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Rapid Loss of Translatable Messenger RNA of Phosphoenolpyruvate Carboxykinase During Glucose Repression in Liver

(actinomycin D/cordycepin/cyclic AMP/insulin)

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ABSTRACT **The rate of synthesis of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) in rat liver decreased with a half-life of 30 min after fasted rats were refed either a chow diet or glucose. A requirement for both glucose and insulin to bring about this rapid deinduction was shown, as well as the ability of dibutyryl adenosine 3′:5′-cyclic monophosphate to block the decrease in enzyme synthesis. Estimates of the stability of messenger RNA of phosphoenolpyruvate carboxykinase were made by using the inhibitors actinomycin D and cordycepin to block further messenger RNA synthesis, and then measuring the decrease in specific enzyme synthesis. It is suggested that the use of actinomycin D yields an overestimation of the template stability. The results with cordycepin imply that the enzyme messenger RNA has a short half-life of approximately 1 hr. Thus, it is possible that deinduction may proceed by way of a decrease in messenger RNA production, leading to a rapid fall in the synthesis of phosphoenolpyruvate carboxykinase.**

The "deinduction" from a high content of a particular enzyme to a low content often occurs at a rate that is comparable to the known degradation rate of that enzyme (1). This observation can be explained either by a transient acceleration of degradation as a result of the new stimulus or by a rapid cessation of synthesis while degradation continues at the previous rate. With several enzymes it has been shown that degradation is not accelerated under conditions when the content of the enzyme is falling, so that a curtailment of enzyme synthesis must be invoked in order to explain the rapidity of the change. This fall in the synthesis rate has been demonstrated directly by specific immunochemical procedures with tyrosine aminotransferase (2, 3), serine dehydratase (4), and phosphoenolpyruvate carboxykinase (PEPCK) [EC 4.1.1.32; GTP:oxaloacetate carboxy-lyase (transphosphorylating)] (5), in either mammalian liver or cultured liver-derived cells.

PEPCK is predominantly a cytosolic enzyme in rat liver, and plays a key regulatory role in gluconeogenesis (6). It increases in activity during starvation and diabetes, and this increase can be overcome by refeeding or insulin maintenance, respectively (7, 8). In this report the rate of deinduction of PEPCK initiated by refeeding fasted rats is examined, and estimates are made of the PEPCK template mRNA stability with the use of transcriptional inhibitors in an attempt to understand the molecular mechanisms underlying the rapid block in enzyme synthesis.

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; cAMP, adenosine 3′:5′-cyclic monophosphate.

MATERIALS AND METHODS

Materials. Actinomycin D, cordycepin, and $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (Na salt) were obtained from Sigma Chemical Co. Crystalline glucagon-free insulin was a gift of the Eli Lilly Co. [4,5-^{3}H]leucine (30–50 Ci/mmol) was purchased from New England Nuclear Corp., and NCS solubilizer from Amersham-Searle Corp. Goat antiserum specific for PEPCK was prepared as described (5).

Animals. Seven-week-old male rats maintained on standard laboratory chow were used in all studies. Fasted animals had food withdrawn 20 hr before the start of each experiment. Refeeding was commenced with the presentation of chow or by intragastric intubation of 2 g of glucose in 4 ml of 0.9% NaCl. Diabetes was induced by injecting alloxan subcutaneously (7.5 mg/100 g of body weight) and allowing 5 days for marked glucosuria to develop. The insulin-treated diabetics were injected subcutaneously with 3 units of insulin at 0 hour and 1 unit each subsequent hour during the experiment. Actinomycin D (100 μg/100 g of body weight) and cordycepin (1.5 mg/100 g) were administered intraperitoneally at 2-hr intervals, while dibutyryl cAMP (3 mg/100 g) was given hourly.

Immunoprecipitation of PEPCK. At various times each animal was injected with 100 μCi of [4,5-^{3}H]leucine intraperitoneally and killed by cervical dislocation 30 min later. The livers were removed, homogenized in 0.25 M sucrose, and centrifuged at 105,000 $\times$ g for 40 min (9). The supernatant fraction was used for the assay of PEPCK (10) and for the measurement of isotope incorporation into total cytosol proteins (11) and PEPCK (5). Radioactivity in PEPCK was determined by incubating 0.18 unit of enzyme with a 40% excess of specific antiserum in a total volume of 0.3 ml for 15 min at 37° and overnight at 5°. The immunoprecipitate was centrifuged, washed three times with 0.9% NaCl at 0°, and dissolved in 0.2 ml of NCS solubilizer. The radioactivity was measured in 10 ml of a scintillation fluid containing 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene. The supernatant from the first antibody–antigen precipitate was used for a second precipitation by addition of 0.18 unit of partially purified nonradioactive PEPCK with a further 40% excess of antiserum. This precipitate was washed and counted as before, and the radioactivity was subtracted from that in the first precipitate. The criteria for specificity of

this procedure were established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and have been reported (5).

RESULTS

Deinduction of PEPCK. Fasted animals were refed, and the change in the rate of PEPCK synthesis was measured during a 30-min pulse. The results are expressed as a percentage of the labeling of total cytosol protein. A fall in the relative rate of PEPCK synthesis occurs after refeeding, with a half-life of 30 min (Fig. 1), but there is an initial lag between the presentation of food and the true cellular stimulus. In the first 90 min of deinduction there is no marked change in the incorporation of [^{3}H]leucine into total cytosol proteins, no alteration in PEPCK activity per total liver, and no measurable alteration in the degradation rate of the enzyme (5).

An involvement of glucose in the deinduction process is apparent from Table 1, where we show that glucose intubated into the stomachs of fasted rats produces a comparable fall in the rate of PEPCK synthesis to that found in animals refed a normal diet. However, glucose alone cannot be the stimulus since PEPCK is synthesized at a high rate in livers of alloxan diabetic rats, animals that have elevated concentrations of blood glucose. When insulin is administered to these diabetic rats an abrupt fall in the relative rate of PEPCK synthesis occurs, with a similar time course to that noted when fasted animals are refed. As glucose intubation or refeeding a chow diet would lead to an increase in the blood glucose level and thereby release of insulin from the pancreas, it is possible that all three examples of deinduction are mediated by a rise in circulating insulin. This is unlikely, however, since insulin is not effective in reducing the PEPCK pulse when the hormone is injected into either normal fasting animals or diabetic fasting animals (Table 1). Clearly, both insulin and glucose are required to elicit the response on PEPCK synthesis.

The ability of dibutyryl cAMP to prevent deinduction of PEPCK by either refeeding of chow or glucose to fasted rats or insulin administration to alloxan diabetics (Table 2) suggests that the fall in the rate of PEPCK synthesis is mediated by a decrease in the intracellular cAMP concentration. Such an effect could be elicited by glucose and insulin directly on the liver, or by an extrahepatic action, such as the suppression of glucagon secretion from the pancreas. There is evidence that the alpha-cell is insulin-sensitive and that both insulin and glucose are required for a block in glucagon secretion (12).

TABLE 1. *Short-term effects on hepatic PEPCK synthesis in vivo*

Treatment	Radioactivity in PEPCK (% of cytosol protein)
Fasted	3.20 ± 0.27
refed glucose 1 hr	1.23 ± 0.21
refed glucose 2 hr	0.49 ± 0.06
Fasted	2.66 ± 0.35
plus insulin 2 hr	2.78 ± 0.13
Diabetic	3.49 ± 0.14
plus insulin 1 hr	1.02 ± 0.18
plus insulin 2 hr	0.37 ± 0.06
Fasted diabetic	2.44 ± 0.17
plus insulin 2 hr	2.04 ± 0.20

The relative radioactivity in liver PEPCK, expressed as a percentage of counts in total cytosol protein, was determined as described in *Methods*. Values are the means ± SEM for four animals in each group.

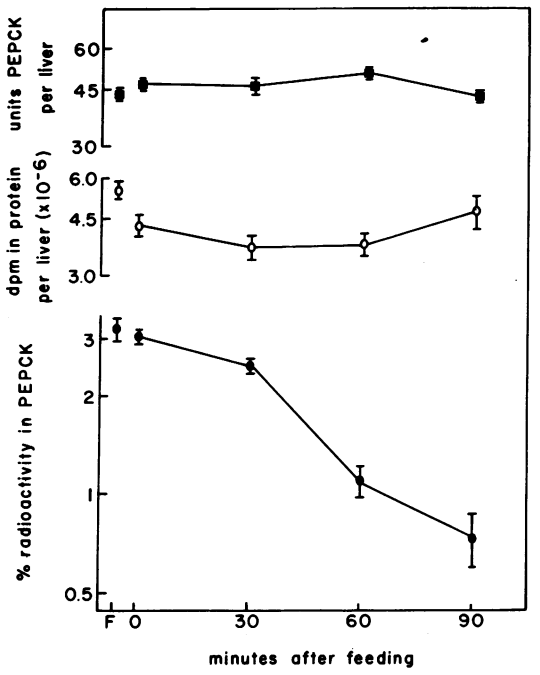

FIG. 1. Deinduction of hepatic PEPCK during refeeding. Animals were fasted for 20 hr and refed a chow diet for periods up to 90 min. At various times during the refeeding periods, each animal was given [^{3}H]leucine for 30 min, and the relative incorporation of radioactivity into PEPCK was determined. (●) % protein radioactivity in PEPCK; (○) dpm per liver in total cytosol protein (values have been multiplied by 10^{-6}); (■) units of PEPCK activity per liver. Values are the means ± SEM for six animals in each group. *F*, fasted.

Stability of PEPCK mRNA and Deinduction. The rapid decrease in the rate of PEPCK synthesis observed during refeeding could be the result of either a transcriptional or a translational block. However, since the observed reduction is measured as a fall in PEPCK synthesis *relative* to total cytosol proteins, the effect must be either specific to PEPCK or possibly shared by a group of proteins that represent a very small fraction of total protein synthesis. Furthermore, the

TABLE 2. *Prevention of deinduction by dibutyryl cAMP*

Treatment	Radioactivity in PEPCK (% of cytosol protein)
Fasted 20 hr	2.34 ± 0.10
refed	0.23 ± 0.11
refed plus dibutyryl cAMP	2.52 ± 0.19
refed glucose	0.48 ± 0.05
refed glucose plus dibutyryl cAMP	3.24 ± 0.26
Diabetic	2.49 ± 0.49
plus insulin	0.42 ± 0.13
plus insulin and dibutyryl cAMP	2.23 ± 0.45

Each animal was given [^{3}H]leucine for 30 min, 2 hr after the various treatments. The radioactivity in PEPCK was determined relative to that in cytosol proteins. Values are the means ± SEM for data on three animals in each group.

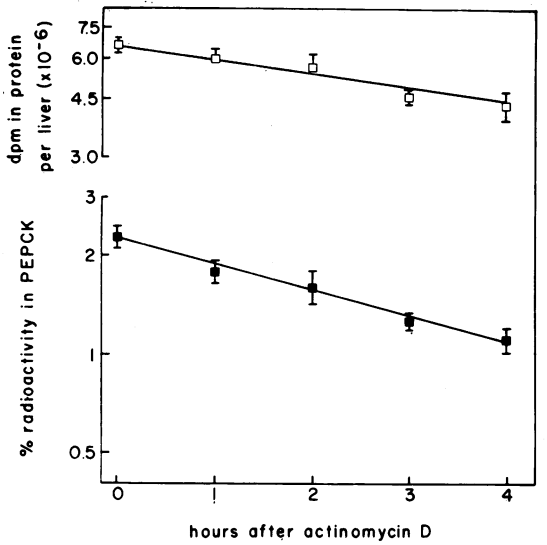

FIG. 2. Effects of actinomycin D on PEPCK synthesis. Fasted rats were injected with actinomycin D at zero time with an additional injection at 2 hr for the later time course, and the rates of protein labeling and PEPCK synthesis were determined at various times. (■) % protein radioactivity in PEPCK; (□) dpm per liver in total cytosol protein ($\times 10^{-6}$). Values are the means ± SEM for five animals in each group.

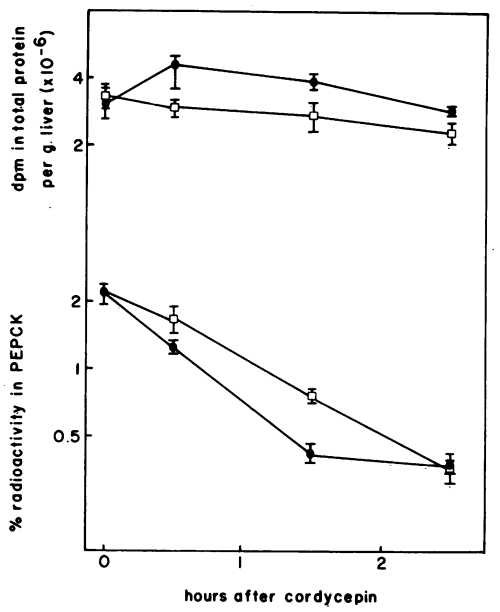

FIG. 3. Effect of cordycepin on PEPCK synthesis. Fasted rats were injected with cordycepin at zero time, with an additional injection at 2 hr for the later time points, and the rates of protein labeling and PEPCK synthesis were determined with 30-min pulses. Cordycepin doses were (●) 1.5 mg/100 g of body weight and (□) 3 mg/100 g. Values are the means ± SEM for six animals in each group.

effect on PEPCK synthesis during deinduction can only occur by a reduction in the rate of transcription of specific mRNA if that mRNA has a half-life of less than 30 min, for even if transcription were halted entirely, the enzyme would continue to be synthesized so long as translatable template was present. Recent measurements of the average half-life of total cellular mRNA show that this RNA is very stable, perhaps with a half-life of several days (13–16). Such experiments are based on the rate of radioactivity loss from poly(A)-containing RNA that had been labeled with radioactive uridine, and do not imply that all mRNA is long lived. By analogy with cell proteins, we should consider that some templates may be short lived, especially those that code for enzymes like PEPCK or tyrosine aminotransferase, which rapidly adapt in content to changing nutritional and hormonal states.

It is not possible to measure directly the rate of turnover of PEPCK mRNA without first being able to isolate it. However, indirect estimates can be made by using inhibitors of transcription such as actinomycin D, to block RNA synthesis, and then following the change in specific enzyme synthesis. Such an approach assumes that actinomycin D is specifically inhibiting transcription, and that translation is being limited by the amount of available mRNA. During 4 hr after the administration of actinomycin D to fasted rats, there is a gradual fall in the extent of [^{3}H]leucine incorporation into liver cytosol proteins (Fig. 2). However, the change in the relative rate of PEPCK synthesis occurs with a half-life of 4 hr, and implies that in the presence of a block in RNA synthesis, translation of PEPCK mRNA continues for a time period considerably longer than the half-life of the deinduction process.

Another explanation is that actinomycin D may stabilize PEPCK mRNA, thus leading to an overestimate of template stability. This possibility is in accord with the post-transcriptional control hypothesis of Tomkins, who envisages that the product of a regulatory gene is a labile inhibitor of translation and functions by promoting mRNA degradation (17). When transcription is blocked by actinomycin D, the inhibitor activity is lost first, due to its greater lability, and thus the more stable mRNA continues to be expressed (18).

To test the possibility that actinomycin D might be increasing the stability of PEPCK mRNA, the drug was administered to diabetic rats 30 min before insulin was injected. The parallel experiment could not be performed by refeeding fasted rats, since actinomycin D was found to interfere with food absorption from the stomach, and this presumably blocked deinduction by an unrelated process (unpublished observations). The data in Table 3 illustrate that actinomycin D prevented the rapid fall in the rate of PEPCK synthesis initiated by insulin, thus suggesting a preservation of some factor required for PEPCK translation that was normally lost during deinduction.

Use of Cordycepin to Estimate PEPCK mRNA Stability. To distinguish between a possible effect of actinomycin D specifically on the translation or stability of PEPCK mRNA and a more general effect due to a transcriptional block, we estimated the apparent stability of the PEPCK mRNA with cordycepin. This compound is generally considered to block the appearance of poly(A)-containing mRNA in the cytosol (13, 19, 20). Thus its action, while not specifically on transcription, should allow an independent assessment of the stability of PEPCK mRNA. This assumes, of course, that the mRNA for PEPCK contains poly(A), which is probable since all mRNAs studied to date, with the exception of histone mRNA, contain poly(A) (21).

Unlike actinomycin D, the administration of cordycepin to fasted rats produced a very rapid fall in the rate of PEPCK synthesis, with a half-life between 40 and 60 min (Fig. 3) at concentrations of cordycepin that produced no inhibition of total cytosol protein synthesis. Also, it did not interfere with the deinduction of PEPCK synthesis during the refeeding of

TABLE 3. *Effect of transcriptional inhibitors on deinduction*

Treatment	Radioactivity in PEPCK (% of cytosol protein)
Diabetic	2.69 ± 0.13
plus insulin	0.29 ± 0.07
plus insulin and actinomycin D	1.13 ± 0.19
plus actinomycin D	1.44 ± 0.36
Fasted 20 hr	2.08 ± 0.17
refed	0.25 ± 0.06
refed with cordycepin	0.21 ± 0.04
plus cordycepin	0.53 ± 0.06
plus actinomycin D	1.39 ± 0.06
plus actinomycin D and cordycepin	1.26 ± 0.15

Actinomycin D and cordycepin were administered 30 min before administration of insulin or refeeding. The animals were pulsed with [^{3}H]leucine 2 hr later, and killed after 30 min. The radioactivity in PEPCK relative to that in cytosol proteins was determined. Values are the means ± SEM for data on four animals in each group.

fasted rats (Table 3). When actinomycin D and cordycepin were administered simultaneously to fasted rats, the rate of synthesis of the enzyme decreased to the same degree as when actinomycin D was given alone (Table 3).

DISCUSSION

In a previous report (5), we showed that changes in PEPCK activity during a fasting-refeeding cycle were due to alterations in the rate of synthesis of the enzyme, while degradation remained unchanged. The fall in the rate of PEPCK synthesis after fasted rats were refed occurs with a half-life of 30 min (Fig. 1). A similar time course is obtained when insulin was administered to alloxan diabetic rats (Table 1), suggesting that the same mediator is responsible for both effects. We inferred that both glucose and insulin are required for deinduction to occur, since neither alone is sufficient to decrease the rate of PEPCK translation (Table 1). The interaction among glucose, insulin, and the synthesis of PEPCK is not clear, although the ability of dibutyryl cAMP to overcome enzyme deinduction suggests that deinduction may be mediated by a fall in cAMP concentration. Cyclic AMP has been shown to increase the rate of PEPCK synthesis in fetal (22) and adult (23) rat liver as well as in hepatoma cells (24). Wicks (24, 25) has suggested that in Reuber H-35 cells cAMP acts post-transcriptionally on several enzymes, including PEPCK. However, Sudilovsky *et al.* (26) have argued against a role for cAMP in the deinduction of serine dehydratase by glucose since no fall in hepatic cAMP concentration was observed.

The rapidity of the decrease in PEPCK synthesis during deinduction led us to question whether there was a very short template half-life for this enzyme. We therefore estimated the template stability with two inhibitors of mRNA synthesis, actinomycin D and cordycepin. While the experiments with actinomycin D gave an approximate half-life for PEPCK mRNA of several hours, the results from the use of cordycepin suggested a much shorter half-life.

Such markedly different results with actinomycin D and cordycepin have been reported elsewhere (27, 28). Grayson and Berry (27) recently showed a significant difference in the estimation of the half-life of a specific mRNA using these two transcriptional inhibitors, with actinomycin D giving the longer half-life. The authors proposed that the discrepancy was the result of the differential sites of action of the inhibitors: that is, actinomycin D inhibits only transcription, leaving a large nuclear pool of heterogeneous RNA that continues to supply cytoplasmic mRNA, while cordycepin is believed to block the conversion of heterogeneous RNA to mRNA, and therefore would prevent any replenishment of the cytoplasmic mRNA pools. That this is not occurring with PEPCK mRNA is suggested from the results of a mixing experiment given in Table 3, where the administration of actinomycin D together with cordycepin gave results identical to actinomycin D alone. This result suggests that actinomycin D is stabilizing the PEPCK mRNA beyond the cordycepin site in inhibition, or alternatively, that actinomycin D is interfering with the action of cordycepin. It has been shown that heterogeneous RNA turns over rapidly in the nucleus (29) and that actinomycin D, at the concentration used in these experiments, accelerates the breakdown of nuclear RNA (30). Such findings argue against a long-lived replenishment from the nucleus of mRNA pools in the presence of actinomycin D.

Butcher *et al.* (28) have reported different responses of tyrosine aminotransferase to cordycepin and actinomycin D. Cordycepin was unable to elicit the "superinduction" of the enzyme, although it was as effective as actinomycin D in preventing the initial induction by cortisol in Reuber H-35 or HTC cells. Actinomycin D appeared to increase the amount of translatable tyrosine aminotransferase mRNA in a manner that is still unresolved.

Singer and Penman (13, 16) have recently reported that in HeLa cells, actinomycin D caused a decrease in the half-life of cytoplasmic mRNA, as measured by the binding of poly(A)-containing RNA to oligo(dT)–cellulose columns. They also observed a biphasic decay in the radioactivity of this mRNA fraction, and proposed that short-lived and long-lived pools of mRNA exist, with approximate half-lives of 7 and 24 hr, respectively (16). On the other hand, Endo *et al.* (31) estimated the turnover of mRNA bound to rat-liver polysomes by measuring the radioactivity susceptible to ribonuclease attack. They showed a prolongation in mRNA half-life in the livers of animals treated with actinomycin D. While it is not possible at this time to resolve these conflicting results, it is evident that actinomycin D is interfering with the normal metabolism of RNA, and that it has limited use in experiments designed to study the kinetics of mRNA turnover.

Unlike prokaryotic cells, where the mRNA is turning over in a few minutes (32), eukaryotic cells have relatively stable mRNA pools (13–16). Nevertheless, it is possible that some proteins may have very short-lived templates, and that their synthesis may be rapidly turned off by a transcriptional block. Our experiment with cordycepin (Fig. 3) showed that in the absence of any effect on total protein synthesis, PEPCK synthesis decreased, with a half-life of 40 to 60 minutes. If cordycepin is interfering with translation, it is doing so in a very specific manner. Thus, it is possible that the fall in PEPCK synthesis brought about by refeeding fasted rats may be due to a block in the appearance of new PEPCK mRNA in the cytoplasm, with the rapid decay of preformed mRNA. The proof of such an hypothesis would depend on demonstrating whether PEPCK mRNA remains in the cell after deinduction.

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