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Mechanism for acute control of fatty acid synthesis by glucagon and 3′:5′-cyclic AMP in the liver cell

(acetyl-CoA carboxylase/citrate)

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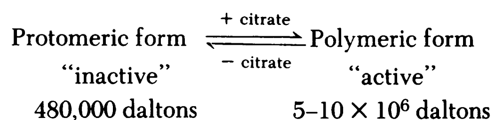
ABSTRACT Labeling experiments with chicken liver cell monolayers and suspensions show that glucagon and N^6,O^2-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP) block fatty acid synthesis from acetate without appreciably affecting cholesterogenesis from acetate or acylglyceride synthesis from palmitate. Neither acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] activity assayed in the presence of citrate nor fatty acid synthetase activity is decreased in extracts of cells treated with glucagon. However, the cytoplasmic concentration of citrate, a required allosteric activator of acetyl-CoA carboxylase, is depressed more than 90% by glucagon or dibutyryl cyclic AMP. Pyruvate or lactate largely prevents the inhibitory action of these effectors on fatty acid synthesis by causing a large increase in cytoplasmic citrate level. Thus, it appears that glucagon, acting via cyclic AMP, inhibits fatty acid synthesis by blocking the formation of citrate, an essential activator of acetyl-CoA carboxylase.

In animal cells the mobilization and storage of reserve fuels, notably glycogen and fat, are acutely regulated by hormones whose actions are mediated by adenosine 3′:5′-cyclic monophosphate (cAMP) (1). The mechanisms by which glycogenolysis, glycogenesis, and lipolysis are modulated via cAMP-dependent protein kinases have now been largely clarified (1, 2). In contrast, the short-term hormonal control of lipogenesis is poorly understood. Although glucagon and cAMP are known to suppress fatty acid synthesis in certain tissues—e.g., liver (3–5)—neither the mechanism nor the site of inhibition by these agents has been elucidated.†

It is generally agreed (6) that acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2], which catalyzes the first committed step of fatty acid synthesis, is a key regulatory enzyme in this pathway. Investigations in this laboratory have shown (10–12) that the homogeneous carboxylases from both liver and adipose tissue exist in either a catalytically inactive protomeric state or an active polymeric filamentous form. Citrate, the allosteric activator of this enzyme, is required for both catalysis and polymerization (13). Thus, it has been proposed (6, 13) that the state of the protomer–polymer equilibrium:



and hence the rate of fatty acid synthesis are determined by cellular citrate concentration. However, compelling evidence implicating citrate as activator of the carboxylase in the intact cell has been lacking. Attempts to assess the correlation between rates of *de novo* fatty acid synthesis and cellular citrate concentration have led to inconclusive results (6). These studies suffer the serious limitation that citrate concentrations were determined for the whole tissue rather than for the extramitochondrial compartment where fatty acid synthesis is localized.

Evidence presented in this paper reveals that cAMP exerts its inhibitory effect on fatty acid synthesis by drastically decreasing the cytoplasmic citrate concentration. The close correlation between the cytoplasmic concentration of citrate and the rate of fatty acid synthesis strongly supports the postulated role of citrate as a "feed-forward" activator of acetyl-CoA carboxylase.

MATERIALS AND METHODS

Cholera enterotoxin was obtained from Schwarz/Mann. Bovine insulin and porcine glucagon were gifts of Dr. Walter N. Shaw, Eli Lilly and Co. Basal medium (Eagle) (10×) with Earle's salts and without L-glutamine and $NaHCO_3$ (BME) was obtained from Grand Island Biological Co.

Hepatocyte Isolation, Culture, and Incubation. White Leghorn chickens (10–15 days old; mixed sex) were fed a high-carbohydrate, low-fat diet ad lib. for at least 48 hr prior to sacrifice. Hepatocytes were prepared by external digestion with collagenase (14). Monolayer cultures [3×10^6 cells per 35-mm cell culture dish in BME supplemented with $NaHCO_3$, penicillin, streptomycin, glucose (final concentration, 25 mM), 5% rooster serum, and amino acids] were incubated at 37° under 90% air/10% CO_2 as previously described (14). Hepatocyte suspensions [5 to 10×10^7 cells in 2–3 ml of Eagle's basal medium with 0.22% $NaHCO_3$ and without amino acids and phenol red, supplemented with glucose (final concentration, 25 mM)] were incubated under 90% air/10% CO_2 in a gyratory bath at 37°. Concentrations of hormones and effectors, when used, were as follows: insulin and glucagon, 5 μg/ml of medium; cholera toxin, 1 μg/ml; N^6,O^2-dibutyryl cAMP (Bt_2cAMP), butyrate, and cAMP, 0.1 mM; and pyruvate, 5 mM.

Fatty Acid and Cholesterol Synthesis. Liver cell monolayers or suspensions were incubated with 5 mM Na [*1*-^{14}C]acetate (0.2 μCi/μmol) or ^3H_2O (1 mCi/ml of medium). After saponification, cholesterol was extracted and isolated as the digitonide; fatty acids were extracted with petroleum ether following acidification (14).

Acylglyceride Synthesis. Liver cell monolayers were incubated with 5 mM Na [*1*-^{14}C]acetate (0.2 μCi/μmol) and 0.3 mM palmitic acid adsorbed to bovine serum albumin (10 mg/ml of medium) or 5 mM Na acetate and 0.3 mM [*U*-^{14}C]palmitic acid adsorbed to albumin (1.7 μCi/μmol). After extraction with chloroform/methanol, 2:1 (vol/vol), lipid classes were resolved

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; BME, basal medium (Eagle) (10×) with Earle's salts and without L-glutamine and $NaHCO_3$; Bt_2cAMP, N^6,O^2-dibutyryl cAMP.

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† It has not been possible to confirm (6) earlier reports that acetyl-CoA carboxylase is inhibited by a cAMP-dependent (7–9) or cAMP-independent protein kinase-catalyzed phosphorylation.

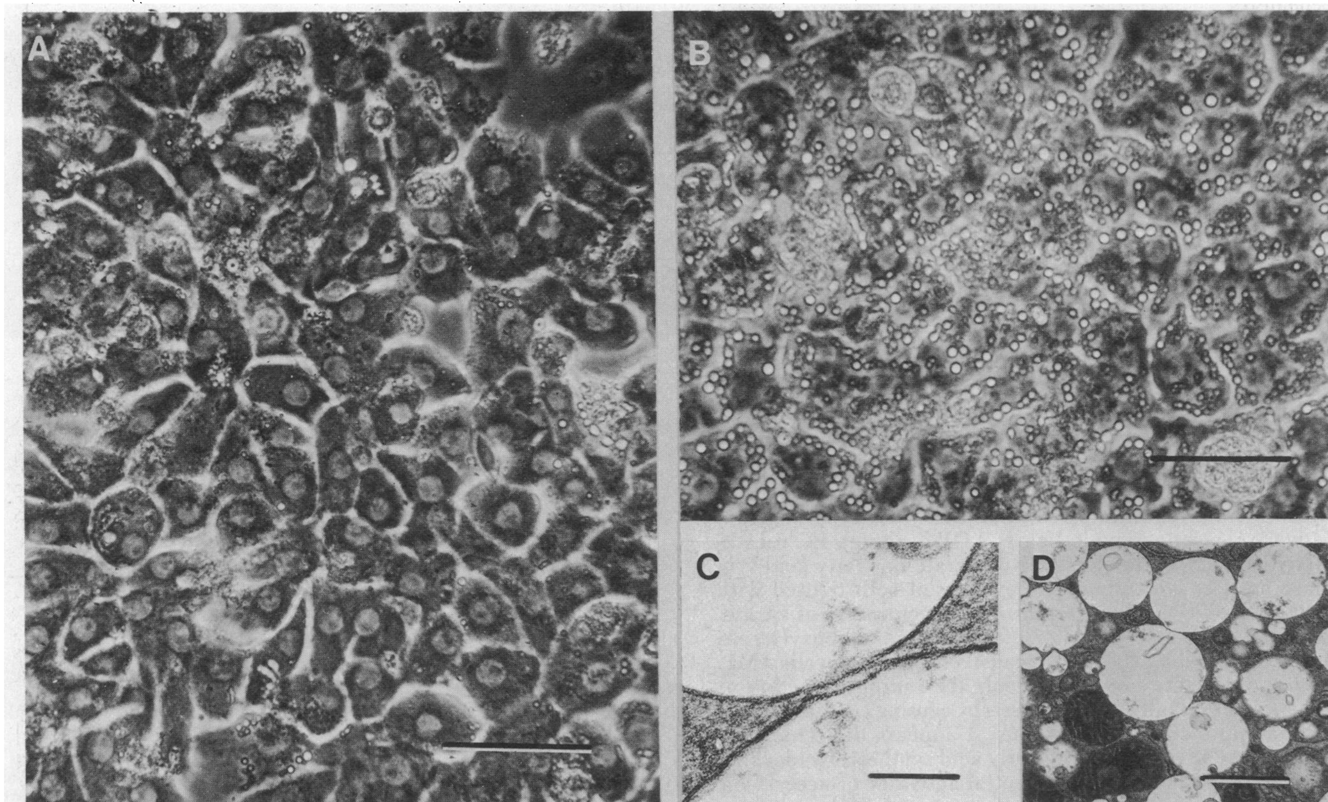

FIG. 1. Liver cells in monolayer culture for 48 hours in the presence of insulin plus glucagon (*A*) or insulin alone (*B*, *C*, and *D*). *A* and *B* are phase-contrast micrographs; *C* and *D* are electron micrographs of thin sections of glutaraldehyde- and osmium tetroxide-fixed cell monolayers embedded in Epon. Scale bars: *A* and *B*, 40 μm; *C*, 1 μm; *D*, 10 μm.

on silica gel thin-layer plates (solvent system: 1,2-dichloroethane/glacial acetic acid, 100:1, vol/vol) and radioactivity was counted (14).

Measurement of Cellular, Mitochondrial, and Cytoplasmic Citrate Levels. To measure total cellular citrate levels, cell suspensions were quenched with cold 6% $HClO_4$, and centrifuged; the supernate was neutralized with KOH. Under these conditions, less than 5% of the citrate contained in cell suspensions is extracellular. Mitochondria and cytoplasm were rapidly separated by a modification of the digitonin disruption-rapid stop technique (15, 16). Cell suspensions were mixed with cold digitonin-containing buffer such that the final concentrations were: sucrose, 0.25 M; potassium morpholinopropane sulfonate, 17 mM (pH 7.0); EDTA, 2.5 mM; hemimellitic acid (benzene-1,2,3-tricarboxylic acid), 8.4 mM; digitonin, 0.8 mg/ml; hexokinase, 0.1 mg/ml; glucose, 18.5 mM. This mixture was incubated for 80–90 sec at 4° and then the mitochondria were centrifuged for 1 min through a layer of dibutyl phthalate into 20% $HClO_4$ in a Beckman Microfuge B. The upper layer ("cytoplasm") was quenched immediately with 6% $HClO_4$ and then both fractions were neutralized. Citrate was determined fluorimetrically (17).

Enzyme Assays. To assay acetyl-CoA carboxylase, cells were homogenized in 2 mM potassium phosphate, pH 7.0/0.5 mM EDTA/15 mM toluene sulfonyl fluoride with a Dounce homogenizer. The composition of the homogenate was immediately changed to 50 mM potassium phosphate, pH 7.0/0.5 mM EDTA/1.0 mM dithiothreitol/10 mM toluene sulfonyl fluoride/50 mM NaF/5 mM potassium citrate. After a 1-min spin in a Beckman Microfuge, the supernate was assayed immediately by the method of Gregolin *et al.* (10) modified in that 10 mM potassium citrate and 2 mM dithiothreitol replaced isocitrate and glutathione, respectively. One unit of carboxylase fixes 1.0 μmol of $H^{14}CO_3^-$ per min at 37°. Cell-free extracts for fatty acid synthetase assay were prepared similarly except that NaF and citrate were omitted; the assay of Chang *et al.* (18) was used with [2-^{14}C]acetyl-CoA replacing [^{14}C]malonyl-CoA. After saponification, extraction of nonsaponifiables, and acidification, labeled fatty acids were extracted with petroleum ether and their radioactivity was counted as described above. One unit of synthetase catalyzes the incorporation of 1.0 μmol of [^{14}C] acetyl-CoA into fatty acids per min at 37°.

RESULTS AND DISCUSSION

Chicken liver cells in nonproliferating monolayer culture under appropriate conditions (14) maintain *in vivo* rates of fatty acid synthesis‡ for 5–6 days. Although 20–30% of the newly synthesized fatty acid is secreted into the medium as triglyceride in very low density lipoprotein (14), the greatest amount accumulated in the form of cytoplasmic triglyceride-rich vesicles (Fig. 1*B* and *D*). Staining with Oil Red EGN revealed that the vesicles had a lipid core; a bilayered membrane surrounding the lipid was evident in electron micrographs (Fig. 1*C*). Labeling experiments with [*1*-^{14}C]acetate showed the lipid content of isolated vesicles to be primarily (>90%) triglyceride with small amounts of phospholipid and cholesterol. The lipid components of the vesicles appeared to arise exclusively via *de novo* synthetic processes because their formation was not diminished by culturing the liver cells in lipid-free medium.

The addition of glucagon dramatically curtailed the formation of triglyceride-rich vesicles (Fig. 1*A*); Bt_2cAMP had the same effect (not illustrated). Both of these agents appeared to block vesicle formation, rather than to accelerate vesicle

‡ In the context of this paper, fatty acids refer to free fatty acids plus saponifiable glyceride acyl groups.

Table 1. Effect of glucagon on incorporation (natom/min per mg cell dry weight) of [1-^{14}C]acetate and ^3H_2O into fatty acids by chicken liver cells

Conditions	[^{14}C]Acetate incorporated	^3H_2O incorporated	
		No acetate	With acetate
Monolayers			
Control	1.9	—	—
Glucagon	0.088 (4.6%)	—	—
Suspensions			
Control	2.3	9.6	17.5
Glucagon	0.073 (3.2%)	0.31 (3.2%)	0.58 (3.3%)

Liver cell monolayers 4 hr after plating or liver cell suspensions immediately after isolation were incubated for 1 hr with medium containing 5 mM [1-^{14}C]acetate, ^3H_2O (1 mCi/ml), or ^3H_2O plus 5 mM unlabeled acetate. Numbers in parentheses are percentages of control values (without glucagon).

turnover, because neither agent caused the disappearance of vesicles already formed. These findings indicated that glucagon and cAMP might have an inhibitory effect on lipogenesis *per se*.

Exposure of liver cells in culture to glucagon caused a dramatic drop in the rate of [1-^{14}C]acetate incorporation into fatty acids (Table 1). Half-maximal inhibition of fatty acid synthesis occurred at a glucagon concentration of 5 ng/ml of medium. The inhibitory effect was not the result of a glucagon-induced dilution of the cytoplasmic [^{14}C]acetyl-CoA precursor pool because tritium incorporation from ^3H_2O into fatty acids was inhibited to the same extent as [^{14}C]acetate incorporation. The specific activity of the ^3H_2O utilized is unaffected by changes in the size of the acetyl-CoA pool (19).

Because glucagon is known to activate adenylate cyclase (20) and to increase intracellular cAMP levels (21), cAMP, Bt_2cAMP, and cholera enterotoxin [a potent activator of adenylate cyclase (22)] would be expected to have similar inhibitory effects on fatty acid synthesis. Bt_2cAMP inhibited [^{14}C]acetate incorporation into fatty acids to the same extent as glucagon; cAMP was somewhat less effective (Fig. 2*A*). This can be attributed to the slower rate of entry of cAMP into animal cells as compared to its dibutyryl derivative (23). Cholera toxin also inhibited fatty acid synthesis from [^{14}C]acetate (Fig. 2*B*), but only after a lag of about 1 hr; a similar delay in the action of cholera toxin is observed in other cell systems (22).

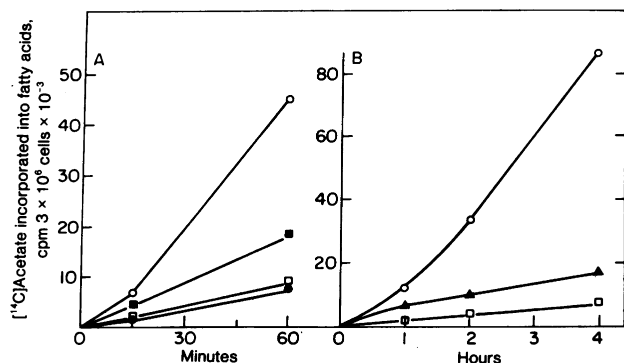

FIG. 2. Effect of glucagon (□), cAMP (■), Bt_2cAMP (●), and cholera toxin (▲) on the incorporation of [^{14}C]acetate into fatty acids by liver cells in monolayer culture. O, control. Four hours after plating, fresh medium containing 5 mM Na [1-^{14}C]acetate and the appropriate effector was added. After incubation, [^{14}C]acetate incorporation into fatty acids of cells plus medium was determined. Each point is the average of duplicate dishes.

Table 2. Effect of glucagon on incorporation of [1-^{14}C]acetate into fatty acids and cholesterol by chicken liver cell monolayers

Condition	[^{14}C]Acetate incorporation (nmol/min per mg cell dry weight)	
	In fatty acids	In cholesterol
Control	1.17	0.037
Glucagon	0.016 (1.4%)	0.025 (68%)

Procedures and data presentation as in Table 1.

Both fatty acid and cholesterol synthesis draw upon the same cytoplasmic acetyl-CoA precursor pool (Fig. 3). Were cAMP (or glucagon) to act at a point between extracellular [^{14}C]acetate and cytoplasmic [^{14}C]acetyl-CoA, by decreasing either the rate of uptake of [^{14}C]acetate or its conversion to [^{14}C]acetyl-CoA, the rates of fatty acid synthesis and cholesterogenesis from acetate should be equally affected. Glucagon blocked [^{14}C]acetate incorporation into fatty acids by 98%, whereas [^{14}C]acetate incorporation into cholesterol was decreased by only 30% (Table 2); Bt_2cAMP had the same effect. Thus, it appears that the action of glucagon or cAMP is focused on the fatty acid pathway, most likely on fatty acid synthesis.

These results, however, do not exclude the possibility that glucagon and cAMP might activate a catabolic pathway—e.g., β-oxidation—that would degrade labeled fatty acids already formed. To distinguish between these alternatives, the effect of glucagon on the rate of incorporation of [U-^{14}C]palmitate into cellular acylglycerides was determined. As shown in Table 3, triglyceride is the major labeled lipid synthesized from either [^{14}C]acetate or [^{14}C]palmitate. Whereas glucagon inhibited triglyceride synthesis from acetate by 97%, palmitic acid incorporation was depressed by only 25%. These results, together with those presented above, indicate that glucagon, acting via a cAMP-mediated process, blocks fatty acid synthesis *per se*.

The fact that the pathway between acetyl-CoA and fatty acid is blocked by glucagon implicates the acetyl-CoA carboxylase- or fatty acid synthetase-catalyzed reaction as possible targets of cAMP action. Some reports (7–9, 24), as yet unconfirmed,† suggest that both the carboxylase and synthetase may be regulated through a phosphorylation-dephosphorylation mecha-

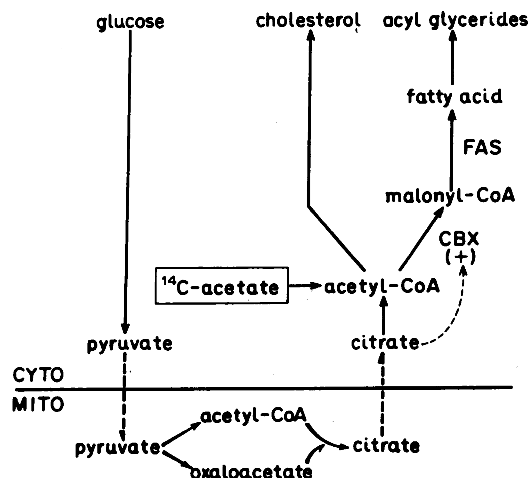

FIG. 3. Summary schema of fatty acid and cholesterol synthesis, showing steps in cytoplasm (CYTO) and mitochondrion (MITO); CBX and FAS refer to acetyl-CoA carboxylase and fatty acid synthetase, respectively.

Table 3. Effect of glucagon on incorporation (nmol/min per mg cell dry weight) of [*U*-^{14}C]palmitate and [*1*-^{14}C]acetate into acylglycerides by chicken liver cells

	[*U*-^{14}C]Palmitate incorporation		[*1*-^{14}C]Acetate incorporation	
Condition	In triglyceride	In phospholipid	In triglyceride	In phospholipid
Control	0.54	0.027	1.24	0.082
Glucagon	0.40	0.020	0.034	0.004

Liver cell monolayers 4 hr after plating were incubated for 1 hr with medium containing either 5 mM [*1*-^{14}C]acetate and 0.3 mM palmitic acid adsorbed to bovine serum albumin (10 mg/ml) or 5 mM acetate and 0.3 mM [*U*-^{14}C]palmitic acid adsorbed to bovine serum albumin. Cells were homogenized and then lipids were extracted with chloroform/methanol, separated by thin-layer chromatography, and assayed for radioactivity.

nism. Our investigations, however, show that neither the acetyl-CoA carboxylase nor the fatty acid synthetase activity of cytoplasmic extracts is affected by prior glucagon treatment of the cells, a condition that leads to >90% suppression of [^{14}C]acetate incorporation into fatty acids. In a typical experiment, carboxylase and synthetase activities (mean ± range) were 25 ± 2 munits (bicarbonate fixation with citrate present) and 4.3 ± 0.4 munits (palmitate formation) per mg of protein, respectively, whether the cytoplasmic extracts were obtained from glucagon-treated or control cells. Care was taken to minimize the time (<3 min at 4°) between cell disruption and assay to avoid reversal of cAMP-induced covalent modification of the enzymes if this had occurred.

Because citrate is a required activator of the avian liver acetyl-CoA carboxylase (6, 10–13), the possibility was considered that inhibition of fatty acid synthesis might result from a cAMP-mediated decrease in cellular citrate concentration. This would be consistent with the finding that glucagon blocks [^{14}C]acetate incorporation into fatty acids without significantly affecting [^{14}C]acetate incorporation into cholesterol (Table 2). As illustrated in Table 4, glucagon or Bt_2cAMP treatment of liver cell suspensions in a medium containing glucose as the sole carbon source decreased cellular citrate concentration and fatty acid synthetic rate to about the same extent; butyrate had no effect (data not shown). The addition of pyruvate, which markedly increased cellular citrate levels, caused a corresponding increase in the rate of incorporation of [^{14}C]acetate into fatty acids. Moreover, in the presence of pyruvate, the inhibitory effect of glucagon or Bt_2cAMP on fatty acid synthesis was largely prevented, as was the effect of these agents in decreasing cellular citrate concentration. Lactate had an effect similar to that of pyruvate (data not shown).

To assess the dependence of fatty acid synthesis on citrate level, the kinetics of [^{14}C]acetate incorporation into fatty acids and changes in cellular citrate concentration were compared. Hepatocytes were used immediately after isolation because such cells have a low citrate content. Both [^{14}C]acetate incorporation into fatty acids and the accumulation of cellular citrate exhibited similar kinetic patterns with comparable lag periods (Fig. 4*A*). A constant rate of fatty acid synthesis was achieved only after 30 min, presumably when sufficient citrate had accumulated to activate acetyl-CoA carboxylase maximally. Glucagon prevented the increase of both citrate level and fatty acid synthesis rate. When glucagon was added after the citrate concentration had increased to a level that supports maximal fatty acid synthesis, both citrate concentration and fatty acid synthetic rate fell abruptly (Fig. 4*B*). Thus, it appears that the

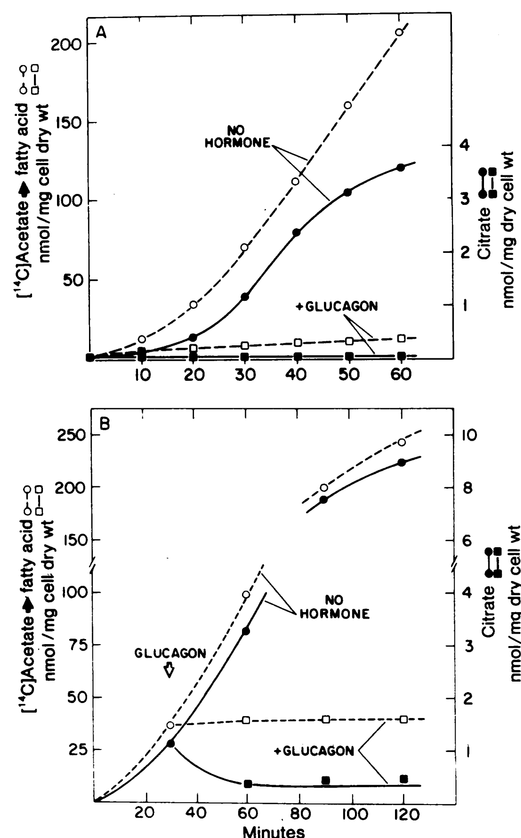

FIG. 4. Effect of glucagon on cellular citrate level and on [^{14}C]acetate incorporation into fatty acids by hepatocyte suspensions. (*A*) Freshly prepared hepatocytes were incubated with 5 mM [*1*-^{14}C]acetate in the presence or absence of glucagon. (*B*) Freshly prepared hepatocytes were incubated with 10 mM [*1*-^{14}C]acetate for 30 min, and then glucagon was added to half of the flasks.

rate of fatty acid synthesis from acetyl-CoA depends on cellular citrate concentration.

Because *de novo* fatty acid synthesis is strictly a cytoplasmic process (6), it was necessary to determine whether the citrate concentration in this cell compartment and fatty acid synthetic rate are similarly affected by glucagon and Bt_2cAMP. Preliminary experiments with conventional cell fractionation techniques yielded poor recoveries of this metabolically labile intermediate. Therefore, a modification of the digitonin disruption-rapid stop technique (15, 16) was used. Cells were incubated and then exposed briefly to 0.08% (wt/vol) digitonin, which disrupts the plasma and outer mitochondrial membranes and leaves the inner mitochondrial membrane intact. Hemimellitic acid (an inhibitor of mitochondrial citrate transport) and an ATP-trapping system (hexokinase and glucose) to block cytoplasmic ATP-citrate lyase also were present during the cell lysis.[§] Mitochondria, and other particulate components were then rapidly sedimented (<20 sec) through a layer of dibutyl phthalate into 20% $HClO_4$, after which the supernate ("cytoplasm") was immediately quenched with $HClO_4$. Under these conditions but without perchloric acid, 92% of the mitochondrial marker, citrate synthase, was recovered in the pellet and 92% of the cytoplasmic marker, lactate dehydrogenase, was in the supernate. Moreover, the recovery of citrate in the mitochondrial and cytoplasmic fractions equals that from cells

[§] Within 10–20 sec, intracellular components become exposed to the hemimellitic acid and the ATP-trapping system; under these conditions, cytoplasmic citrate levels were found to remain constant for at least 5 min.

Table 4. Effect of glucagon and dibutyryl cAMP on cellular citrate levels and fatty acid synthesis in chicken liver cells

Exp.	Treatment	Total cellular citrate and fatty acid synthesis: Cellular citrate level (nmol/mg cell dry weight)	[1-^{14}C]Acetate incorporation into fatty acids (nmol/min per mg cell dry weight)	Intracellular distribution of citrate: Cytoplasm (nmol/mg cell dry weight)	Mitochondria (nmol/mg cell dry weight)	Recovery, %
1	None	1.37	0.65	1.15 (83%)	0.24 (17%)	≥100
	Bt_2cAMP	0.12	0.05	0.12 (71%)	0.05 (29%)	≥100
2	None	1.13	0.87	0.93 (84%)	0.18 (16%)	97
	Glucagon	0.21	0.10	0.17 (81%)	0.04 (19%)	≥100
3	Pyruvate	4.11	2.28	3.06 (80%)	0.77 (20%)	94
	Pyruvate + Bt_2cAMP	6.93	1.52	5.66 (80%)	1.44 (20%)	≥100
4	Pyruvate	6.71	1.57	4.74 (72%)	1.86 (28%)	98
	Pyruvate + glucagon	7.91	1.20	6.18 (73%)	2.30 (27%)	≥100

Liver cell suspensions were incubated for 1 hr with 5 mM [1-^{14}C]acetate and appropriate additions. Sodium pyruvate was added at a level of 5 mM. After removal of an aliquot for assessment of [^{14}C]acetate incorporation into fatty acids, suspensions were either mixed with 6% $HClO_4$ and assayed for total cellular citrate or subjected to the digitonin disruption-rapid stop technique and the resultant fractions assayed for citrate. Numbers in parentheses are percentages of the total citrate (cytoplasmic plus mitochondrial) in the cell compartment.

quenched immediately with $HClO_4$. After a 1-hr incubation of the hepatocytes with glucose or glucose and pyruvate as carbon sources, about 75% of the cellular citrate was found in the cytoplasm (Table 4). Like total cellular citrate concentration, the cytoplasmic level was drastically decreased by glucagon or Bt_2cAMP with glucose as primary carbon source. When pyruvate was present, however, the effects of these agents were prevented.

Previous work (16, 10–13) established that tricarboxylic acid activator is an absolute requirement for catalysis by avian liver acetyl-CoA carboxylase. Taken in conjunction with this earlier evidence, the present investigation provides compelling evidence that cytoplasmic citrate is a required activator of fatty acid synthesis from acetyl-CoA in the intact liver cell. Thus, citrate serves dual functions in the cytoplasm, both as the precursor of fatty acids and cholesterol and as feed-forward activator of acetyl-CoA carboxylase (see Fig. 3). Importantly, cAMP, a pleiotropic effector of reserve fuel mobilization and storage, appears to regulate fatty acid synthesis in the liver cell by modulating cytoplasmic citrate levels. As yet, the specific site at which cAMP acts to depress cellular citrate concentration has not been identified. The observation that pyruvate prevents the decrease in citrate concentration caused by Bt_2cAMP when glucose is substrate suggests that the block occurs prior to pyruvate in the pathway between glucose and citrate.

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