and tyrosine aminotransferase activities (Wicks, 1968; Fuller and Snoddy, 1970; Barnett and Wicks, 1971) presumably through an inhibition of phosphodiesterase (Butcher and Sutherland, 1962), produced a twofold increase in the relative synthesis rate of P-enolpyruvate carboxykinase. Isobutyrate was also effective while the stimulation of enzyme synthesis by isobutyrate and theophylline together was approximately additive. The maximum effect of dibutyryl cAMP on increasing enzyme synthesis was seen at a concentration of $5 \times 10^{-4}$ $M$ while the effect of $5 \times 10^{-6}$ $M$ dibutyryl cAMP was no different from that produced by theophylline alone (see Figure 5). The optimum concentration of dexamethasone was found to be in the range of $10^{-8}$–$10^{-7}$ $M$, with higher concentrations producing a variable and sometimes inhibitory effect. The maximum increase in the relative rate of enzyme synthesis produced by dexamethasone (2–3-fold) was less than that produced by dibutyryl cAMP (sevenfold), a result in agreement with the effects of these two inducers on enzyme activity (Barnett and Wicks, 1971). The fact that there is an increase in synthesis rate before a detectable increase in enzyme activity (Table I) is to be expected if P-enolpyruvate carboxykinase induction is the result of a specific increase in the synthesis rate of enzyme protein (Granner et al., 1970).

The synthesis rates of P-enolpyruvate carboxykinase presented in Table I are the same order as those previously published by us for rat liver in vivo (Ballard and Hopgood,

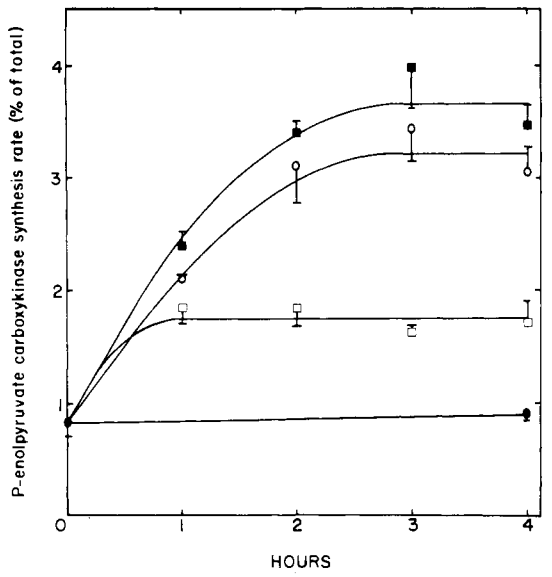


FIGURE 2: Time course of the effect of dibutyryl cAMP and dexamethasone on the relative synthesis rate of P-enolpyruvate carboxykinase. The experimental procedure is described in the text: (●) control; (○) 0.5 m*M* dibutyryl cAMP plus 1 m*M* theophylline; (□) 0.2 $\mu M$ dexamethasone; (■) 0.5 m*M* dibutyryl cAMP plus 1 m*M* theophylline plus 0.2 $\mu M$ dexamethasone. Each value is the mean ±SEM of duplicate determinations on four individual Petri dishes.

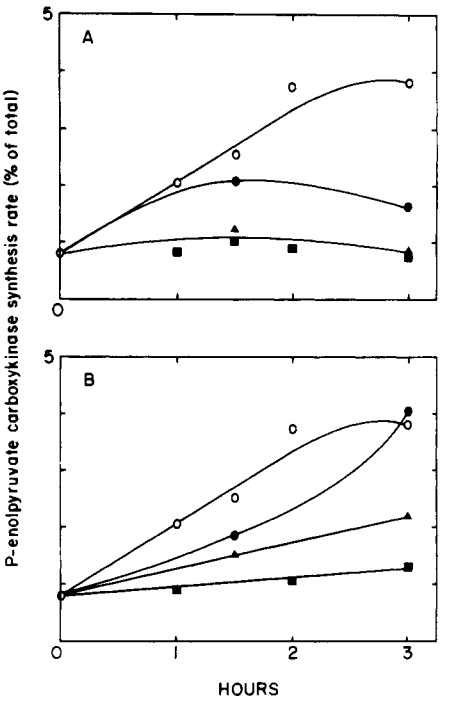


FIGURE 3: Effect of actinomycin D and cordycepin on the induction of P-enolpyruvate carboxykinase synthesis by dibutyryl cAMP. Various concentrations of actinomycin D (A) and cordycepin (B) were added to cells in a serum-free medium. Twenty minutes later, 0.5 m*M* dibutyryl cAMP plus 1 m*M* theophylline were added. At the various times indicated [$^3$H]leucine was added for 15 min and the relative rate of P-enolpyruvate carboxykinase synthesis determined as described in Experimental Procedures. (A) Actinomycin D at 0 (○), 0.4 (●), 1.0 (▲), or 5.0 (■) $\mu$g/ml; (B) cordycepin at 0 (○), 1 (●), 5 (▲), 25 or 50 (■) $\mu$g/ml. Each point is the mean of duplicate determinations on five similarly treated flasks.

1973; Hopgood et al., 1973; Tilghman et al., 1974), but are tenfold greater than the only other reported value for Reuber H-35 cells (Wicks and McKibbin, 1972). The basal synthesis rate of the enzyme under steady-state conditions (i.e., fed animals) is less than 1% of total cytosol proteins. In starved or diabetic animals, which have elevated hepatic cAMP levels (Jefferson et al., 1968; Exton et al., 1971, 1972, 1973; Park et al., 1972), this rate increases to approximately 3%. The rates of P-enolpyruvate carboxykinase synthesis in Reuber H-35 cells increased from 0.5% to a maximum of 3.6%, the final rate depending on the concentration of dibutyryl cAMP employed. There is good agreement between the concentrations of cyclic nucleotide employed here (e.g., $5 \times 10^{-5}$ *M*, Figure 5) and the concentrations of cAMP either generated in the perfused liver by glucagon or used in that system to promote gluconeogenesis and glycogenolysis (Exton et al, 1971; Park and Exton, 1972; Park et al., 1972). Also, since the circulating level of glucocorticoid is almost $10^{-7}$ *M* (Allen and Kendall, 1967), the optimal concentration of glucocorticoid found here is clearly physiological. These arguments, together with the data in Figure 1 suggest that Reuber H-35 cells in culture are a satisfactory model system for studying the regulation of hepatic P-enolpyruvate carboxykinase.

*Kinetics of Induction of P-enolpyruvate Carboxykinase Synthesis.* Dibutyryl cAMP produces a rapid increase in the relative rate of enzyme synthesis to a maximum value 2–3 hr after addition (Figure 2). If dexamethasone is added together with the cyclic nucleotide, the rate of increase in enzyme synthesis is similar. Although the two series of observations are not significantly different from each other, the fact that combinations of steroid and dibutyryl cAMP always produced a greater response than the nucleotide alone probably accounts for the additive, sometimes synergistic, effect of the two agents on enzyme activity (Wicks et al., 1973, 1974). Dexamethasone added alone produced an equally rapid increase in the relative rate of P-enolpyruvate carboxykinase synthesis over the first hour after addition (Figure 2). Thereafter enzyme synthesis remains constant for at least 4 hr at a rate approximately twofold greater than the controls.

The kinetics of the change in P-enolpyruvate carboxykinase activity in the presence of transcriptional inhibitors has led to the proposal that dibutyryl cAMP acts translationally (Wicks and McKibbin, 1972). A similar conclusion has been reached for tyrosine aminotransferase (Holt and Oliver, 1969; Barnett and Wicks, 1971; Butcher et al., 1971, 1972). Thus, actinomycin D at 0.2–0.4 $\mu$g/ml and cordycepin at 10–25 $\mu$g/ml only partially prevent an increase in enzyme activity produced by dibutyryl cAMP. In fetal liver, however, actinomycin D completely blocks the effect of the nucleotide on enzyme activity (Yeung and Oliver, 1968) as well as the increase in the synthesis rate of P-enolpyruvate carboxykinase (Hanson et al., 1973). In Reuber H-35 cells (Figure 3) the effect of actinomycin D and cordycepin on the dibutyryl cAMP stimulated increase in P-enolpyruvate carboxykinase synthesis is concentration dependent. This concentration dependence did not appear to be due to a nonspecific translational effect of the inhibitors because the mean rates of general cytosol protein synthesis were only reduced 20% by actinomycin D and 30% by cordycepin. Expression of the data in terms of the relative rate of P-enolpyruvate carboxykinase synthesis compensates for these general effects of the inhibitors.

One explanation for Figure 3 is that dibutyryl cAMP has a transcriptional effect on P-enolpyruvate carboxykinase synthesis and there is incomplete inhibition of RNA synthesis at the lower concentrations of inhibitor employed. How-

Table II: Effect of Cycloheximide on the Induction of P-enolpyruvate Carboxykinase Synthesis by Dibutyryl cAMP.[a]

| Group | Pretreatment (180 min) | Final addition (60 min) | Radioactivity in cytosol protein (cpm/mg × $10^{-3}$) | Radioactivity in P-enolpyruvate carboxykinase cpm/mg protein | Radioactivity in P-enolpyruvate carboxykinase % of total |
|---|---|---|---|---|---|
| A | None | None | 71.36 | 298 | 0.41 |
| | None | Dibutyryl cAMP | 52.93 | 520 | 0.98 |
| | None | Cycloheximide | 3.08 | 16 | |
| | None | Dibutyryl cAMP + cycloheximide | 6.01 | 45 | |
| B | Cycloheximide | None | 43.51 | 272 | 0.63 |
| | Dibutyryl cAMP + cycloheximide | None | 44.67 | 829 | 1.86 |
| | | Dibutyryl cAMP | 37.59 | 1130 | 3.01 |

[a] Cells were placed in a serum-free medium overnight. The following morning, additions were made to the flasks in group B as indicated for a pretreatment period of 3 hr. Dibutyryl cAMP and theophylline were added together to a final concentration of 0.5 and 1 m*M*, respectively, and cycloheximide to $10^{-5}$ *M*. The cells were then washed three times with serum-free medium with a 5-min incubation at 37° between each wash, and the final additions made as indicated to the flasks in both groups. One hour later the flasks were labeled for 15 min with [$^3$H]leucine and the radioactivity in P-enolpyruvate carboxykinase determined as described in the text. Each value is the mean of duplicate determinations on five similarly treated flasks.

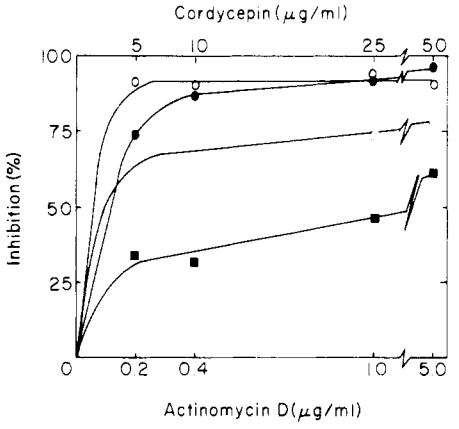


FIGURE 4: Concentration effects of actinomycin D and cordycepin on total RNA and mRNA synthesis. Actinomycin D (circles) and cordycepin (squares) at the concentrations indicated were added to cells in a serum-free medium. Two hours later 50 μCi of [$^3$H]orotic acid was added to each Petri dish for 1 hr and the labeling in total RNA and mRNA determined as described in Experimental Procedures. Closed symbols represent total RNA and open symbols mRNA. Each point is the mean of duplicate determinations on 5–6 similarly treated Petri dishes, and a representative experiment is illustrated.

ever, actinomycin D and cordycepin at 0.4 and 5 μg/ml, respectively, inhibited the synthesis of mRNA to a greater extent than total RNA synthesis (Figure 4). While higher concentrations of the antibiotics caused an additional inhibition of total RNA synthesis, mRNA synthesis was not further affected in the case of actinomycin D and only slightly increased by higher concentrations of cordycepin. Assuming that the effects of these inhibitors on P-enolpyruvate carboxykinase mRNA synthesis are similar to their effects on total mRNA synthesis, we suggest that mRNA synthesis is probably not required for dibutyryl cAMP action on P-enolpyruvate carboxykinase synthesis. Therefore, considering only the lower concentrations of these inhibitors, dibutyryl cAMP stimulation of P-enolpyruvate carboxykinase synthesis might be a post-transcriptional or translational event.

We have not resolved the cause of the concentration effects of the inhibitors. It is possible that P-enolpyruvate carboxykinase mRNA synthesis and total mRNA synthesis are not inhibited to the same extent by a given concentration of actinomycin D or cordycepin. However, in view of the known effects of actinomycin D on the initiation of protein synthesis and peptide chain elongation (Singer and Penman, 1972; Palmiter and Schimke, 1973), there may be a concentration dependent inhibition of an increase in template translation relative to that of other proteins. Experiments of the type depicted in Figures 3 and 4 indicate the caution with which inhibitor studies should be conducted and interpreted.

If dibutyryl cAMP is acting to increase template translation, it can be predicted that following preincubation of cells in the presence of the nucleotide and cycloheximide, there will be no stimulation of the relative rate of P-enolpyruvate carboxykinase synthesis when both inhibitor and inducer are removed (Wicks et al., 1973). On the other hand, if dibutyryl cAMP acts transcriptionally, or proximal to the cycloheximide block, to increase the amount of translatable P-enolpyruvate carboxykinase mRNA, such an experiment will lead to an increase in enzyme synthesis.

Cycloheximide at $10^{-5}$ *M* reduced general cytosol protein synthesis to 10% or less of the control value and reduced basal and dibutyryl cAMP induced rates of incorporation of label into P-enolpyruvate carboxykinase (Table II, group A). Preincubation in the presence of cycloheximide alone had no effect on the rate of P-enolpyruvate carboxykinase synthesis following removal of the inhibitor (Table II, group B). Under these conditions general protein synthesis recovered to 61% of the control value 1 hr after removal of cycloheximide. However, if dibutyryl cAMP is also included during the preincubation period and then washed out, the rate of incorporation of radioactivity into enzyme is increased threefold. Furthermore, readdition of the cyclic nucleotide to these flasks stimulates P-enolpyruvate carboxykinase synthesis to a greater extent then that usually seen after an hour's exposure to dibutyryl cAMP. Similar results were obtained with a cycloheximide concentration of $10^{-6}$ *M*.

Taken together, these data are indicative of an increase in translatable mRNA activity for P-enolpyruvate carboxykinase relative to that of other proteins. Therefore, dibutyryl cAMP is probably acting transcriptionally or proximal to the cycloheximide block at peptide chain elongation (Wettstein et al., 1964; Colombo et al., 1965; Stanners, 1966). The data in Figures 3 and 4 show that the nucleotide

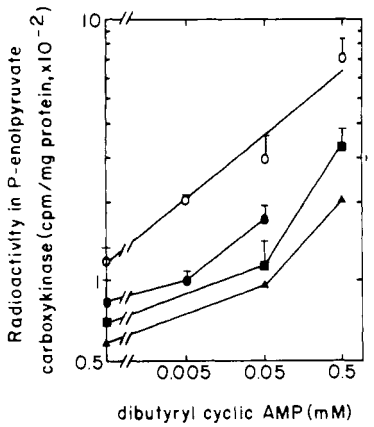


FIGURE 5: Concentration dependence of the insulin–dibutyryl cAMP interaction. Different concentrations of insulin together with the concentrations of dibutyryl cAMP (plus 1 m*M* theophylline) indicated were added to cells in a serum-free medium. Three hours later [$^3$H]leucine was added for 15 min and P-enolpyruvate carboxykinase synthesis determined as described in Experimental Procedures. The insulin additions were (○) none; (●) $5 \times 10^{-10}$ *M*; (■) $5 \times 10^{-9}$ *M*; and (▲) $5 \times 10^{-8}$ *M*. Each point is the mean (±SEM as indicated) of duplicate determinations on one or more groups of five similarly treated flasks. The data are presented as a log–log plot for convenience.

probably does not increase P-enolpyruvate carboxykinase mRNA synthesis. Furthermore, previous work from our laboratory (Tilghman et al., 1975) indicates that dibutyryl cAMP might act to increase translation of a relatively unchanged pool of mRNA. Therefore, cAMP might exert some post-transcriptional effect on P-enolpyruvate carboxykinase synthesis at a point between the inhibitory sites for cordycepin and cycloheximide, that is, between poly(A) addition to mRNA (Penman et al., 1970; Darnell et al., 1971; Singer and Penman, 1972) and peptide chain elongation. One suitable control point would be at initiation of protein synthesis. However, a definitive statement concerning the effect of cAMP on P-enolpyruvate carboxykinase awaits the isolation and quantitation of mRNA specific for the enzyme.

Induction of P-enolpyruvate carboxykinase and tyrosine aminotransferase by glucocorticoids in Reuber H-35 cells in culture is blocked by actinomycin D (Lee et al., 1970; Wicks and McKibbin, 1972) suggesting that these hormones act transcriptionally. We also found that actinomycin D at 0.4 μg/ml almost completely suppressed the normal increase in the synthesis rate of P-enolpyruvate carboxykinase seen with dexamethasone.[2] However, glucocorticoids have only a small effect, compared to dibutyryl cAMP, on P-enolpyruvate carboxykinase activity and synthesis rate (Figure 2), so that inhibition would be difficult to distinguish from controls.

*Effect of Insulin on P-enolpyruvate Carboxykinase Synthesis.* Insulin partially prevents the increase in P-enolpyruvate carboxykinase activity produced by dibutyryl cAMP and dexamethasone in Reuber H-35 cells in culture (Barnett and Wicks, 1971), a result in keeping with the in vivo effects of the hormone. Experiments using the perfused liver have indicated that the insulin effect on lowering cAMP levels depends on the concentration of insulin employed as well as the initial intracellular concentrations of the nucleotide (Park et al., 1972). We therefore investigated the concentration dependence of the interaction between insulin, glucocorticoid, and dibutyryl cAMP on the synthesis rate of P-enolpyruvate carboxykinase (Figure 5 and Table III).

Table III: Concentration Dependence of the Interaction between Insulin and Dexamethasone.[a]

| | Insulin Concn (*M*) | | |
|---|---|---|---|
| Addition (*M*) | 0 | $5 \times 10^{-10}$ | $5 \times 10^{-9}$ |
| None | 0.60 ± 0.07 | 0.70 ± 0.15 | 0.34 ± 0.20 |
| Dexamethasone | | | |
| $2 \times 10^{-6}$ | 1.22 ± 0.07 | | 0.70 ± 0.01 |
| $2 \times 10^{-7}$ | 1.34 ± 0.11 | 0.93 ± 0.11 | 0.73 ± 0.02 |
| $2 \times 10^{-8}$ | 1.17 ± 0.06 | 1.01 ± 0.08 | |

[a] Experimental details are described in the text. Dexamethasone and insulin were added at the concentrations indicated at time zero, and the data are reported as the relative rate of P-enolpyruvate carboxykinase synthesis measured 3 hr later. Each value is the mean ±SEM of duplicate determinations on two or more groups of five similarly treated flasks.

As the concentration of cyclic nucleotide is increased from $5 \times 10^{-6}$ to $5 \times 10^{-4}$ *M* a greater concentration of insulin is required in order to prevent an increase in the synthesis rate of P-enolpyruvate carboxykinase (Figure 5). Clearly the final rate of enzyme synthesis depends on the concentrations of both the inhibitor and inducer. Since concentrations of $10^{-5}$ *M* cAMP and $10^{-10}$ *M* insulin are within a physiological range, such an antagonistic interaction probably occurs in vivo. These concentration effects are not as clearly seen with dexamethasone (Table III) because of the similarities in the rates of synthesis of P-enolpyruvate carboxykinase at all three concentrations of the glucocorticoid tested. Nevertheless, $5 \times 10^{-9}$ *M* insulin was more effective at inhibiting an increase in enzyme synthesis than was a concentration of hormone an order of magnitude lower. Addition of insulin at a later stage of induction (one or more hours after the inducers) either prevents any further increase[2] or causes a decrease in enzyme synthesis (Tilghman et al., 1975). Thus insulin effectively antagonises the actions of both dexamethasone and dibutyryl cAMP on the synthesis rate of P-enolpyruvate carboxykinase.

Previous studies in whole animals (Tilghman et al., 1974) suggested a requirement for glucose in the manifestation of the insulin effect on P-enolpyruvate carboxykinase synthesis since injection of the hormone into fasted-diabetic animals did not lower the synthesis rate of the enzyme. Thus, the action of insulin on enzyme synthesis may require some degree of concomitant glucose utilization. However, in Reuber H-35 cells this is not the case (Figure 6). As the glucose concentration is lowered from 15 m*M* to zero the basal and dibutyryl cAMP stimulated synthesis rates of P-enolpyruvate carboxykinase remain the same, as does the insulin effect. With dexamethasone, glucose removal increases the glucocorticoid-enhanced relative rate of enzyme synthesis, but the degree of inhibition caused by insulin remains the same. This apparent increase in the magnitude of the glucocorticoid response obscures an actual decrease (of approximately 50%) in the radioactivity incorporated into cytosol protein and P-enolpyruvate carboxykinase in those dishes containing zero glucose. This was not due to a decrease in amino acid uptake.[2]

These data therefore demonstrate a direct effect of insulin per se on enzyme induction and shows that glucose is not necessary for the manifestation of the insulin effect on P-enolpyruvate carboxykinase synthesis rate. This result contrasts to those found previously in vivo (Tilghman et al., 1974). However, it is possible that the glucose effect in vivo

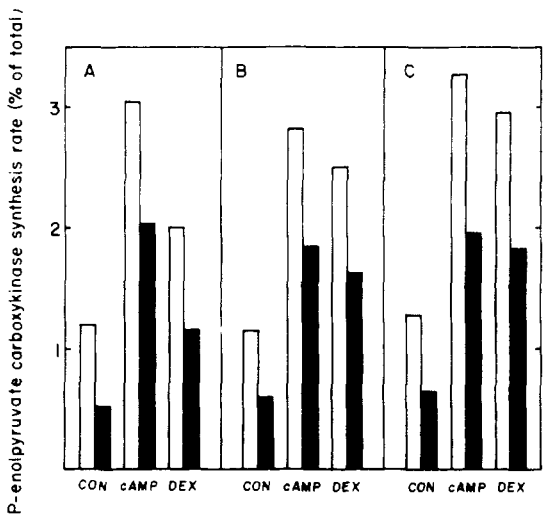


FIGURE 6: Effect of acute changes in glucose concentration of the medium on the insulin inhibition of P-enolpyruvate carboxykinase synthesis rate. The medium in a series of Petri dishes was changed for one containing ca. 15 m*M* (A), 5 m*M* (B), or zero glucose (C). In the latter case the medium was made with dialyzed serum and was fortified with 5 m*M* alanine and 5 m*M* pyruvate. Six hours later the medium was changed again for one of identical composition, but containing no serum. The next morning either 0.5 m*M* dibutyryl cAMP plus 1 m*M* theophylline (cAMP) or 0.2 $\mu M$ dexamethasone (Dex) was added and the relative synthesis rate of P-enolpyruvate carboxykinase measured 3 hr later as described in Experimental Procedures. Insulin, $5 \times 10^{-9}$ *M*, was added at the same time as the inducers to half the dishes (filled bars). The glucose concentration in the medium was measured at the end of the experiment and found to be 14.2 m*M*, 4.6 m*M*, and <0.1 m*M* for the dishes in groups A, B, and C, respectively. A representative experiment is shown, each value being the mean of duplicate determinations on a single Petri dish.

is linked to insulin release and suppression of glucagon release both of which are apparently interrelated by negative feedback controls modulated in turn by the blood glucose concentration (Samols et al., 1972). Since hypoglycemia is a potent stimulator of glucagon release (Unger and Lefebvre, 1972), it is not surprising that the suppressive action of insulin on P-enolpyruvate carboxykinase synthesis in vivo also requires glucose.

These and other studies (Hopgood et al., 1973; Tilghman et al., 1974, 1975) indicate that hepatic P-enolpyruvate carboxykinase activity in vivo is controlled by a mutually antagonistic interaction of cAMP and insulin exerted on the synthesis rate of the enzyme. Since hepatic enzyme activity (Shrago et al., 1963; Reshef and Hanson, 1972) and cAMP concentration (Exton et al., 1971; Park and Exton, 1972) are both increased by glucagon, enzyme activity in vivo will ultimately depend on the circulating insulin/glucagon ratio. Insulin has been shown to antagonize the effect of glucagon on hepatic cAMP concentration and to decrease the level of the cyclic nucleotide in the perfused liver and in diabetic rats in vivo (Park et al., 1972). Thus the inhibitory effect of insulin on dibutyryl cAMP action may be due to a decrease in the intracellular concentration of the cyclic nucleotide. However, several reports (Goldberg et al., 1967; Pitot and Jost, 1968; Sudilovsky et al., 1971; Nichols and Goldberg, 1972; Treadow and Khairallah, 1972; Sudilovsky and Pitot, 1973) argue that the glucose/insulin effect has no obligatory relationship with a fall in cAMP concentration. Therefore, the mechanism whereby insulin decreases the synthesis rate of P-enolpyruvate carboxykinase remains to be resolved.

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# Binding of Hormones to Receptors. An Alternative Explanation of Nonlinear Scatchard Plots†

Simeon I. Taylor*

ABSTRACT: Binding of $^{125}$I-labeled hormones to receptors can usually be inhibited by addition of unlabeled hormone. In analyzing such binding-inhibition data, it is commonly assumed that both labeled and unlabeled hormones are bound with equal affinity. When this assumption is made incorrectly, an artifactually nonlinear Scatchard plot results. Equations to describe these nonlinear Scatchard plots are derived. These results are discussed with regard to previously published observations of nonlinear Scatchard plots for binding of insulin to its receptor.

Interactions of polypeptide hormones with membrane receptors are the subject of intense research interest (Roth, 1973). One approach to the investigation of these interactions has been to study the binding of radioactively labeled hormone analogs to receptors. Such binding data are routinely displayed graphically by plotting the ratio of bound to free hormone as a function of the concentration of hormone bound—i.e., a Scatchard plot (Scatchard, 1949). In the case of certain hormones—insulin (Hammond et al., 1972; House, 1971; Freychet et al., 1972; Kahn et al., 1974) among others (Lefkowitz et al., 1970; Bockaert et al., 1972; Shlatz and Marinetti, 1972)—nonlinear Scatchard plots have been reported. Many hypothetical models have been proposed to account for these nonlinearities: cooperative binding interactions (House, 1971; De Meyts et al., 1973), polymerization of hormone (House, 1971; Nichol et al., 1969), heterogeneity of receptors (Hammond et al., 1972; Freychet et al., 1972; Kahn et al., 1974; Klotz and Hunston, 1971), or heterogeneity of labeled hormone. It is the purpose of this communication to propose another possible explanation of apparent nonlinearity in the Scatchard plot. In addition, the plausibility of this model will be evaluated insofar as it applies to the binding of one hormone, insulin, to its receptor.

In a typical binding study, a low concentration of $^{125}$I-labeled hormone is allowed to bind to receptors. Varied concentrations of unlabeled hormone are added and inhibition of $^{125}$I binding is observed. The data are analyzed with the aid of a Scatchard plot—assuming that iodinated and na-

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received November 4, 1974.*

* Supported by The Insurance Medical Scientist Scholarship Fund and Massachusetts Mutual Life Insurance Company, Springfield, Massachusetts. Please address correspondence to Dr. S. I. Taylor, 18 Ware Street, Cambridge, Massachusetts 02138.