Regulation of Phosphoenolpyruvate Carboxykinase (GTP) Synthesis in Rat Liver Cells

RAPID INDUCTION OF SPECIFIC mRNA BY GLUCAGON OR CYCLIC AMP AND PERMISSIVE EFFECT OF DEXAMETHASONE*

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Isolated rat liver cells maintained in suspension culture for 4 to 5 h synthesize the gluconeogenic cytosolic enzyme phosphoenolpyruvate carboxykinase at a rate approximately 5-fold lower than the in vivo hepatic rate. Glucagon rapidly re-induces phosphoenolpyruvate carboxykinase synthesis in such cells. The rate of enzyme synthesis doubles in 40 min and plateaus at a level 6- to 13-fold higher than in control cells 120 min after glucagon addition at maximal concentration. Consistent with the presumed role of cyclic AMP as a mediator of enzyme induction, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, added simultaneously with glucagon, shifts the hormone dose-response curve 2 log units to the left. Moreover, cyclic AMP supplied exogenously to the cells mimics the inductive effect of glucagon. Total cellular RNA isolated from hepatocytes induced by glucagon contains an increased level of mRNA coding for phosphoenolpyruvate carboxykinase, as determined by translational assay. The kinetics and extent of the rise in mRNA level are adequate to explain the stimulation of enzyme synthesis. Although glucagon on its own induces a build-up of phosphoenolpyruvate carboxykinase mRNA and a commensurate stimulation of enzyme synthesis, the glucagon induction is very markedly amplified when the cells are first preincubated with dexamethasone. The glucocorticoid by itself, however, does not have any substantial effect on the level of phosphoenolpyruvate carboxykinase mRNA or on the rate of enzyme synthesis. Its role can therefore be characterized as permissive.

Glucagon plays a key role in the adaptive response of hepatic metabolism during the transition from the fed to the fasting states. The actions of glucagon in the liver include a stimulation of glycolysis, gluconeogenesis, and fatty acid oxidation, and conversely an inhibition of glycogen synthesis, glycolysis, and fatty acid synthesis (1, 2). These metabolic changes are acutely caused by alterations of enzyme activities due to phosphorylation, as exemplified by the regulation of phosphorylase, glycogen synthase, and pyruvate kinase (3, 4). A second type of regulatory process, operating over a longer time range, consists of hormonal effects on the synthesis, and eventually on the content, of various enzymes (5-7). A classical example is the glucagon-induced increase in cytosolic P-enolpyruvate carboxykinase (phosphoenolpyruvate carboxykinase (GTP) EC 4.1.1.32) (8), the rate-limiting enzyme of gluconeogenesis, as a consequence of a selective stimulation of the enzyme synthesis (9). The accumulation of P-enolpyruvate carboxykinase is considered as an important factor to sustain active gluconeogenesis during fasting.

Our interests lie in the sequence of events which lead to an alteration of the synthesis rate of specific hepatic proteins, in particular P-enolpyruvate carboxykinase, following the interaction of glucagon with its membrane receptor. As in the case of enzyme regulation by phosphorylation, this sequence is currently thought to involve the initial activation of adenylate cyclase, resulting in a rise of intracellular cyclic AMP. In earlier studies, it was shown that the injection of glucagon or dibutyl cyclic AMP to the rat results in the rapid accumulation in the liver of functional mRNA coding for P-enolpyruvate carboxykinase (10-13). Similarly, the cyclic AMP-dependent induction of hepatic tyrosine aminotransferase synthesis was reported to result from an increase in translatable enzyme mRNA (14, 15). These data strongly suggest a role of cyclic AMP at the transcriptional or post-transcriptional levels, as opposed to the translational level, in the regulation of gene expression in the mammalian liver. However, the molecular processes underlying this regulation remain to be elucidated.

Previous studies on the regulation of hepatic P-enolpyruvate carboxykinase synthesis by glucagon or cyclic AMP were carried out either in vivo in the whole rat or in vitro in hepatoma derived cell culture systems. Both experimental approaches have obvious limitations. First, the synthesis of P-enolpyruvate carboxykinase in the rat liver is affected by a variety of hormones, including epinephrine, the glucocorticoids, insulin, and possibly still other factors, in addition to glucagon (16). The interaction between these multiple factors makes it difficult to define the mechanism of action of individual inducers in vivo. Furthermore, experiments involving precursor incorporation techniques or the use of inhibitors of macromolecule synthesis are often technically difficult or unsatisfactory in the whole animal. On the other hand, hepatoma cells cultured in vitro may display defective or abnormal mechanisms of response to hormones. For these reasons, we have undertaken a study of the regulation of P-enolpyruvate carboxykinase synthesis in normal rat liver cells maintained in suspension culture. The present report describes the rapid, large, and consistent induction of enzyme synthesis elicited in this system by glucagon or cyclic AMP, as a consequence of the accumulation of functional P-enolpyruvate carboxykinase mRNA. The system is also used to investigate the permissive

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role of the glucocorticoids in the cyclic AMP-mediated induction of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase from Clostridium histolyticum (Type IV, batch 100F-6810) was purchased from Sigma. Bovine serum albumin (Fraction V from Sigma) was extensively dialyzed against 5 mM Na phosphate buffer, pH 7.4, and lyophilized before its addition to the incubation media. Crystallized highly purified porcine glucagon was a generous gift of Dr. Jorgen Schlittkrull of the Novo Research Institute. It was dissolved in 0.003 N HCl, 2% (w/v) glycerol, and 0.1% (v/v) phenol and stored as a stock solution at 2 °C. Dexamethasone was purchased from Sigma. It was dissolved in 0.02% (w/v) sulfosalicylic acid at a concentration of 14.5 mM and diluted with water to a concentration of 0.44 mM immediately before addition to incubated cells. 3-isobutyl-1-methylxantine (from Sigma) was dissolved in 0.1 N NaOH at a concentration of 18.1 mM and neutralized with 1 N HCl immediately before addition to the incubation medium. All other biochemicals were supplied by Sigma, Boehringer Mannheim, or Bio-Rad. Polyvinylidene difluoride (PVDF) membranes were from Millipore. Solubilizer was from Amersham. New England Nuclear or Amersham International Ltd. NCS Tissue Solubilizer (TSA) was used.

**Animals**—Male Wistar rats, bought from Kleintierfarm Madoerin Inc. (Fuellbornsdorf, Switzerland) and weighing between 180 and 260 g at the time of the experiments, were used. Twenty-four h before the experiments, food was removed from the cages. After 22 h of fasting, the animals were fed 7% of glucose/kg of body weight by gavage and offered 0.5% of glucose as a drink instead of water. After gavage, the usefulness of the fasting-refeeding cycle in bringing about a uniformly low rate of hepatic P-enolpyruvate carboxykinase synthesis has been shown previously (10). For experiments with adrenalectomized rats, the removal of the adrenal gland was performed under ether anesthesia 3 to 5 days before hepatocyte isolation. After surgery, the rats received 1% (w/v) NaCl to drink instead of water.

**Liver Cell Isolation**—Surgery and liver perfusion were carried out basically as described by Seglen (17). The calcium-free buffer, collagenase buffer, suspending and washing buffers were similar to those of Seglen, except that all were supplemented with 5 mM glucose, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine. Nineteen amino acids were also added to provide final concentrations mimicking plasma levels in the rat (18). Moreover, bovine serum albumin was present at 1% (w/v) in suspension and washing buffers. All the solutions were thoroughly filtered with 0.22-μm polyethersulfone filters and were used. Care was taken to avoid bacterial contamination throughout the perfusion, isolation, and incubation procedures. The liver perfusion was performed in two steps, first using the calcium-free buffer and subsequently a recirculating collagenase buffer containing 0.045% (w/v) collagenase. The digestion time was shortened to 25-30 min. After perfusion, the liver was transferred to a petri dish containing 75 ml of suspension buffer and the cells were dispersed using a dog comb. The cells were filtered once through a 250-μm hole nylon mesh and centrifuged at 200 rpm (i.e. 7.2 × gmean) for 2.5 min in a bench top centrifuge. The cells were washed three times in 80 ml of washing buffer, centrifugation between washes being performed as above. A stock suspension of cells was made up by dispensing the final cell pellet in an incubation medium containing: 137 mM NaCl, 5.36 mM KCl, 1.22 mM CaCl₂, 0.64 mM MgCl₂, 1.10 mM KH₂PO₄, 0.70 mM Na₂SO₄, 25 mM NaHCO₃, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). The medium was supplemented before use with 10 mM glucose as well as with glutamine, other amino acids, and antibiotics at the concentrations indicated above. Dialyzed bovine serum albumin was also added at 2% (w/v). The medium was saturated with O₂/CO₂ (95:5) before use. The cells were counted in a Neubauer haemocytometer. In twenty-five experiments, the cell yield per liver was 489 ± 37 × 10⁶ cells (mean ± S.E.). The stock suspension of cells was made up to contain approximately 20 × 10⁶ cells/ml.

**Incubation of Cells**—In the initial series of experiments, in which only the relative rate of P-enolpyruvate carboxykinase synthesis was measured, the 6 × 10⁶ cells (i.e. 0.4 ml of stock suspension) distributed into individual suspension flasks (25 ml) with ethylene vials for scintillation counting containing 4.0 ml of supplemented incubation medium. In subsequent experiments, in which both P-enolpyruvate carboxykinase mRNA level and relative rate of enzyme synthesis were simultaneously determined, 60 × 10⁶ cells were incubated in individual 250-ml Erlenmeyer flasks containing 90 ml of incubation medium. In all experiments, incubation was carried out at 37.5 °C under a gas phase consisting of O₂/CO₂ (95:5) in a metabolic incubator with reciprocating shaking at about 100 cycles/min. Hormones and other additions to the cells were made from concentrated stock solutions and control cells received the vehicle only. The viability of the cells after isolation and at various times of incubation was determined by the trypan blue exclusion test at a final dye concentration of 0.25% (w/v).

**Pulse-labeling of Cells**—In the experiments involving the measurement of the relative rate of P-enolpyruvate carboxykinase synthesis only, the cells were pulse-labeled with [³H]leucine by Method A, as follows. At chosen times, 5 × 10⁶ cells were transferred from the incubation flasks to 15-ml polystyrene tubes, spun at 1,000 rpm for 3 min in the bench top centrifuge and washed once at room temperature in 2 ml of leucine-free incubation medium. The washed cell pellet was suspended for protein labeling in 1 ml of leucine-free incubation medium containing 30 μCi/ml of [³H]leucine. Incubation in this medium was continued for 20 min at 37.5 °C, followed by two washes in 5 ml of washing buffer without albumin. The final cell pellet was suspended in 1 ml of a homogenization buffer containing 10 mM Tris/HCl, pH 7.4, 200 mM sucrose, 60 mM NaCl, and 1 mM EDTA. Cell homogenization was accomplished by three cycles of freezing-thawing, using Liquid N₂. In the experiments involving the determination of the relative rate of mRNA synthesis rate and enzyme yield, samples of 200 × 10⁶ cells were pulse-labeled using Method B. The cells were dispersed into 12-ml tubes containing 10 ml of leucine-free incubation medium and spun at 300 rpm for 2 min at room temperature. The supernatant was discarded and 0.2 ml of leucine-free incubation medium containing 30 μCi/ml of [³H]leucine was added. The cells were incubated for 20 min in this medium, washed once in 5 ml of washing buffer without albumin and homogenized as described above in 0.2 ml of homogenization buffer. The cell extracts were stored at −80 °C until further analysis.

**Determination of Amino Acid Incorporation into Total Cellular and Soluble Proteins and into P-enolpyruvate Carboxykinase**—The incorporation of [³H]leucine into total cell protein was measured by trichloroacetic acid precipitation of a sample of homogenate, as described by Ballard et al. (19). The homogenate was then centrifuged at 111,000 × gmean, for 45 min at 2 °C. The particle-free supernatant was used for the assay of P-enolpyruvate carboxykinase activity (20-22), as well as for the determination of [³H]leucine incorporation into soluble proteins by trichloroacetic acid precipitation and into P-enolpyruvate carboxykinase by direct immunoprecipitation. The latter procedure was performed using 0.4 ml of high speed supernatant after pulse-labeling by Method A or 0.15 ml of high speed supernatant after pulse-labeling by Method B. In the first case, the total amount of P-enolpyruvate carboxykinase in the antigen-antibody reaction was comprised between 60 and 100 milliunits, consisting of the endogenous enzyme present in the cell extract and, when required, of exogenous unlabeled P-enolpyruvate carboxykinase added in the form of rat liver cytosol. In the second case, the amount of exogenous P-enolpyruvate carboxykinase was negligible and 60 milliunits of carrier enzyme were added. A specific antibody against cytosolic P-enolpyruvate carboxykinase (23) was added in 20% excess (in terms of precipitable milliunits) over the enzyme amount, so as to precipitate P-enolpyruvate carboxykinase quantitatively. The antigen-antibody reaction mixture, containing 0.6% (w/v) Triton X-405, was incubated at 37 °C for 30 min and 2 °C for 3 h. At the completion of the reaction, the mixture was dispensed on top of 0.5 ml of 1 M sucrose dissolved in 10 mM Na phosphate buffer, pH 7.4, 150 mM NaCl, 5 mM leucine, and 2 ml of washing buffer (w/v) Triton X-405. The immunoprecipitate was pelleted through the sucrose cushion by centrifugation at 1,750 × gmean and 2 °C for 30 min. The pellet was washed three times in resuspension in the detergent-containing solution. The washed immunoprecipitate was submitted to electrophoresis in cylindrical SDS-polycrylamide gels as described previously (10). Slab gel electrophoresis analysis of pulse-labeled soluble proteins was performed according to the method of Laemmli (24). For these experiments, the cells were pulse-labeled by Method B. The slab gels were processed for fluorography as described (23). The protein concentration in total homogenate and high speed supernatant was determined by the Lowry method (25). The total protein content of liver cells was 968 ± 37 μg/10⁶ cells and the soluble protein content 413 ± 15 μg/10⁶ cells (means ± S.E., 18 determinations).

**Isolation of Total Cellular RNA**—Fifty-five × 10⁶ cells were used

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
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for each RNA extraction. At appropriate times of incubation, the cells were pelleted by centrifugation at 300 rpm for 2 min in the bench top centrifuge and washed once in 50 ml of ice-cold, albumin-free washing buffer. The washed cell pellet was used immediately for the extraction of total cellular RNA, or alternatively was quickly frozen in liquid N₂ and stored at -80 °C until RNA isolation. The RNA was isolated by the guanidinium thiocyanate procedure, exactly as described by Chirgwin et al. (26), using initially 7 ml of the guanidinium thiocyanate solution for the homogenization of 55 × 10⁶ cells. The isolated RNA was precipitated twice from 0.2 M K acetate, pH 5.2, with two volumes of ethanol and finally dissolved in boiled deionized water at a concentration of 5 mg/ml. The RNA samples were stored at -80 °C.

The yield of total RNA from 55 × 10⁶ cells was 955 ± 25 μg (mean ± S.E., 30 preparations).

Messenger RNA Translation Assay in the Wheat Germ Cell-free Protein Synthesis System—Total cellular RNA was translated in a wheat germ cell-free protein synthesis system, using the incubation conditions described in detail previously (27, 28). After the incubation, a high speed supernatant of the reaction mixture was obtained by centrifugation at 110,000 × gₚₑₙ and 2 °C for 35 min. A sample of high speed supernatant was precipitated with trichloroacetic acid for the determination of [³H]leucine incorporation into total released poly-peptides, which served as an estimate of the overall template activity of the RNA. The remainder of the high speed supernatant was used for the direct immunoprecipitation of P-enolpyruvate carboxykinase which was synthesized by the wheat germ extract at the direction of liver cell RNA. The immunoprecipitation was accomplished as described above with respect to the liver cell extracts. The amount of [³H]leucine incorporated into immunoprecipitable P-enolpyruvate carboxykinase served as an estimate of the concentration of functional mRNA coding for the enzyme in total cellular RNA. The incorporation of leucine into total poly-peptides and into P-enolpyruvate carboxykinase was dependent on the RNA input within a range of concentrations comprised between 0 and 15 μg of total cellular RNA in a reaction volume of 80 μl. Routinely, the assay was performed using 6 or 9 μg of RNA.

Radioactivity Measurements—Protein precipitates were dissolved in NCS Tissue Solubilizer and the tritium radioactivity measured by liquid scintillation spectrometry with an efficiency of about 50%. Cylindrical SDS-polyacrylamide gels containing immunoprecipitated P-enolpyruvate carboxykinase were fractionated in 2-mm portions. The radioactivity in the gel fragments was measured after elution in the liquid scintillation mixture described in Ref. 29. Ninety-five % of the radioactivity was released from the gel pieces and counted at an efficiency of about 50%. Prior to fractionation, the gels were scanned spectrophotometrically at 280 nm in order to localize the position of P-enolpyruvate carboxykinase. The amount of [³H]leucine incorporated into P-enolpyruvate carboxykinase was computed by adding the radioactivity of the fractions corresponding to the enzyme band and subtracting a background estimated from the radioactivity of the neighboring fractions.

RESULTS

Viability and Protein Synthesis Capacity of Incubated Liver Cells—Using the two-step collagenase perfusion method of Seglen (17) for the isolation of liver cells, we routinely obtained 500 × 10⁶ hepatocytes from the liver of adult male rats weighing about 250 g. Immediately after isolation, 97 ± 0.15% of the cells (mean ± S.E., 10 experiments) were viable, as estimated by the trypan blue exclusion test. The apparent viability of the cells incubated in suspension remained excellent for several hours, 94.5 ± 0.48% of the cells still excluding the dye after 5 h and 90.1 ± 0.66% of the cells after 7 h of incubation. As others (30, 31), we noticed a tendency of suspended cells to aggregate during incubation. In general, this tendency became manifest around 5 h of incubation and resulted in the formation of small clusters (less than 15 cells), so that accurate cell counting was still possible at 7 h. However, larger clumps preventing cell enumeration at the end of the incubation formed in about 16% of the experiments without obvious reason.

Consistent with the high viability figures, the capacity of the cells for protein synthesis was well preserved throughout the incubation. Cells were pulse-labeled with [³H]leucine for 20 min periods starting at 15 min, 4.5 and 6.5 h of incubation, in order to determine the amino acid incorporation into total cellular proteins and into the soluble fraction of these proteins. As may be seen in the second and third columns of Table I, the cells maintained a nearly steady level of leucine incorporation during incubation. In subsequent series of experiments we sometimes noted small (less than 30%) but significant decreases in the total protein synthetic activity of the cells during the first 4.5 h of incubation. In all cases, however, the rate of amino acid incorporation remained stable from 4.5 h until the end of the experiments.

In the same experiments, we also measured the incorporation of [³H]leucine into P-enolpyruvate carboxykinase by immunoprecipitation of the enzyme (Table I, fourth column). From these data, the relative rate of P-enolpyruvate carboxykinase synthesis, i.e. the ratio of the radioactivity in the enzyme over the radioactivity in soluble protein, was computed. The data, expressed in ppm, are given in the last column of Table I. In contrast to the stability of total protein synthesis, the relative rate of P-enolpyruvate carboxykinase synthesis decreased 7-fold in 4.5 h of incubation and a further 2-fold during the next 2 h.

This sharp decline in enzyme synthesis does not reflect a general depression in the pattern of protein synthesis in the hepatocyte, as shown by electrophoretic analysis of the total soluble proteins synthesized at various times of incubation (Fig. 1, tracks 1–3). Within the limits of resolution of conventional SDS-polyacrylamide gel electrophoresis, there was no obvious qualitative change in protein synthesis during the course of a 6.5-h incubation. These data agree with earlier reports demonstrating the ability of liver cells in suspension culture to synthesize several plasma proteins at relatively constant rates for 12 h or more (30, 32).

Glucagon Effect on P-enolpyruvate Carboxykinase Synthesis—We reasoned that the extremely low rate of P-enolpyruvate carboxykinase synthesis in liver cells preincubated in hormone-free medium for a few hours would constitute an advantageous feature for enzyme induction studies. Glucagon was added at a concentration of 10⁻⁷ m to cells preincubated for 4.5 h and the incubation was continued in the presence of the hormone for a further 2 h, after which the cells were pulse-labeled with [³H]leucine. Extracts of cells incubated without glucagon were reacted with the anti-P-enolpyruvate carboxykinase antibody and the immunoprecipitates were analyzed on SDS-polyacrylamide gels, as shown in Fig. 2. Clearly, glucagon elicited a marked stimulation of [³H]leucine incorporation into P-enolpyruvate carboxykinase.

| Incubation time (h) | [³H]Leucine incorporation into P-enolpyruvate carboxykinase | Relative rate of PEPC, P-enolpyruvate carboxykinase |
|---------------------|-------------------------------------------------|-----------------------------------------------|
| 4.5                 | 2283 ± 446                                     | 2821 ± 312                                   |
| 6.5                 | 207 ± 50                                       | 375 ± 91                                     |
| 8                   | 61 ± 17                                        | 149 ± 58                                     |
The hormonal effect was selective, as demonstrated by gel electrophoresis of the total complement of soluble proteins made by control or glucagon-treated cells (Fig. 1, tracks 3 and 4). There is no apparent difference due to glucagon in the synthesis rate of the proteins separated by this method. In such gels, P-enolpyruvate carboxykinase migrates with the front of the prominent 70,000 molecular weight polypeptide, tentatively identified as pro-albumin, and is therefore masked by the latter.

The time-course of the glucagon-dependent induction of P-enolpyruvate carboxykinase in preincubated liver cells is presented in Fig. 3. Glucagon was present at a concentration of 10^{-7} M and cells were pulse-labeled at 40-min intervals after its addition. From a starting value of 428 ppm, the relative rate of enzyme synthesis rose approximately 7-fold to 2,723 ppm in 120 min and plateaued at this level. A clear-cut increase in P-enolpyruvate carboxykinase synthesis was noticeable as early as 40 min following hormone addition. On the contrary, the rate of enzyme synthesis in control cells incubated without glucagon slowly declined to barely detectable levels by the end of the incubation. In both treated and control cells, the rate of amino acid incorporation into total protein remained unchanged throughout the experiment.

Dose-Response Relationship of Glucagon Effect—The next experiments were performed in order to investigate the concentration-dependence of the glucagon induction. As in previous experiments, the cells were preincubated without hormone for 4.5 h. Glucagon was then added to provide concentrations ranging from 4 \times 10^{-10} to 4 \times 10^{-7} M and the effect on P-enolpyruvate carboxykinase synthesis was measured 2 h later. It should be mentioned that glucagon is degraded by liver cells (33) and that the hormone concentration might well have decreased during the 2-h incubation. The data in Fig. 4 show that a 2-fold increase in the relative rate of enzyme synthesis occurred at a nominal glucagon concentration of 1.6 \times 10^{-9} M and that maximal induction took place with concentrations comprised between 10^{-7} and 4 \times 10^{-7} M. Total protein synthesis was not affected by glucagon at any concentration.

In order to assess the presumed role of cyclic AMP as a mediator of the inductive effect of glucagon, the cells were also challenged with the hormone in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. As may be seen in Fig. 4, the latter compound strongly potentiated the effect of submaximal concentrations of glucagon.

Cyclic AMP Effect on P-enolpyruvate Carboxykinase Synthesis—In preliminary experiments dealing with the direct effect of cyclic AMP added externally to liver cells, we found that concentrations of 0.3 mM cyclic AMP and 0.5 mM 3-isobutyl-1-methylxanthine would elicit a maximal induction of P-enolpyruvate carboxykinase synthesis in preincubated liver cells. Cyclic AMP at 1 mM or higher, on the other hand, caused a consistent inhibition of total protein synthesis, accompanied by a decrease in the relative rate of P-enolpyruvate carboxykinase compared to the maximally induced rate (data not shown). Fig. 5 shows the kinetics of induction occurring when maximal concentrations of cyclic AMP and 3-isobutyl-1-methylxanthine were provided to liver cells 5.5 h after isolation. From a value of 332 ppm at the time of inducer addition, the relative rate of enzyme synthesis reached 3,980 ppm in 2 h. In these experiments, the time course of action of the cyclic nucleotide was somewhat delayed relative to the glucagon effect (compare Figs. 5 and 3). The short lag might have been due to a diffusion barrier to the penetration of the nucleotide in the liver cell. We determined that the maximal increase in intracellular cyclic AMP following the addition of glucagon occurred in 4 to 5 min (experiments not shown). It is conceivable that the build-up of an effective intracellular cyclic AMP level would take more time after the external addition of the nucleotide. After 2 h, however, the cells exposed to cyclic AMP had a rate of enzyme synthesis identical to cells incubated in the presence of 10^{-7} M glucagon (Fig. 5).

Glucocorticoid-Glucagon Interaction in the Regulation of Hepatic P-enolpyruvate Carboxykinase—The glucocorticoids...
play a complex role in the regulation of P-enolpyruvate carboxykinase synthesis in the whole animal. Whereas they induce the enzyme in the kidney (34) as a consequence of an increase in specific P-enolpyruvate carboxykinase mRNA (23, 28, 29), they are ineffective to stimulate the synthesis of hepatic P-enolpyruvate carboxykinase in the intact rat, possibly because of the inhibitory effect of insulin released after glucocorticoid administration (35). Taking advantage of our hormone-responsive in vitro system, we attempted to clarify the role of the glucocorticoids in the regulation of hepatic P-enolpyruvate carboxykinase. We first asked whether the presence of a glucocorticoid in the incubation medium would prevent the fall of P-enolpyruvate carboxykinase synthesis normally seen in hepatocytes maintained in vitro. freshly isolated liver cells were incubated with 10^{-7} M dexamethasone or, as before, in hormone-free medium. After 4.5 h, they were pulse-labeled in order to measure enzyme synthesis. From an initial relative rate of 2362 ± 436 ppm, P-enolpyruvate carboxykinase synthesis dropped to 450 ± 183 ppm (mean ± S.E., 5 experiments) in the presence of dexamethasone, not significantly different from 224 ± 101 ppm in control cells. Thus, the glucocorticoid was clearly unable to sustain P-enolpyruvate carboxykinase synthesis at the level prevailing at the start of the incubation.

The second question was to know whether cells preincubated with dexamethasone would exhibit a stronger inductive response to glucagon. That this was indeed the case is shown in Table II. Glucagon caused a 13-fold stimulation of P-enolpyruvate carboxykinase in control cells and a 43-fold stimulation in cells incubated with dexamethasone. The amplification of the response to glucagon due to dexamethasone was therefore, approximately 3-fold (43 + 13). The relative rate of enzyme synthesis in cells induced by glucagon in the presence of dexamethasone is comparable to the maximal rate seen in

![Graphs](image-url)

**Fig. 3 (left). Time-course of glucagon effect on P-enolpyruvate carboxykinase synthesis.** The cells were preincubated for 4.5 h prior to the addition of glucagon (10^{-7} M). Zero time is the time of glucagon addition. Pulse-labeling of cells and measurements of [3H]leucine incorporation into proteins and P-enolpyruvate carboxykinase were performed as described under "Experimental Procedures." •—•, relative rate of P-enolpyruvate carboxykinase synthesis after glucagon addition. ○—○, relative rate of enzyme synthesis in control cells. Values are means ± S.E. of 9 experiments, except at 160 min where only 4 determinations were made. PEPC, P-enolpyruvate carboxykinase.

**Fig. 4 (center). Dose-response relationship of glucagon effect on P-enolpyruvate carboxykinase synthesis and effect of 3-isobutyl-1-methylxanthine.** The cells were preincubated without hormone for 4.5 h prior to the addition of glucagon or glucagon plus 3-isobutyl-1-methylxanthine (0.8 mM). The relative rate of synthesis of P-enolpyruvate carboxykinase was measured 2 h after drug addition. ○—○, relative rate of enzyme synthesis in the presence of glucagon. Values are means ± S.E. of 7 experiments. ●—●, relative rate of enzyme synthesis in the presence of glucagon plus 3-isobutyl-1-methylxanthine. Values are means ± S.E. of 4 experiments. PEPC, P-enolpyruvate carboxykinase.

**Fig. 5 (right). Time-course of cyclic AMP effect on P-enolpyruvate carboxykinase synthesis.** The cells were preincubated in hormone-free medium for 5.5 h prior to the addition of cyclic AMP (0.3 mM) plus 3-isobutyl-1-methylxanthine (0.5 mM). The relative rate of P-enolpyruvate carboxykinase synthesis was measured as described under "Experimental Procedures." ■—■, enzyme synthesis after addition of cyclic AMP plus 3-isobutyl-1-methylxanthine. ○—○, enzyme synthesis in control cells. ●, enzyme synthesis after addition of glucagon (10^{-7} M). Values are means ± S.E. of 4 or 5 determinations. PEPC, P-enolpyruvate carboxykinase.

**Table II**

| Time (h) | Hormone during preincubation | Hormone during experimental period | [3H]Leucine incorporation into Soluble protein | PEPC | Relative rate of PEPC synthesis |
|---------|-----------------------------|----------------------------------|-----------------------------------|------|--------------------------------|
|         |                             |                                  | cpm/mg protein × 10^{-3} | cpm/mg protein | ppm |
| 0       | None                        | Glucagon                         | 759.2 ± 42.2                 | 173 ± 45   | 224 ± 101 |
| 2       | None                        | Glucagon                         | 732.0 ± 171.4                | 2,328 ± 452 | 3,018 ± 659 |
| 0       | Dexamethasone               | None                             | 837.3 ± 94.3                 | 92 ± 24   | 122 ± 41 |
| 2       | Dexamethasone              | Dexamethasone + glucagon         | 847.9 ± 101.3                | 339 ± 103  | 450 ± 183 |
| 2       | Dexamethasone              | Dexamethasone                    | 606.5 ± 96.5                 | 11,262 ± 1,368 | 19,304 ± 2,026 |
| 0       | Dexamethasone              | Dexamethasone                    | 736.2 ± 90.4                 | 262 ± 114  | 366 ± 143 |

**Effect of dexamethasone on glucagon-dependent induction of P-enolpyruvate carboxykinase**

Liver cells from intact, fasted, glucose-re-fed (cf. Table III) rats were preincubated for 4.5 h in hormone-free medium or in the presence of dexamethasone (10^{-7} M). Glucagon (10^{-7} M) or vehicle were then added and the incubation continued for an experimental period of 2 h. Samples of cells were pulse-labeled with [3H]leucine after the preincubation period (0 time) and at the end of the experimental period (2 h). The cells were processed for the determination of radioactive leucine incorporation into total soluble proteins and into P-enolpyruvate carboxykinase as described under "Experimental Procedures." The data are the means ± S.E. of 5 experiments. PEPC, P-enolpyruvate carboxykinase.
Glucagon added to these cells following a preincubation in hormone-free medium caused a 2.8-fold stimulation of P-enolpyruvate carboxykinase synthesis in 2 h (Fig. 6). Although more modest than in normal cells, the induction was nevertheless obvious in all the experiments. When the cells were primed with dexamethasone, glucagon produced an 18-fold increase in the relative rate of P-enolpyruvate carboxykinase synthesis, corresponding to a 6-fold enhancement over the effect of glucagon acting alone (18 + 2.8). As in normal cells, the glucocorticoid by itself was devoid of any marked effect on P-enolpyruvate carboxykinase synthesis.

**Effects of Glucagon and Dexamethasone on P-enolpyruvate Carboxykinase mRNA**—Several studies in the whole rat have shown that an injection of dibutyryl cyclic AMP results in the build-up of P-enolpyruvate carboxykinase mRNA in the liver, supporting the view that the cyclic AMP-mediated induction of the enzyme is achieved by a transcriptional or post-transcriptional mechanism (10–13). Earlier in vitro experiments using Reuber H-35 hepatoma cells as a model system suggested, however, that cyclic AMP might also stimulate the synthesis of P-enolpyruvate carboxykinase by a translational mechanism because RNA synthesis inhibitors appeared not to prevent enzyme induction (37, 38). To explain the synergism between dexamethasone and glucagon observed in the present experiments, we reasoned that dexamethasone might induce the accumulation of P-enolpyruvate carboxykinase mRNA, but that this mRNA could not be translated efficiently in the cell in the absence of a stimulus capable of raising the intracellular concentration of cyclic AMP. This hypothesis was tested by measuring the level of P-enolpyruvate carboxykinase mRNA in cells preincubated for 5 h with or without dexamethasone (10^{-6} M) and subsequently challenged with glucagon (10^{-7} M) for a period of 2 h. Total cellular RNA was isolated from such cells and assayed in a wheat germ translation system for its capacity to direct the incorporation of amino acids into total polypeptides and specifically into P-enolpyruvate carboxykinase. The level of functional P-enolpyruvate carboxykinase mRNA was expressed as the ratio of mRNA-directed leucine incorporation into enzyme over mRNA-directed leucine incorporation into total products (Table III). Three important results emerge from these experiments. First, glucagon caused a 10-fold increase in the level of P-enolpyruvate carboxykinase mRNA in liver cells preincubated in hormone-devoid medium. Second, the level of enzyme mRNA was not different in cells incubated with or without dexamethasone. Third, P-enolpyruvate carboxykinase mRNA was induced 30-fold by glucagon in the presence of dexamethasone, as compared to 10-fold in its absence. Thus, the glucocorticoid amplified the induction of the enzyme mRNA by a factor of 3. Since the glucagon-dependent effects on the level of P-enolpyruvate carboxykinase mRNA were quantitatively similar to the hormonally induced alterations in enzyme synthesis reported in Table II, we concluded that enzyme induction was not achieved by a translational mechanism, neither in the absence of dexamethasone nor after preincubation with the glucocorticoid.

The next experiments were performed to determine whether changes in the rate of enzyme synthesis could be accounted for by equivalent changes in mRNA level throughout the induction time-course. The kinetics of mRNA accumulation was established in cells induced by glucagon or cyclic AMP after preincubation in the presence of dexamethasone. At all time points, the ongoing rate of P-enolpyruvate car-

![Fig. 6. Effect of dexamethasone on glucagon-dependent induction of P-enolpyruvate carboxykinase in liver cells from adrenalectomized animals.](image-url)
An attractive feature of the system is the very low rate of enzyme synthesis (Table I).

In the adult rat, the rate of hepatic P-enolpyruvate carboxykinase synthesis is maintained at this level only as long as the liver is exposed to the internal milieu of the whole animal. Under these circumstances, even considerable increases of enzyme synthesis would not be expected to result in any substantial changes in the assayable level of P-enolpyruvate carboxykinase over short periods of time. The same argument probably also explains why Oliver et al. (42) did not observe acute effects of glucagon on P-enolpyruvate carboxykinase activity in freshly plated hepatocyte monolayers, while they and other authors (43) reported a rapid rise in enzyme level in older cultures with a much reduced intracellular enzyme pool.

Two lines of evidence support the contention that cyclic AMP is the mediator of the glucagon induction. First, the inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine, strongly potentiated the hormonal effect. The half-maximum concentration of glucagon was shifted leftward from approximately $2.5 \times 10^{-8}$ to $4 \times 10^{-10}$ M in the presence of the phosphodiesterase inhibitor. Second, cyclic AMP itself was able to induce the synthesis of P-enolpyruvate carboxykinase directly. At maximal concentrations, cyclic AMP and glucagon were equipotential inducers.

Glucagon, acting via cyclic AMP, induces the synthesis of P-enolpyruvate carboxykinase in the liver cell as the result of an increase in enzyme mRNA level. In the present experiments, we noted that the stimulation of enzyme synthesis caused by glucagon or directly by cyclic AMP was always accompanied by a commensurate increase in the level of functional P-enolpyruvate carboxykinase mRNA, as measured by translation assay. Previous in vivo studies from several laboratories, using cDNA hybridization as well as translation assays, have shown that the mRNA coding for P-enolpyruvate carboxykinase accumulates in the rat liver following an injection of dibutyryl cyclic AMP, concomitantly with the induction of enzyme synthesis (10–13, 44, 45). Taken together, these data discount the possibility of a translational type of induction mechanism, which would call for effects on P-enolpyruvate carboxykinase synthesis not paralleled by equivalent changes in specific mRNA level.

An important objective of this work was to clarify the role of the glucocorticoids in the regulation of hepatic P-enolpyruvate carboxykinase synthesis. In the intact rat, the enzyme is not induced after an injection of hydrocortisone or triamcinolone. There is, however, a slight stimulation of the already enhanced rate of P-enolpyruvate carboxykinase synthesis in the diabetic animal following glucocorticoid administration (35). These observations suggested that the glucocorticoid
show that the glucocorticoids by themselves are not effective inducers of P-enolpyruvate carboxykinase synthesis in the rat liver cell. The level of mRNA encoding the enzyme does not increase in hepatocytes incubated for several hours in the presence of dexamethasone. While ineffective when acting alone, the glucocorticoids nevertheless play an important regulatory role for the expression of P-enolpyruvate carboxykinase mRNA. This role becomes manifest after addition of glucagon or cyclic AMP. Under the concerted actions of a glucocorticoid and of glucagon or its second messenger, the level of enzyme mRNA and the relative rate of enzyme synthesis increase 30- to 50-fold. The rate of enzyme synthesis observed in vitro is then at least equal to the fully induced rate measured in vivo in the liver of fasting rats (36). In the absence of dexamethasone, on the contrary, glucagon causes a more limited accumulation of mRNA and the rate of P-enolpyruvate carboxykinase synthesis remains far lower than the induced rate in the liver of the starved animal. Recently, Kletzien et al. (46) reported data on the regulation of P-enolpyruvate carboxykinase activity in hepatocytes maintained in monolayer culture which are entirely consistent with the hormonal effects on enzyme mRNA level and rate of synthesis described here. Their data and ours demonstrate the permissive role of the glucocorticoids in the regulation of hepatic cytosolic P-enolpyruvate carboxykinase.

The molecular basis for the glucocorticoid-cyclic AMP interaction in the regulation of P-enolpyruvate carboxykinase mRNA in the liver remains to be elucidated. The hormonally induced accumulation of functional mRNA may result from a stimulation of the transcription of the structural gene, a stimulation of the processing of the primary transcript into mature mRNA, a stabilization of the mRNA against degradation, or from a combination of several of these mechanisms. The glucocorticoids are thought to regulate the transcription of specific genes, probably following the interaction of the hormone-receptor complex with DNA sequences in the vicinity of the concerned genes (47, 48). Cyclic AMP is also involved in the regulation of gene expression in eucaryotic cells by influencing the synthesis of specific mRNAs (49, 50). It will be of considerable interest to see whether the two co-inducers of hepatic P-enolpyruvate carboxykinase mRNA act cooperatively to enhance the rate of transcription of the enzyme gene, or whether each one acts at a different step in the gene expression pathway.

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REFERENCES
1. Exton, J. H. (1981) in Glucagon Physiology, Pathophysiology and Morphology of the Pancreatic A Cells (Unger, R. H., Ordi, L., eds) pp. 195-219, Elsevier, New York
2. McGarry J. D. and Foster, D. W. (1980) Annu. Rev. Biochem. 49, 395-420
3. Exton, J. H. Cherrington, A. D., Blackmore, P. F., Dehaye, J.-P., Suckling, W. G., Gordon, J. E. and Chrisman, T. D. (1981) in Protein Phosphorylation, Cold Spring Harbor Conferences on Cell Proliferation, Volume 8 (Rosen, O. M. and Krebs, E. G., eds) pp. 513-528, Cold Spring Harbor Laboratory, Cold Spring Harbor
4. Blair, J. B. and Kletzien, R. F. (1981) in Protein Phosphorylation, Cold Spring Harbor Conferences on Cell Proliferation, Volume 8 (Rosen, O. M. and Krebs, E. G., eds) pp. 575-590, Cold Spring Harbor Laboratory, Cold Spring Harbor
5. Holten, D. and Kenney, F. T. (1967) J. Biol. Chem. 242, 4372-4377
6. Jost, J.-P., Khairallah, E. A. and Pitot, H. C. (1968) J. Biol. Chem. 243, 3057-3066
7. Lyons, R. T. and Pitot, H. C. (1976) Arch. Biochem. Biophys. 174, 262-272
8. Shrago, E., Lardy, H. A., Nordlie, R. C. and Foster, D. O. (1963) J. Biol. Chem. 238, 3188-3192
9. Hanson, R. W., Fisher, L. Ballard, F. J. and Reshef, L. (1973) Enzyme (Basel) 15, 97-110
10. Iynedjian, P. B. and Hanson, R. W. (1977) J. Biol. Chem. 252, 665-672
11. Garcia Ruiz, J. P., Ingram, R. and Hanson, R. W. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4189-4193
12. Nelson, K., Cimbal, M. A., and Hanson, R. W. (1980) J. Biol. Chem. 255, 8099-8115
13. Beale, E. O., Katzen, C. S. and Graner, D. K. (1981) Biochemistry 20, 4878-4883
14. Ernest, M. J. and Feigelson, P. (1976) J. Biol. Chem. 251, 319-322
15. Nomuchi, T., Dieterhaft, M. and Graner, D. (1978) J. Biol. Chem. 253, 1322-1335
16. Thiruman, S. M., Ballard, F. J. and Hanson, R. W. (1976) in Glucogenogenesis. Its Regulation in Mammalian Species (Hanson, R. W. and Mehlman, M. A., eds), pp. 47-91, John Wiley, New York
17. Seglen, P. O. (1976) Methods Cell Biol. 13, 29-88
18. Seglen, P. O. and Wollman, R. (1976) Nature (Lond.) 202, 603-604
19. Ballard, F. J., Hopgood, M. F. Reshef, L., Tlgman, S. and Hanson, R. W. (1974) Biochem. J. 144, 199-207
20. Chang, H. C. and Lane, M. D. (1966) J. Biol. Chem. 241, 2413-2420
21. Ballard, F. J. and Hanson, R. W. (1969) J. Biol. Chem. 244, 5625-5630
22. Iynedjian, P. B. (1979) Enzyme (Basel) 24, 366-373
23. Iynedjian, P. B. and Jacot, M. M. (1980) Eur. J. Biochem. 111, 89-98
24. LasenI, U. K. (1970) Nature (Lond.) 227, 680-685
25. Lowey, O. H., Rosebrugh, N. J., Farr, A. L. and Randall, R. L. (1951) J. Biol. Chem. 193, 265-275
26. Chirgwin, J. M., Przybyla, A. E., McDonald, R. J. and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
27. Iynedjian, P. B. (1979) in Techniques in the Life Sciences, Biochemistry (Kornberg, J. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. and Tipton, K. F., eds) Vol. B 2/1, B 299, pp. 1-27, Elsevier, Amsterdam
28. Iynedjian, P. B. and Jacot, M. M. (1981) J. Biol. Chem. 256, 7068-7076
29. Iynedjian, P. B. and Hanson, R. W. (1977) J. Biol. Chem. 252, 8350-8403
30. Jeejeebhoy, K. N., Ho, J., Greenberg, G. R., Phillips, M. J., Bruce- Robertson, A. and Sodtke, U. (1975) Biochem J. 146, 141-155
31. Carlson, S. A., Schneill, E., Weigel, P. H. and Roseman, S. (1981) J. Biol. Chem. 256, 8058-8062
32. Chen, C. L. C. and Feigelson, P. (1978) Biochemistry 17, 5308-5313
33. Canivet, B., Gorden, P., Carpentier, J. L., Orci, L. and Freychet, P. (1981) Mol. Cell. Endocr. 21, 311-329
34. Iynedjian, P. B., Ballard, F. J. and Hanson, R. W. (1975) J. Biol. Chem. 250, 5596-5603
35. Gunn, J. M., Hanson, R. W., Meyuhas, O., Reshef, L. and Ballard, J. F. (1975) Biochem J. 150, 195-203
36. Hopgood, M. F., Ballard, F. J., Reshef, L. and Hanson, R. W. (1973) Biochem J. 134, 445-453
37. Gunn, J. M., Tilghman, S. M., Hanson, R. W., Reshef, L. and Ballard, J. F. (1975) Biochemistry 14, 2350-2357
38. Tilghman, S. M., Gunn, J. M., Fisher, L. M., Hanson, R. W., Reshef, L. and Ballard, F. J. (1975) J. Biol. Chem. 250, 3322-3329
39. Gunn, J. A. and Potter, V. R. (1980) Ann. N. Y. Acad. Sci. 349, 59-68
40. Fehlmann, M., Morin, O., Kitabgi, P. and Freychet, P. (1981) Endocrinology 109, 253-261
41. Kelley, D. S., Shull, J. D. and Potter, V. R. (1980) J. Cell. Physiol. 103, 159-168
42. Gunn, J. T., Edwards, A. M. and Pitot, H. C. (1978) Eur. J. Biochem. 87, 221-227
43. Schudt, C. (1980) Biochim. Biophys. Acta 628, 277-285
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44. Yoo-Warren, H., Cimbala, M. A., Felz, K., Monahan, J. E., Leis, J. P. and Hanson, R. W. (1981) J. Biol. Chem. 256, 10224-10227
45. Beale, E. G., Hartley, J. L. and Granner, D. K. (1982) J. Biol. Chem. 257, 2022-2028
46. Kletzien, R. F., Weber, C. A. and Stumpo, D. J. (1981) J. Cell. Physiol. 109, 83-90
47. Yamamoto, K. R. and Alberts, B. M. (1976) Annu. Rev. Biochem. 45, 721-746
48. Payvar, F., Wrangle, O., Carlstedt-Dirke, J., Okret, S., Gustafsson, J. A. and Yamamoto, K. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6628-6632
49. Williams, J. G., Tsang, A. S. and Mahbubani, H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7171-7175
50. Chung, S., Landfear, S. M., Blumberg, D. D., Cohen, N. S. and Lodish, H. F. (1981) Cell 24, 785-797