Hormonal Control of Cyclic 3'5'-AMP Levels and Gluconeogenesis in Isolated Hepatocytes from Fed Rats*

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Glucagon can stimulate gluconeogenesis from 2 mM lactate nearly 4-fold in isolated liver cells from fed rats; exogenous cyclic adenosine 3':5'-monophosphate (cyclic AMP) is equally effective, but epinephrine can stimulate only 1.5-fold. Half-maximal effects are obtained with glucagon at 0.3 nM, cyclic AMP at 30 μM and epinephrine at 0.2 μM. Insulin reduces by 50% the stimulation by suboptimal concentrations of glucagon (0.5 nM). A half-maximal effect is obtained with 0.3 mM insulin (45 microunits/ml).

Glucagon in the presence of theophylline (1 mM) causes a rapid rise and subsequent fall in intracellular cyclic AMP with a peak between 3 and 6 min. Some of the fall can be accounted for by loss of nucleotide into the medium. This efflux is suppressed by probenecid, suggesting the presence of a membrane transport mechanism for the cyclic nucleotide.

Glucagon can raise intracellular cyclic AMP about 30-fold; a half-maximal effect is obtained with 1.5 nM hormone. Epinephrine (plus theophylline, 1 mM) can raise intracellular cyclic AMP about 2-fold; the peak elevation is reached in less than 1 min and declines during the next 15 min to near the basal level.

Insulin (10 nM) does not lower the basal level of cyclic AMP within the hepatocyte, but suppresses by about 50% the rise in intracellular and total cyclic AMP caused by exposure to an intermediate concentration of glucagon. No inhibition of adenylate cyclase by insulin can be shown.

Basal gluconeogenesis is not significantly depressed by calcium deficiency but stimulation by glucagon is reduced by 50%. Calcium deficiency does not reduce accumulation of cyclic AMP in response to glucagon but diminishes stimulation of gluconeogenesis by exogenous cyclic AMP. Glucagon has a rapid stimulatory effect on the flux of "Ca++" from medium to tissue.

The hormonal control of gluconeogenesis can be studied with greater precision and convenience in isolated liver cells than in other preparations. In previous studies, these cells were shown to respond to glucagon and epinephrine by a rise in the content of cyclic AMP1 (1-4) and the rate of gluconeogenesis (1, 2, 5-11), but the sensitivity to gluconeogenic hormones was very much less than observed in perfused liver. It was suggested that this low sensitivity is due to loss of hormone receptors or increased rates of hormone degradation, or both (2).

Using a slightly modified method (11) for cell preparation, we have studied the effect of various concentrations of glucagon, insulin, epinephrine, and cyclic AMP on lactate gluconeogenesis in the hepatocyte. We have attempted to correlate the effects on gluconeogenesis with changes in the level of intracellular cyclic AMP. The isolated hepatocytes permit a more detailed analysis than is possible using the perfused rat liver.

We also have examined the role of calcium ions in the control of lactate gluconeogenesis.

EXPERIMENTAL PROCEDURE

Materials—Mono component insulin and glucagon were obtained from the Eli Lilly Co., epinephrine from Park Davis; probenecid (sodium salt) from Sigma, and cyclic [3H]AMP (specific activity, 33 Ci/mmol) from New England Nuclear. Protein kinase was purified from rabbit skeletal muscle (12, 13).

Animal—Fed, male Sprague-Dawley rats weighing 175 to 300 g were used.

Preparation of Isolated Liver Cells—Cells were prepared by a modification (11) of the method of Berry and Friend (14). Briefly, this entailed perfusion of livers from male rats fed ad libitum with Krebs-Henseleit bicarbonate buffer with 0.25% fraction V albumin (Pentex), 5 mM glucose, 150 mg/liter of collagenase (Worthington type I), 400 mg/liter of hyaluronidase (Sigma), but without calcium ions or EDTA. The medium was gassed by vigorously bubbling 95% O2/5% CO2 directly into the perfusion reservoir. After 30 to 40 min the cells were dispersed as described by Berry and Friend (14) and the suspension was shaken for 10 min in the above medium with constant gassing. The suspension was filtered through nylon mesh and washed twice with Krebs buffer containing 1% albumin and 2.5 mM calcium chloride. The cells were resuspended in this latter buffer (usually in about 80 to 100 ml/liver) and incubated without substrate with gassing for 30 to 40 min in a 250-ml Erlenmeyer
flask. Trypan blue was excluded by 90 to 95% of the cells. Plastic labware was used throughout and all procedures were performed at or about 0°C.

**Determination of Rate of Gluconeogenesis**—One-milliliter aliquots of cell suspension were added to plastic tubes (17 x 100 mm) (Falcon Plastics) which contained appropriate hormone and substrate additions. The tubes were gassed for 1 min with 95% O₂/5% CO₂, stopped, and shaken (200 cycles/min) in a 37°C bath. The rates of gluconeogenesis were obtained as linear for at least 60 min for the amount of cells (about 100 μg of DNA) employed (11). Reactions are stopped with 0.5 ml of 5% ZnSO₄, 0.5 ml of 0.5 N Ba(OH)₂, and 2 ml of water then were added and the precipitate was removed by centrifugation. [¹°C]Glucose was separated from charged compounds by the procedure of Exton and Park (11, 15). Aliquots of the resin-treated supernatants were added to 10 ml of Aquasol (New England Nuclear) and counted in a Packard Tri-Carb liquid scintillation counter.

**Expression of Results**—Rates of gluconeogenesis were expressed as micromoles of lactate converted to glucose per 30 min per mg of DNA. The number of micromoles was obtained by dividing the total radioactivity incorporated into lactate by the specific activity of the labeled lactate. No correction was made for loss of ¹⁴CO₂ via randomization of oxalate in the Krebs' cycle and subsequent decarboxylation to phosphoenolpyruvate.

**Determination of Cyclic AMP in Hepatocyte Suspensions**—The amount of intracellular, extracellular (medium), and total (intracellular + medium) cyclic AMP was determined. After a suitable incubation period, cells and medium from 1 ml of hepatocyte suspension were separated by centrifugation for 10 s at room temperature. The medium was decanted into a test tube containing 500 μl of 0.6 N PCA and cyclic [³H]AMP (10,000 cpm, added to estimate the recovery of cyclic AMP). 10 μl of 500 μl of 0.6 N PCA and cyclic [³H]AMP (10,000 cpm) were added. Water (1 ml) was added to the cell pellet to make the volumes of the cell sample and medium about equal. The samples were purified with Dowex 50 cation exchange chromatography and assayed for cyclic AMP by the protein kinase binding assay, as described by Gilman (17). If total cyclic AMP were to be determined, 500 μl of 0.6 N PCA + cyclic [³H]AMP (10,000 cpm) were added to 1 ml of cell suspension and the samples then were purified and assayed as noted above.

**Preparation of Plasma Membranes**—Plasma membranes were prepared by a modification (16) of the method of Song et al. (17). Ten milliliters of cell suspension (generally about 400 to 500 mg of cells) were sedimented at 1000 x g for 2 min and resuspended in 10 ml of 1 mM NaHCO₃, pH 7.4, at 0°C. The cells were homogenized in the cold with 30 strokes in a tight fitting Dounce homogenizer. The homogenate was diluted to 40 ml with cold NaHCO₃ and a crude membrane fraction was sedimented by centrifugation at 1500 x g for 10 min. The pellet was resuspended in 2.8 ml of cold NaHCO₃ and 5.5 volumes of 300 μl of 1 mM NaHCO₃ and 5.5 volumes of 100 μl of 1 mM NaHCO₃ and centrifugation at 48.2% sucrose and then 4 ml of 42.4% sucrose. The tube was centrifuged at 0-4°C in the number 30 rotor of the Beckman L2-50 ultracentrifuge for 60 min at 66,000 x g. The light gray layer at 42.4%/48.4% interface was collected and washed three times by resuspension in 40 ml of 1 mM NaHCO₃ and centrifugation at 12,000 x g for 10 min. The membranes then were suspended in 500 μl of 1 mM NaHCO₃. They could be stored at -70°C for at least 1 month without loss of adenylate cyclase activity. The yield of membranes from 400 to 500 mg of cells was usually about 200 to 300 μg of membrane protein.

**Assay of Adenylate Cyclase and Phosphodiesterase Activity**—Adenylate cyclase activity of liver cell plasma membranes was estimated by the method of Johnson and Sutherland (18) and Pilkis et al. (16). Phosphodiesterase activity was assayed by the method of Beavo et al. (19).

**Analysis of [³Ca⁺]²⁺ Uptake by Isolated Liver Cells**—At various times after [³Ca⁺]²⁺ addition, 100-μl samples were transferred to the top of a 12-ml centrifuge tube (at 0°C) containing 9 ml of 7% bovine serum albumin in 150 mM NaCl and 5 mM CaCl₂ as a lower layer and 2 ml of the NaCl-CaCl₂ as the upper layer. After 2 min centrifugation the cells formed a button at the bottom, the extracellular radioactivity remaining in the upper 2 ml. The extracellular radioactivity was removed by a device which aspirated the upper 5 ml and then rinsed the walls from the lip down to the point of aspiration with NaCl-CaCl₂ solution. The remaining fluid above the pellet then was removed and the cells were dissolved in 1% sodium dodecyl sulfate, transferred to a scintillation vial containing 10 ml of Aquasol (New England Nuclear), and counted in a Packard Tri-Carb liquid scintillation counter.

**DNA Content of Hepatocytes**—DNA content of isolated liver cells was determined by a modification (11) of the diphenylamine method of Burton (20). One gram of liver tissue contains about 2.5 mg of DNA (11).

**RESULTS**

**Basal Levels of Cyclic AMP**—To estimate the basal level of cyclic AMP, a suspension of cells was incubated and sampled at intervals over a 15-min incubation. The total content of cyclic AMP in cells plus medium was stable at a value of 220 ± 10 pmol/mg of DNA in this particular experiment (Fig. 1). This is equivalent to about 0.45 nmol/g of liver, or a concentration of about 1 μM, assuming uniform distribution in an intracellular water content which is 50% of liver wet weight. This value is about the same as that obtained for whole rat liver (97, 99). In experiments with other batches of cells, the basal level of cyclic AMP ranged from 180 to 250 pmol/mg of DNA.

**Time Course of Effect of Glucagon or Epinephrine on Level of Cyclic AMP**—The time course of the change in the content of cyclic AMP in the medium plus cells in response to a maximally effective concentration of epinephrine (50 μM) plus theophylline (1 mM) shown in Fig. 1. The peak elevation was seen at the time of the earliest measurement (1 min), and the content was returned to the control value by 15 min. The response was somewhat variable and the peak was usually only about 2 times the basal level. It was therefore difficult to follow changes by the protein kinase binding assay unless large aliquots of cell suspension were used.

Fig. 2 shows the time course of the effect of increasing concentrations of glucagon in the presence of theophylline (1 mM) on total, intracellular, and extracellular levels of cyclic AMP. At the lowest concentration of hormone, total and intracellular cyclic AMP reached peaks at the earliest time of measurement (3 min) and declined to near basal levels by 40 min. However, at higher glucagon concentrations, the levels of cyclic AMP peaked later (5 to 6 min) and did not decline fully to the baseline by 40 min. At the highest glucagon concentration (70 nM), total cyclic AMP did not decline at all. Inclusion

**Fig. 1.** Time course of the effect of epinephrine on total cyclic AMP levels. Hepatocytes were incubated with 50 μM epinephrine plus 1 mM theophylline for various times and the incubation was terminated with 500 μl of 0.6 N PCA plus cyclic [³H]AMP. The resulting precipitate was sedimented and the supernatant fraction was chromatographed on Dowex 50 and assayed for cyclic AMP as described under "Experimental Procedure" (12, 16). Total cyclic AMP levels refer to cyclic AMP in the cells plus medium.
Glucagon (10 nM) elevated the nucleotide about 10-fold over the basal level at this time had increased 30-fold to about 30 mM. By 20 min, the ratio had fallen to 0.8, and by 40 min to 0.2. At this time, the concentrations of cyclic AMP in the isolated, perfused liver can lower the level of cyclic AMP most strikingly between 6 and 12 min. This effect was most easily seen by calculating the ratio of intracellular to extracellular cyclic AMP (Fig. 3 and inset), where the ratio was about 14:1 at 3 min with a concentration of 10 nM glucagon. The intracellular level at this time had increased 30-fold to about 30 μM. By 20 min, the ratio had fallen to 0.8, and by 40 min to 0.2. At this time, the concentrations of cyclic AMP in the medium had risen to 0.25 kM as compared to an almost undetectable concentration at zero time. However, since the intracellular cyclic AMP level was still above 1 μM, there remained a substantial concentration gradient across the plasma membrane from inside to outside.

The data from Fig. 2 also show that half-maximal stimulation was obtained with a glucagon concentration of about 1.5 nM. A glucagon concentration as low as 0.15 nM, in the presence of theophylline, consistently gave a 2 to 4-fold increase in the intracellular cyclic AMP content of cells plus medium (Fig. 3). This agent in the presence of glucagon enhanced the elevation of cyclic AMP inside and lowered the level outside of the cell. The cyclic AMP content of cells plus medium (Fig. 3) was not significantly altered by probenecid up to 40 min, suggesting that it did not affect production or metabolism of cyclic AMP. In separate experiments, 0.1 mM probenecid did not affect the basal- or glucagon-stimulated adenylate cyclase activity of liver plasma membranes (data not shown). The above results indicate that probenecid reduces efflux of cyclic AMP.

Effect of Insulin on Basal and Glucagon Elevated Levels of Cyclic AMP—Exton and Park (24) have shown that insulin in the isolated, perfused liver can lower the level of cyclic AMP when it has been elevated by glucagon or epinephrine. Insulin (10 nM) had no detectable effect on the basal level of cyclic AMP (375 ± 20 pmol/mg of DNA) and theophylline (1 mM) had no further significant effect (425 ± 35 pmol/mg of DNA). The effect of various concentrations of insulin on the total content of cyclic AMP in a cell suspension treated with an intermediate concentration of glucagon (1.5 nM) was tested after a 12-min incubation. Low concentrations of insulin (<1 nM) had no effect. Insulin at 1, 10, and 100 nM inhibited the glucagon stimulation 42 ± 2%, 57 ± 5%, and 54 ± 8%, respectively. Fig. 4 shows that insulin lowered the intracellular content of cyclic AMP most strikingly between 6 and 12 min. This

Control, zero time 180 ± 30; 2.5 min 170 ± 20; 10 min 170 ± 20; 20 min 170 ± 20; 40 min 170 ± 30. Insulin zero time 170 ± 10; 2.5 min 170 ± 20; 10 min 150 ± 10; 20 min 170 ± 10; 40 min 180 ± 20 pmol of cyclic AMP/mg of DNA.

![Fig. 2. Time course of the effect of glucagon on total, intracellular, and medium cyclic AMP levels. Ten milliliters of hepatocytes were incubated in 25-ml plastic Erlenmeyer flasks with increasing concentrations of glucagon (0.75, 1.5, 7.5, and 75 nM) for various times with continuous gassing. In order to distinguish between intracellular and medium cyclic AMP, aliquots (1 ml) of cells and medium were separated by rapid centrifugation (10 s) and 0.6 N PCA containing tritiated cyclic AMP was added to the cell pellet and to the medium. In this experiment total cyclic AMP was obtained by adding medium and intracellular values. The incubation medium contained 1 mM theophylline.](http://www.jbc.org/)

![Fig. 3. Effect of probenecid on cyclic AMP efflux from isolated liver cells. Hepatocytes (~80 μg of DNA/ml) were incubated with glucagon (10 nM) in the presence and absence of 0.1 mM probenecid. Cyclic AMP levels in the cells and in the medium were determined as described in the methods (see also Fig. 2). The incubation medium contained 1 mM theophylline. In the inset to the figure, I/E represents the ratio of intracellular cyclic AMP to extracellular cyclic AMP. C represents cells incubated with glucagon alone, and P represents cells incubated with glucagon plus probenecid.](http://www.jbc.org/)
effect was also accompanied by a lower content of the nucleotide in the medium.

If cells were first incubated with 15 nM insulin for 10 min and then 1.5 nM glucagon was added for 3 min, there was a 51% inhibition of the elevation of total cyclic AMP obtained with glucagon alone (data not shown). When insulin was added with the glucagon at zero time the inhibition was only 29%. These data suggest that insulin requires several minutes to reach a maximal effect.

**Effect of Insulin and Glucagon on Adenylate Cyclase and Phosphodiesterase Activity**—The effects of glucagon and insulin on adenylate cyclase and phosphodiesterase activity were investigated. The adenylate cyclase activity of plasma membranes prepared from isolated hepatocytes (see "Experimental Procedure") was compared with that of membranes from whole liver (Table I). Both preparations required either Mg$^{2+}$ or Mn$^{2+}$. Activity as a function of these ions varied in a complex way depending on the concentration of the ion and the presence or absence of hormones or other activators. Details will be presented separately. In general, the properties of the adenylate cyclase from isolated liver cells or from whole liver were similar. Both preparations were stimulated strongly by 10 mM fluoride but membranes from isolated hepatocytes were consistently more sensitive to glucagon. Insulin did not inhibit basal or glucagon-stimulated adenylate cyclase activity in either preparation, tested with Mg$^{2+}$ or with Mn$^{2+}$ (Table I).

Neither glucagon or insulin alone or in combination affected the phosphodiesterase activity of a 30,000 × g supernatant fraction of cells that had been preincubated with the hormones for 10 min. Phosphodiesterase was assayed with 1 μM cyclic AMP which has been shown to be satisfactory for demonstrating insulin effects in adipocytes (26-29).

**Sensitivity of Gluconeogenesis in Isolated Cells to Glucagon, Epinephrine, and Cyclic AMP**—Garrison and Haynes (2) reported that the sensitivity of isolated liver cells to stimulation of gluconeogenesis by glucagon was almost 3 orders of magnitude less than that of the perfused liver, unless peptides were added to the incubation to protect the hormone from degradation. Epinephrine sensitivity was 2 orders of magnitude lower, and sensitivity to cyclic AMP was about 25% of that in the perfused liver.

The dose-response curves for glucagon and cyclic AMP in our preparation, using 2 mM [U-14C]lactate as the gluconeogenic substrate are shown in Fig. 5. The cells were sensitive to concentrations of glucagon as low as 0.1 nM, and the concentration for half-maximal stimulation was about 0.3 nM. These values agree well with those found for the perfused rat liver (30, 31). No protective agents were needed; bacitracin, which has been reported to inhibit glucagon degradation (32), was without effect (data not shown). The cells responded detectably to cyclic AMP at concentrations as low as 10 nM, and the concentration for half-maximal effect was about 30 nM. These values also agree with those found for the perfused rat liver (31, 32). Epinephrine at optimal concentration stimulated gluconeogenesis much less effectively than glucagon. A half-maximal response was obtained at 0.2 μM. Optimal concentrations of glucagon (10 nM) and epinephrine (10 μM) were not additive (Fig. 5, inset).

Effect of Insulin on Glucagon Stimulated Gluconeogenesis—
FIG. 5. Effect of glucagon, cyclic AMP, and epinephrine on gluconeogenesis in hepatocytes. Cells were incubated with 2 mM [U-\(^{14}\)C]lactate for 30 min. \[^{14}\text{C} \]Glucose was separated from \[^{14}\text{C} \]lactate by procedures described under "Experimental Procedure." The arrows indicate the concentration of effector giving half-maximal stimulation. The inset shows rates of gluconeogenesis in micromoles/30 min/mg of DNA; B is basal, E is epinephrine (10 \(\mu\)M), and G is glucagon (10 nM). Data for glucagon and cyclic AMP are from one batch of cells; another batch was used for the epinephrine curve but a concurrent glucagon curve was exactly as shown in the figure. Three aliquots of cells were analyzed for each point.

Fig. 6 illustrates the effect of increasing insulin concentrations on gluconeogenesis from 2 mM lactate in the absence and presence of glucagon. The concentration of glucagon (0.5 nM) was chosen to give a submaximal stimulation. Insulin had little effect on the basal rate but inhibited partially glucagon action. The half-maximal effect of insulin was obtained at about 0.3 nM and a maximal effect was approached at about 10 nM hormone. In studies to be reported in detail later, \(^*\) insulin (10 nM) completely prevented the stimulation of lactate gluconeogenesis observed with low concentrations of glucagon (0.1 nM) or exogenous cyclic AMP (10 \(\mu\)M).

Role of Ca\(^{2+}\) in Glucagon Stimulation of Gluconeogenesis—The mechanism whereby glucagon stimulates gluconeogenesis from lactate is unknown but the participation of calcium has been suggested (33-35) from studies using the perfused liver. We have now tested the role of Ca\(^{2+}\) in the isolated cell preparation where control of the ion concentration is more precise.

When EGTA was added at a concentration slightly above that of the Ca\(^{2+}\) in the medium, the stimulation by glucagon was partially inhibited at high but not at low concentration of hormone. Similarly, EGTA suppressed partially the effect of an optimal concentration of exogenous cyclic AMP but did not affect the action of a low, intermediate concentration (Fig. 7). With a higher concentration of EGTA (4 mM), however, the glucagon response was diminished at all hormone concentrations (Fig. 8). In experiments not shown, 4 mM EGTA inhibited both epinephrine- and cyclic AMP-stimulated gluconeogenesis in a similar manner. EGTA did not reduce detectably the basal rate of gluconeogenesis (Figs. 7 and 8). When calcium was added to liver cells in excess of added EGTA, the inhibition by


Fig. 6. Effect of insulin concentration on gluconeogenesis. Hepatocytes were incubated for 30 min with 2 mM [U-\(^{14}\)C]lactate with and without 0.5 nM glucagon in the absence and presence of 10 nM insulin. The glucose synthesized was determined as described under "Experimental Procedure." EGTA of the glucagon stimulation was prevented (data not shown).

To investigate further the role of Ca\(^{2+}\), cells were first prepared and preincubated in the absence of Ca\(^{2+}\) and were then incubated with 2 mM [U-\(^{14}\)C]lactate and various concentrations of Ca\(^{2+}\) in the presence or absence of 10 nM glucagon (Fig. 9). Calcium had little effect on the basal rate of gluconeogenesis, but enhanced the stimulation by glucagon substantially. A half-maximal effect was obtained with a concentration of 0.5 mM calcium. It can also be seen (Fig. 9) that stimulation of gluconeogenesis by cyclic AMP was partially dependent on Ca\(^{2+}\). Calcium ion also enhanced the effect
Fig. 7. Effect of EGTA on gluconeogenesis. Hepatocytes were incubated with 2 mM [U-14C]lactate for 30 min with or without the following additions: glucagon, 0.3 or 30 nM, cyclic AMP, 30 μM or 0.3 mM; and EGTA, 2.8 mM. The calcium in the medium was 2.5 mM. Glucose was determined as described under "Experimental Procedure." Each value is the mean obtained from three aliquots from a single preparation of cells.

Fig. 8. The effect of EGTA on basal and glucagon-stimulated gluconeogenesis. Hepatocytes were incubated with 2 mM [U-14C]lactate for 30 min with increasing concentrations of glucagon (zero to 0.1 nM) in the presence and absence of 4 mM EGTA. Glucose was determined as described under "Experimental Procedure." Each value is the mean from three aliquots from a single preparation of cells.

Fig. 9. The effect of calcium on gluconeogenesis in hepatocytes. Hepatocytes were prepared as usual but were preincubated for 30 min in a medium which did not contain calcium. Gluconeogenesis from labeled lactate (2 mM) in increasing calcium concentrations was estimated. Cells were incubated with additions as shown. Glucose was estimated as described under "Experimental Procedure." Each value is the mean from three aliquots from a single preparation of cells.

**DISCUSSION**

The low sensitivity of isolated hepatocytes to hormones reported by Garrison and Haynes (2) was presumably due to proteases leaking from damaged cells or carried over from the collagenase treatment (1, 2, 6, 7). In our cells, the sensitivity to glucagon, cyclic AMP, epinephrine, and insulin was equal to that of the perfused liver (30, 31). This suggests that the membrane receptors as well as the added hormones are reasonably well preserved. Consistent with this suggestion was the finding that plasma membrane-bound adenylate cyclase prepared from isolated liver cells was as responsive to glucagon as adenylate cyclase prepared from whole liver, which was not subjected to collagenase digestion. The lack of any potentiation of low concentrations of glucagon by bacitracin suggests that there is little proteolytic activity in the incubation medium. Poor sensitivity and response in earlier studies (1, 2, 6, 7) were probably caused in part by use of high, unphysiological substrate concentrations and use of cells from fasted animals.

The maximum effect of opsinprhin on liver cell cyclic AMP and on gluconeogenesis was much less than could be obtained with glucagon. This contrasts with earlier observations with the perfused liver in which the hormones were equally effective on gluconeogenesis from 20 mM lactate (30, 31). In isolated hepatocytes the maximum effect of glucagon diminishes as the substrate concentration is increased. For example, with 1 mM lactate glucagon has a 4-fold stimulatory effect while at 20 mM the glucagon enhancement is only 2-fold. In cells from fasted rats the rates of gluconeogenesis are 3-fold higher than those observed in cells from fed rats and the glucagon stimulation is only 40%.
glycogenolysis (S. B. Lewis, J. H. Exton, and C. R. Park, unpublished additive. The reason for the discrepancy is unknown. However, it found that glucagon and epinephrine effects on gluconeogenesis were incubated for 2 hours during which time there may have been varying should be noted that in the studies of Tolbert and Fain cells were (results).

Fig. 10. Effect of calcium on basal and glucagon elevated levels of cyclic AMP. Liver cells were prepared and preincubated as described in Fig. 9. Total cyclic AMP was estimated (see "Experimental Procedure") in cells that were incubated for 5 min with and without glucagon and calcium as shown. The experiment was carried out simultaneously and with the same cell preparation as that described in Fig. 9. Each value is the mean from three aliquots from one batch of cells.

hapatocytes from fed animals incubated with this level of lactate, the differential between epinephrine and glucagon was diminished but not abolished, and in cells prepared from fasted rats optimal concentrations of glucagon and epinephrine were equally effective.* The effects of optimal concentrations of glucagon and epinephrine on gluconeogenesis from 2 mM lactate were not additive* (Fig. 5). It now seems likely that a 2-fold elevation of cyclic AMP by epinephrine is not sufficient to activate fully gluconeogenesis in cells from fed rats.

Cyclic AMP appears in the perfusion medium in the isolated rat liver preparation (16, 31, 36, 37), in the incubation medium of rat epididymal fat pads (38), rat isolated fat cells (39, 40), platelets (41), and cerebellar slices (42). It has been suggested (43) in the case of the fat cell that this leakage may reflect damage to the plasma membranes as a consequence of the preparative procedure. Compounds which increase membrane permeability, such as filipin, increased the efflux of cyclic AMP from adipocytes (43) and this efflux is not inhibited by probenecid (40). It is unlikely, however, that the efflux from isolated liver cells is due to damage, although some leakage on this basis cannot be excluded. The inhibition of efflux by probenecid seen in this study points specifically to a transport system. The agent inhibited efflux but did not significantly affect the total amount of cyclic AMP during the first hour of incubation. Thereafter, probenecid caused a fall in total cyclic AMP (Fig. 3). This fall, also seen in pigeon erythrocytes (23), may be due to an induction or activation of phosphodiesterase as a consequence of elevated cyclic AMP (44-46).

Under normal circumstances, cyclic AMP exit is driven both by the cyclic AMP concentration gradient and the electrical gradient. No metabolic energy is required for transport of the nucleotide out of the cell. Given a physiological pH of 7.2 to 7.4, a pK 1 for cyclic AMP, and a liver membrane potential of -40 mv (35), the Nernst equation predicts an equilibrium concentration ratio, out/in, of 5. For example, according to the Nernst equation, the minimum external concentration of cyclic AMP required to double the internal concentration (to 2 M) would be about 10 M. In line with these considerations, 10 M is about the concentration of exogenous cyclic nucleotide for metabolic effects to become clearly apparent (Fig. 5). However, the above calculation ignores the effect of phosphodiesterase activity which will raise further the external concentration required for a given internal concentration to be reached.

Insulin partially counteracts the stimulation of gluconeogenesis by glucagon when both hormones are present in the incubation medium at concentrations presumed to occur in the portal vein under physiological conditions (Fig. 6). This effect correlates well with the ability of the hormone to inhibit the rise in cyclic AMP as a result of glucagon addition (Fig. 4). This result is consistent with the theory that insulin suppresses gluconeogenesis only when the level of cyclic AMP has first been elevated (21). The mechanism whereby insulin lowers the level of cyclic AMP in isolated liver cells and in other liver systems (24, 31, 47, 48) is controversial (16, 49, 51) with both inhibition of adenylate cyclase (52, 53) and activation of phosphodiesterase (26-29, 54, 55) postulated as actions of the hormone. Our data exclude a direct action of insulin on adenylate cyclase (Table 1). The inhibition of the rise in cyclic AMP levels by insulin is not due to loss into the medium as the level of cyclic AMP in the medium was also depressed (Fig. 4). However, a possible exception to the generalization that insulin acts solely by lowering cyclic AMP levels is the

![Graph](https://via.placeholder.com/150)

**Fig. 11. Effect of glucagon on calcium flux.** Liver cells were preincubated with 2.5 mM calcium in Krebs-Henseleit buffer for 40 min. They then were incubated with "Ca"+ (1 μCi/ml; specific activity, 30 Ci/g of calcium) with and without 10 μM glucagon. "Ca"+ taken up by the cells was determined as described under "Experimental Procedure." Note that the vertical scale is logarithmic.
observation that insulin inhibits gluconeogenesis stimulated
by the α-component system of the catecholamines, which may
not employ cyclic AMP as the intracellular mediator (9, 56).

It has been postulated (33–35) that glucagon activates or
inhibits steps in the gluconeogenic pathway, or both, by
modifying ion fluxes across cellular membranes. A role for
calcium in the action of the hormone is suggested by the ability
of EGTA to diminish the stimulation of gluconeogenesis by
cyclic AMP, glucagon, and epinephrine without altering the
basal rate. While it is recognized that EGTA may have effects
on cells by mechanisms other than chelation of Ca2+, the
observation that addition of Ca2+ to cells prepared without
calcium augments the glucagon response favors a role for
Ca2+. This is supported by the observation that addition of
Ca2+ in excess of EGTA completely restores the glucagon
stimulation to that seen in the absence of EGTA. Moreover,
glucagon directly stimulates a flux of 4Ca2+ in isolated
hepatocytes. It is clear from earlier studies in the perfused
liver (34) that the glucagon effect on Ca2+ flux is mediated by
cyclic AMP. However, the presence or absence of Ca2+ does
not affect the basal or glucagon elevated level of cyclic AMP. A
number of suggestions have been made as to how Ca2+ could
affect the basal or glucagon elevated level of cyclic AMP. A
large number of suggestions have been made as to how Ca2+
could activate gluconeogenesis (6, 33, 57). It is interesting to
note that a calcium effect on gluconeogenesis can be demonstrated
for only a part (about 50%) of the total glucagon stimulation
and that Ca2+ has almost no effect on the basal rate (Fig. 9).
This may be due to an inability to completely deplete the cell
of calcium, but this observation could also fit with the idea
that activation of at least two steps, only one of which is
affected by Ca2+, is necessary for a full glucagon effect.

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