HORMONAL REGULATION OF ACETYL-CoA CARBOXYLASE ACTIVITY IN THE LIVER CELL

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INTRODUCTION

Fatty acid synthesis in animal cells, such as the hepatocyte and adipocyte, is under rigorous hormonal control.[13,21,22,42] Evidence to be presented in this paper indicates that this control is, at least in part, exerted at the level of acetyl-CoA carboxylase. To appreciate the physiological basis for regulation at the carboxylase-catalyzed step, it is necessary to consider this reaction in metabolic context. Outlined in Figure 1 is the pathway of lipogenesis in animal tissues leading from glucose to triglyceride and phospholipid. Since several biosynthetic pathways utilize cytoplasmic acetyl-CoA, among them fatty acid and cholesterol synthesis, this precursor occupies a branch-point position in cytoplasmic metabolism. Hence, the first committed step of fatty acid synthesis beyond the branch-point is catalyzed by the carboxylase. It is not unexpected, then, that regulation might be exerted at this site.

It has been established[21,22] that acetyl-CoA carboxylase from animal tissues, either in homogeneous form or in cell-free extracts, requires the presence of tricarboxylic acid activator (e.g., citrate or isocitrate) for catalytic activity. Citrate appears to function as a feed-forward allosteric activator of the carboxylase (Figure 1). In addition, long chain fatty acyl-CoA derivatives, which are potent inhibitors of the carboxylase, in vitro appear to act as negative feedback control agents.[21] Compelling evidence, however, implicating citrate or fatty acyl-CoAs as effectors in the physiological state 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.[21] 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 occurs.

CHICK LIVER CELL CULTURE SYSTEM

Several years ago, we began to work on the development of a primary chick liver cell culture system to study the regulation of acetyl-CoA carboxylase and lipogenesis in a controlled physiological setting. Chick liver cells are particularly well adapted for studies on lipogenesis. The liver accounts for nearly all *de novo* fatty acid synthesis in the chick,[28] as it does in man,[12,34-36] and provides a substantial fraction of the fatty acid utilized by other tissues, including adipose tissue.

A technique for the preparation of viable liver cells from the chick (2 to 3 weeks of age) for monolayer and suspension culture which avoids hepatic perfusion and which is performed under sterile conditions was developed in our laboratory.[39] Hepatocytes are dissociated from minced liver tissue by external digestion with collagenase. When freshly prepared, 90 to 95% of the cells exclude Trypan blue, while 95 to 98% of the cells exclude the dye after 24 hr in monolayer culture. Short-term experiments (1 to 4

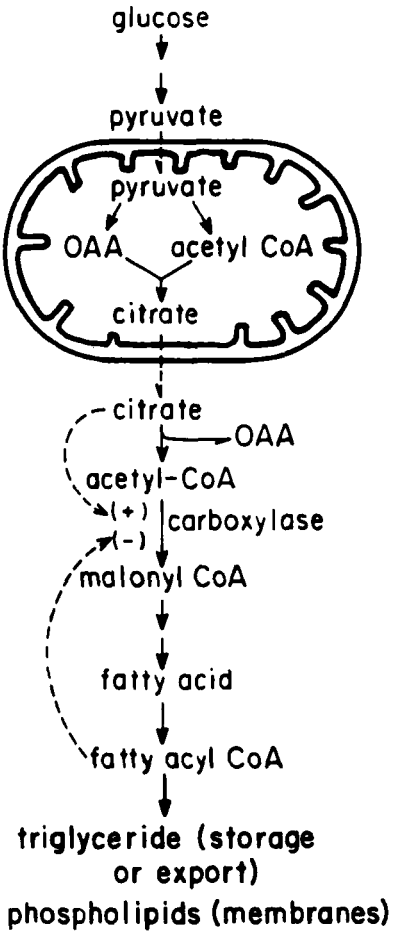

FIGURE 1. Pathway of *de novo* acyl glyceride synthesis in the liver cell.

hr) can be conducted with hepatocyte suspensions, whereas longer-term experiments require the use of liver cells in monolayer cultures. In both cases, cells are incubated at 37°C in Basal Eagles Medium supplemented with glucose (final concentration, 25 m*M*), 5% rooster serum, amino acids, and insulin (5 μg/m*l* of medium) under 90% air-10% CO_2, unless otherwise indicated.

When cell monolayers are prepared, more than 95% of the cells attach firmly to the culture dish within 2 to 4 hr after plating.[39] Upon attachment, cells begin to lose their refractile appearance, flatten, and spread out on the dish; confluency is achieved within 12 to 24 hr. Illustrated in Figure 2 is a confluent monolayer of avian liver cells in culture with insulin for 48 hr. Electron micrographs of these cells indicate polygonal cell boundaries and normal organelle morphology.[39] In monolayer culture, the cells carry out fatty acid synthesis, lipogenesis, cholesterogenesis, and very low-density lipoprotein synthesis and secretion at physiological rates for several days.[39] Avian liver cells cultured under these conditions do not proliferate, but, as with their counterparts in vivo, remain in the quiescent state.

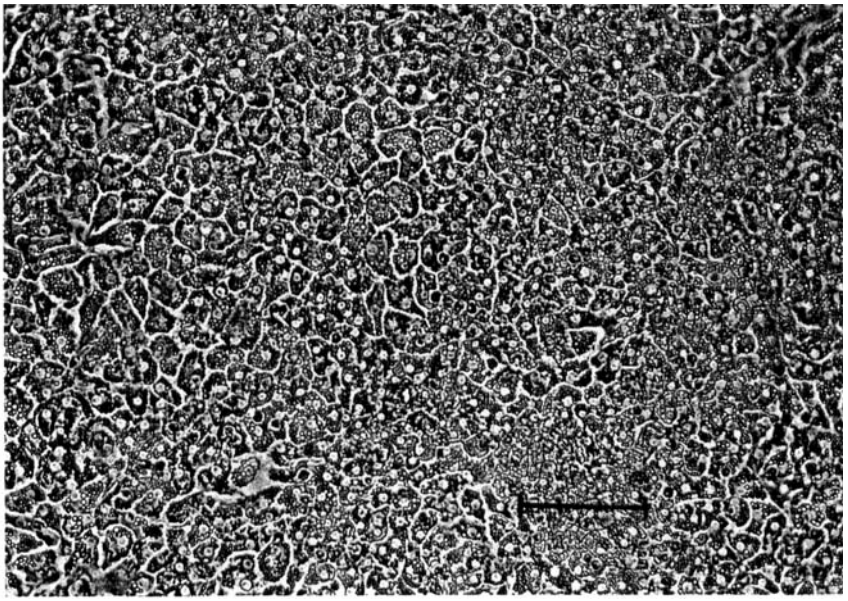

FIGURE 2. Photomicrograph of chick liver cell monolayer at 48 hr after plating in standard culture medium. Liver cells from fed 15-day-old chicks were isolated and plated; confluent monolayers of polygonal cells are obtained between 12 to 24 hr after plating. Scale bar is equivalent to 100 μm. (From Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., and Lane, M. D., *J. Cell Biol.*, 73, 332, 1977. With permission.)

EFFECT OF HORMONES AND Bt_2cAMP* ON FATTY ACID SYNTHESIS

Liver cells derived from chicks of differing nutritional state, i.e., from either fed or fasted animals, carry out fatty acid synthesis in monolayer culture at rates comparable to their counterparts in vivo.[39,44] Moreover, as illustrated in Figure 3, changes in apparent physiological state can be brought about in culture by treatment with appropriate hormones.[39] It has been established[21,22] that fasting or rendering an animal diabetic drastically curtails fatty acid synthesis.** As with fasting or the diabetic state, where the plasma insulin/glucagon ratio is depressed,[41] deleting insulin from the medium causes an 80 to 85% loss of fatty acid synthetic capacity of liver cells in culture within 48 hr (Figure 3A). The level of insulin required to maintain one half of the maximal fatty acid synthetic rate is 10 to 20 ng/mℓ of culture medium (Figure 4), which is five to ten times higher than the level in plasma.[8] Glucagon or Bt_2cAMP (not shown) greatly accelerate the decline in fatty acid synthetic capacity (Figure 3A). This pattern is consistent with the fact that hepatic cAMP levels rise during fasting or as a result of diabetes.[19] The level of glucagon required for one half of the maximal inhibition of fatty acid synthesis, about 4 ng/mℓ of culture medium (Figure 5), approximates the level in plasma in the fasting state.[9] Similar to the situation in vivo,[21] liver cells in monolayer culture derived from fasted chicks exhibit a greatly reduced fatty acid synthetic capacity (Figure 3B). Fatty acid synthesis can be restored to normal levels in culture by insulin with or without triiodothyronine, although the combination of both hormones accelerates recovery.[39] Glucagon or Bt_2cAMP (results not shown) blocks the increase in the [^{14}C]acetate incorporation rate in the presence of insulin or insulin plus

* Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; Bt_2cAMP, N^6, O^2-dibutyrylcAMP.
** Fatty acids in the context of this paper refer to free fatty acids plus saponifiable glyceride acyl groups.

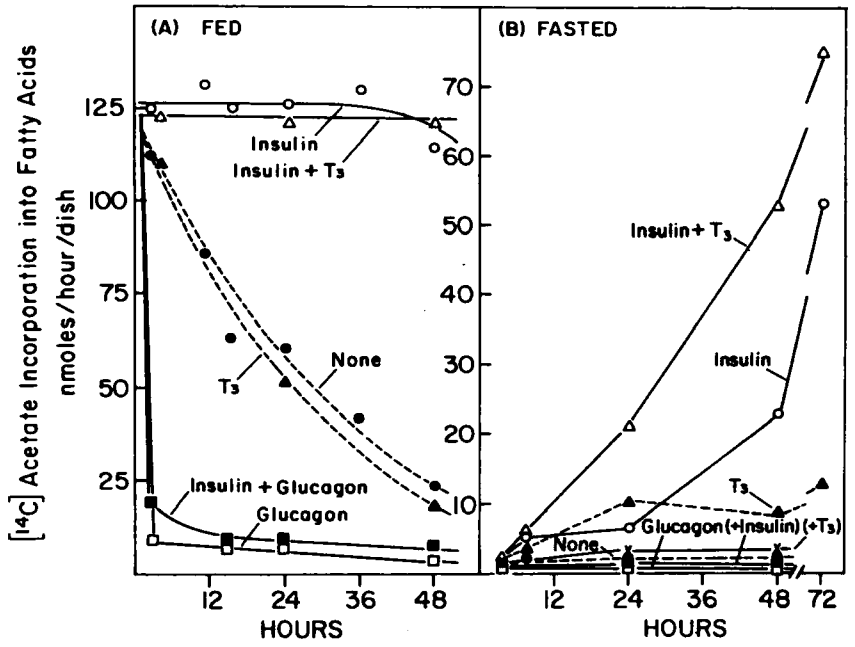

FIGURE 3. The effect of insulin, triiodothyronine, and glucagon on fatty acid synthesis by liver cells in monolayer culture derived from fed and fasted chickens. Liver cells (2.8×10^6 cells/35-mm dish) from (A) fed or (B) 36-hr fasted chickens were maintained in monolayer culture in the presence of the hormones indicated. At the times indicated, fresh medium containing 5 m*M* [1-^{14}C]acetate was added; the cells were incubated for 1 hr, and incorporation of [^{14}C] activity into total fatty acyls of cells plus medium was determined. Insulin, glucagon, and triiodothyronine were added at levels of 5 μg, 5 μg, and 1 μg/m*l* of medium, respectively, where indicated. (From Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., and Lane, M. D., *J. Cell Biol.*, 73, 332, 1977. With permission.)

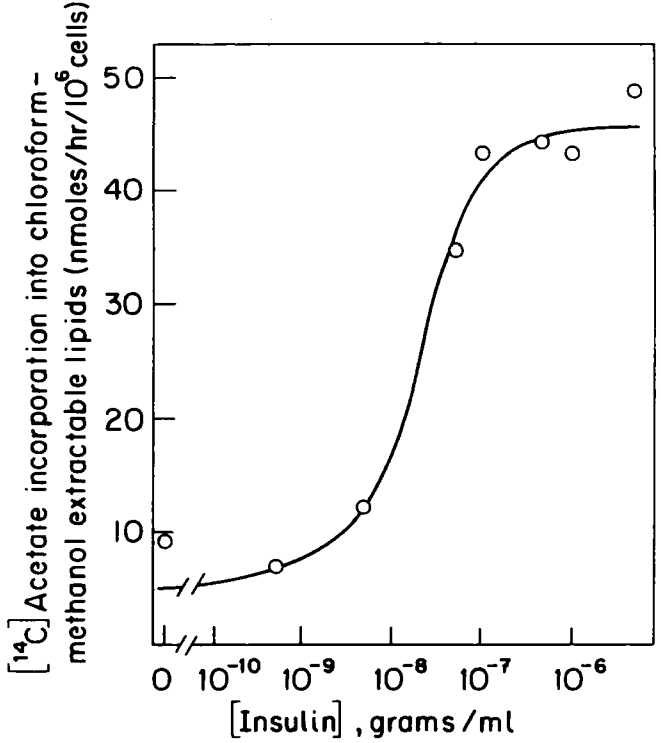

FIGURE 4. Concentration of insulin required for maintenance of lipid synthesis by chick liver cells in monolayer culture. Liver cells from fed chicks were maintained in monolayer culture with the level of insulin indicated. After 48 hr, 5 m*M* [1-^{14}C]acetate was added, the cells were incubated for 1 hr, and incorporation of [^{14}C] activity into chloroform-methanol extractable lipids in cell plus medium was determined; approximately 90% of the [^{14}C] activity in the chloroform-methanol extractable lipids is saponifiable glyceride acyl groups plus free fatty acids.

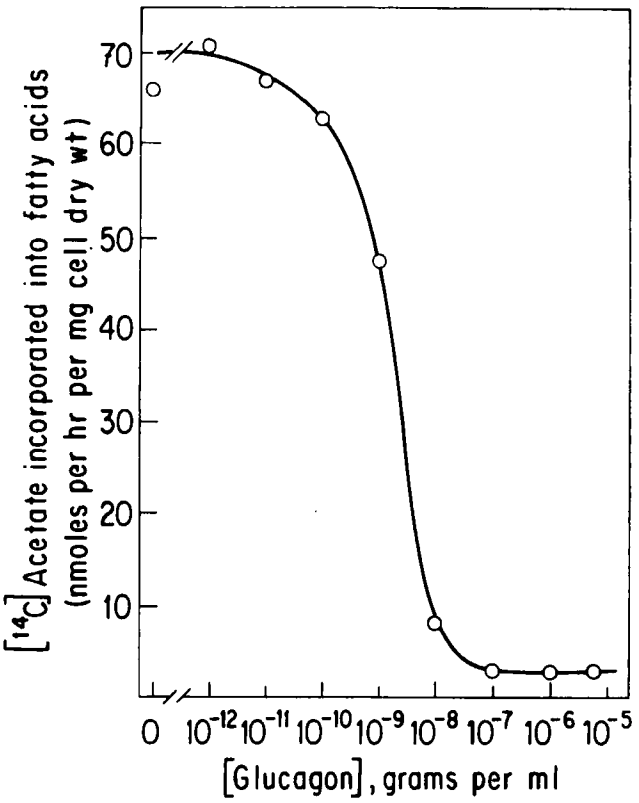

FIGURE 5. Concentration of glucagon required to inhibit fatty acid synthesis by chick liver cells in monolayer culture. Liver cells from fed chicks were maintained in monolayer culture for 4 hr with glucagon present. 5 m*M* [1-^{14}C] acetate was added; the cells were incubated for 1 hr, and incorporation of [^{14}C] activity into fatty acids of cells and medium was determined.

triiodothyronine (Figure 3B). Thus, fatty acid synthesis by chick liver cells in culture accurately reflects the nutritional state of the donor animal and responds to hormones and effectors, i.e., cAMP, known to affect this process in vivo.

When liver cells from chicks fed normally are maintained in monolayer culture for 48 hr in the presence of insulin, large amounts of lipid, primarily triglyceride, are synthesized.[39,44] Although an appreciable amount of this lipid is secreted into the medium,[39] a substantial amount accumulates in refractile cytoplasmic vesicles (Figure 6B). These vesicles contain triglyceride almost exclusively, hence the name triglyceride-rich vesicles.[39] Figure 7 shows an electron micrograph which reveals that the vesicles are bounded by a membrane bilayer varying in size from 0.1 to 2 μm. Examination of the electron micrographs suggests that vesicle expansion occurs by membrane fusion with both smaller osmiophilic lipoprotein-containing vesicles and smaller triglyceride-rich vesicles (see arrows, Figure 7). The vesicles can be isolated by flotation at low centrifugal force after cell lysis by gentle homogenization in hypotonic medium.[39]

Of particular interest is the fact that the addition of glucagon (or Bt_2cAMP, not shown) to the insulin-containing medium totally prevents the accumulation of triglyceride-rich vesicles (Figure 6A). It is evident that both of these agents act to block vesicle formation, rather than to accelerate vesicle turnover, since neither agent causes the disappearance of vesicles once formed. These findings, as well as those presented

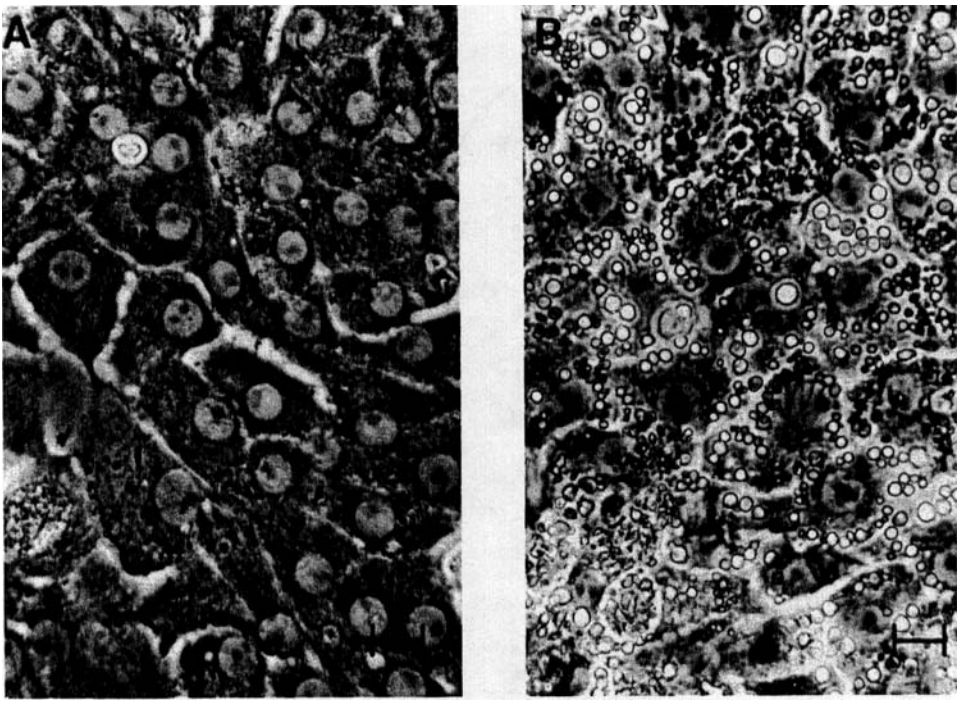

FIGURE 6. Liver cells from fed chickens maintained in monolayer culture for 48 hr with (A) insulin plus glucagon or (B) insulin. Note the presence of cytoplasmic triglyceride-rich vesicles in B and their absence in A. (From Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., and Lane, M. D., *J. Cell Biol.*, 73, 332, 1977. With permission.)

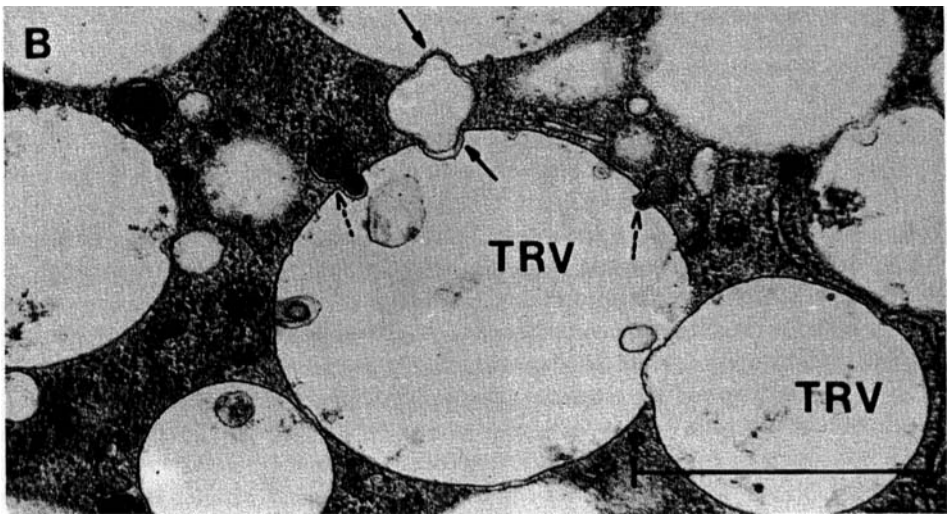

FIGURE 7. Electron micrograph of membrane-enclosed triglyceride-rich vesicles (TRV). Liver cells from fed chickens in monolayer culture for 24 hr in standard medium. Large triglyceride-rich vesicles appear to be undergoing fusion with smaller triglyceride-rich vesicles (unbroken arrows) and lipoprotein-containing vesicles (broken arrows); the latter contain osmiophilic particles having the properties of very low-density lipoprotein. The triglyceride-rich vesicles are bounded by a membrane bilayer. Scale bars are 1 μm. (From Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., E. E., and Lane, M. D., *J. Cell Biol.*, 73, 332, 1977. With permission.)

TABLE I

Effect of Glucagon on Fatty Acid and Cholesterol Synthesis by Chicken Liver Cells

A. Incorporation of [1-^{14}C] Acetate and $^{3}H_2O$ into Fatty Acids by Liver Cell Monolayers and Suspensions

Hormone treatment	^{14}C-acetate incorporated (nanoatoms/min/mg of cell dry weight)	$^{3}H_2O$ incorporated (nanoatoms/min/mg of cell dry weight)	
		Minus acetate	Plus acetate
Monolayers			
None	1.9	—	—
Glucagon	0.088 (4.6%)	—	—
Suspensions			
None	2.3	9.6	17.5
Glucagon	0.073 (3.2%)	0.31 (3.2%)	0.58 (3.3%)

B. Incorporation of [1-^{14}C] Acetate into Fatty Acids and Cholesterol by Liver Cell Monolayers

Hormone treatment	^{14}C-acetate incorporated into (nmol/min/mg of cell dry weight)	
	Fatty acids	Cholesterol
Monolayers		
None	1.17	0.037
Glucagon	0.016 (1.4%)	0.025 (68%)
Suspensions		
None	1.93	0.106
Bt_2cAMP	0.17 (8.8%)	0.077 (73%)

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

From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.

above, indicated that glucagon and cAMP have an inhibitory effect on lipogenesis per se.

Further investigation[44] showed that exposure of chick liver cells in culture to glucagon caused a >95% inhibition of the rate of [1-^{14}C]acetate incorporation into fatty acids (Table 1). This inhibitory effect was not the result of a glucagon-induced dilution of the cytoplasmic [^{14}C]acetyl-CoA pool with unlabeled precursors, since tritium incorporation from $^{3}H_2O$ into fatty acids was inhibited by glucagon to the same extent as [^{14}C]acetate incorporation (Table 1). The specific activity of the $^{3}H_2O$ utilized is unaffected by changes in the size of the acetyl-CoA pool.[43]

Since glucagon is known to activate adenylyl cyclase[29] and raise intracellular cAMP levels,[10] cAMP, Bt_2cAMP, and cholera enterotoxin, a potent activator of adenylyl cyclase,[1] would be expected to have similar inhibitory effects on fatty acid synthesis. As illustrated in Figure 8A, Bt_2cAMP inhibits [^{14}C]acetate incorporation into fatty acids

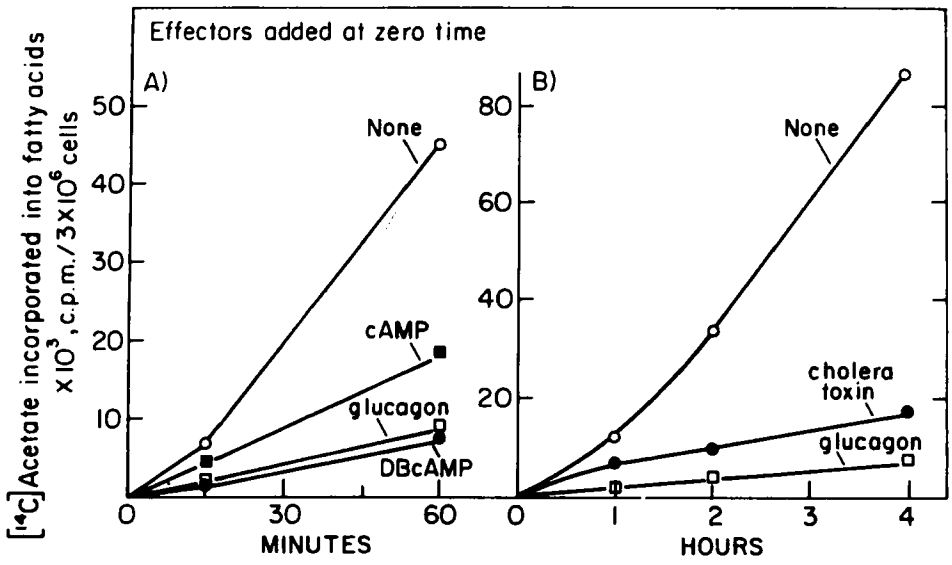

FIGURE 8. Effect of glucagon, cAMP, Bt_2cAMP, and cholera toxin on the incorporation of [^{14}C]acetate into fatty acids by liver cells in monolayer culture. Four hr after plating, fresh medium containing 5 m*M* sodium [1-^{14}C]acetate and the appropriate effector was added. Following incubation, [^{14}C]acetate incorporation into fatty acids of cells plus medium was determined. (From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.)

to the same extent as glucagon; cAMP is somewhat less effective. This can be ascribed to the slower rate of uptake of cAMP by animal cells compared to its dibutyryl derivative.[32] Cholera toxin also inhibits fatty acid synthesis from [^{14}C]acetate (Figure 8B). After a short lag, there is nearly complete suppression of fatty acid synthesis. A similar delay in the action of cholera toxin has been observed with other cell systems.[1]

EFFECT OF GLUCAGON OR Bt_2cAMP ON CHOLESTEROGENESIS, KETOGENESIS, AND ACYLGLYCERIDE SYNTHESIS

As illustrated in Figure 9, fatty acid and cholesterol synthesis utilize the same cytoplasmic acetyl-CoA precursor pool. Were cAMP (or glucagon) to act at a site between extracellular acetate and cytoplasmic acetyl-CoA, either by decreasing the rate of uptake of [^{14}C]acetate or its conversion to [^{14}C]acetyl-CoA, the rates of fatty acid and cholesterol synthesis from acetate should be equally affected. Glucagon and Bt_2cAMP, however, block [^{14}C]acetate incorporation into fatty acids by >90%, while [^{14}C]acetate incorporation into cholesterol is reduced by only 30% in either cell monolayers or suspensions (Table 1B). Thus, it appears that the actions of glucagon and cAMP are specifically focused on the fatty acid pathway, most likely fatty acid synthesis.

The results presented to this point do not exclude the possibility that glucagon and cAMP might activate a catabolic pathway, e.g., β-oxidation, which would degrade labeled fatty acids, once formed. To distinguish between these alternatives, the effect of glucagon on the rate of incorporation of [U-^{14}C]palmitate into cellular acylglycerides and of incorporation of [1-^{14}C]acetate into acetoacetate and β-hydroxybutyrate were determined. As shown in Table 2A, triglyceride is the major labeled lipid synthesized from either [^{14}C]acetate or [^{14}C]palmitate. Whereas glucagon inhibits triglyceride synthesis from acetate by 97%, palmitic acid incorporation is depressed by only 25%. Table 2B shows that under conditions where glucagon inhibits fatty acid synthesis by 95%, [1-^{14}C]acetate incorporation into ketones (acetoacetate + β-hydroxybutyrate) is

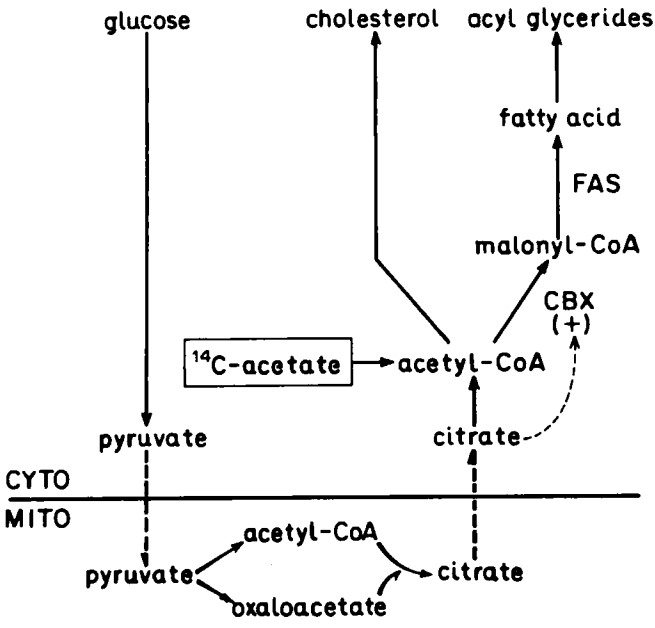

FIGURE 9. Summary schema of fatty acid and cholesterol synthesis, showing the steps in cytoplasm (CYTO) and mitochondrion (MITO); CBX and FAS refer to acetyl-CoA carboxylase and fatty acid synthetase, respectively. (From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.)

stimulated by only 27%. This increase in ketogenesis accounts for only a small diversion of [^{14}C]-labeled fatty acid synthesized into the β-oxidation-ketogenesis pathway. These results, together with those presented above, indicate that glucagon, acting via a cAMP-mediated process, blocks fatty acid synthesis per se.

EFFECT OF GLUCAGON AND Bt_2cAMP ON ACETYL-CoA CARBOXYLASE AND FATTY ACID SYNTHETASE ACTIVITY

Since the pathway between acetyl-CoA and fatty acid is blocked by glucagon, the two enzymes, acetyl-CoA carboxylase and fatty acid synthetase, which carry out the intervening reactions are implicated as direct or indirect targets of cAMP action. It has been reported by Kim's group[3-5,24] and by Porter's group[30] that acetyl-CoA carboxylase and fatty acid synthetase, respectively, are inactivated via a protein phosphorylation mechanism. Neither report has been confirmed. Investigations in our laboratory[38] show that while acetyl-CoA carboxylase does become phosphorylated by a cAMP-independent process when chick liver cells are incubated with $^{32}P_i$, the phosphorylated form of the enzyme is fully active catalytically. More to the point, however, it was found[44] (Table 3) that neither acetyl-CoA carboxylase nor fatty acid synthetase activity of cytoplasmic extracts is affected by prior glucagon (or Bt_2cAMP, not shown) treatment of the cells — a condition which leads to a >90% suppression of [^{14}C]acetate incorporation into fatty acids. In these experiments, care was taken to minimize the time (<3 min at 4°C) between cell disruption and assay to avoid reversal of cAMP-induced covalent modification of the enzymes had it occurred. In other experiments, exposure of cells to glucagon or Bt_2cAMP for up to 10 hr had no effect on carboxylase activity in cell-free extracts. While these results show that carboxylase and fatty acid synthetase activities in cell extracts are not altered by glucagon or Bt_2cAMP, they do

TABLE 2

Effect of Glucagon on the Incorporation of [U-^{14}C]palmitate and [1-^{14}C]acetate into Acylglycerides by Chicken Liver Cells

A. Incorporation of [U-^{14}C]Palmitate and [1-^{14}C]Acetate into Triglyceride and Phospholipid

Hormone treatment	[U-^{14}C]Palmitate incorporated into (nmol/min/mg of cell dry weight)		[1-^{14}C]Acetate incorporated into (nmol/min/mg of cell dry weight)	
	Triglyceride	Phospholipid	Triglyceride	Phospholipid
None	0.54	0.027	1.24	0.082
Glucagon	0.40	0.020	0.034	0.004

B. Incorporation of [1-^{14}C]Acetate into Fatty Acids and Ketones (Acetoacetate plus β-Hydroxy-butyrate)

Hormone treatment	[1-^{14}C]acetate incorporated into (nmol/min/mg of cell dry weight)	
	Fatty acids	Ketones
None	1.9	1.1
Glucagon	0.1	1.4

Note: In A, liver cell monolayers 4 hr after plating were incubated for 1 hr with medium containing either 5 m*M* [1-^{14}C]acetate and 0.3 m*M* palmitic acid adsorbed to bovine serum albumin (10 mg/m*l*) or 5 m*M* acetate and 0.3 m*M* [U-^{14}C]palmitic acid adsorbed to bovine serum albumin. Cells were homogenized, after which lipids were extracted with chloroform-methanol, separated by thin layer chromatography, and counted. In B, liver cell monolayers were incubated with 5 m*M* [1-^{14}C]acetate for 4 hr and the incorporation of [^{14}C] activity into total fatty acyls and ketones (acetoacetate plus D(−)-β-hydroxybutyrate) in cells plus medium determined.

From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.

TABLE 3

Effect of Glucagon on Acetyl-CoA Carboxylase and Fatty Acid Synthetase Activities of Chicken Liver Cells

Additions	[^{14}C] Acetate incorporated into fatty acid (nmol/hr/mg of cell dry weight)	Cytoplasmic enzyme activity: Acetyl-CoA carboxylase (nmol/min/mg of protein)	Cytoplasmic enzyme activity: Fatty acid synthetase (nmol acetyl-CoA/min/mg of protein)
None	109	26.4	4.3
		23.9	4.2
+ Glucagon	13.1	24.2	4.2
		25.9	4.5

Note: Liver cell suspensions were incubated for 1 hr in the presence or absence of glucagon. Cells were lysed, postmitochondrial supernates prepared, and the supernates assayed within 3 min after cell rupture.

not exclude the possibility that these activities are altered *in situ* by a cAMP-mediated change in the level of a dissociable allosteric effector of one of these enzymes.

EFFECT OF GLUCAGON AND Bt_2cAMP ON INTRACELLULAR CITRATE CONCENTRATION

Since citrate is an essential activator of acetyl-CoA carboxylase from animal tissues,[14,20,22,27] it appeared possible that inhibition of fatty acid synthesis might result from a cAMP-mediated decrease in cellular citrate concentration. Investigations in this laboratory[14,20,27] have shown that the homogenous carboxylases from both liver and adipose tissue exist in either a catalytically inactive protomeric state or an active polymeric filamentous form. Figure 10 shows an electron micrograph of the catalytically active filamentous form of the enzyme. The carboxylase filament is a twisted helical structure with dimensions of 70 to 100 Å in width by up to 5000 Å in length[14,20,23,27] and mol wt[16] in the range of 5 to 10 × 10^6. When placed in medium without citrate, the filamentous form depolymerizes reversibly to catalytically inactive protomers, as evidenced by electron microscopy,[14,22,23] changes in sedimentation equilibrium and velocity,[15,16,23,26] and decreased intrinsic viscosity.[26] The mol wt of the protomeric unit is about 480,000.[15-17] Assuming that an average carboxylase filament is composed of 20 protomeric units, it can be calculated[26] from the specific activity and liver content of the enzyme that a single hepatocyte would contain about 50,000 filaments.

Citrate, the allosteric activator of this enzyme, is required for both catalysis and polymerization.[26] Thus, it has been proposed[22,26] that the state of the protomer-polymer equilibrium (see Reaction 1), hence the rate of fatty acid synthesis, is determined by cellular citrate concentration.

$$\underset{\text{"inactive"}}{\text{Protomeric form}} \xleftrightarrow[-\text{ citrate}]{+\text{ citrate}} \underset{\text{"active"}}{\text{Polymeric filamentous form}} \qquad (1)$$

480,000 daltons — 5 to 10 million daltons

Compelling evidence, however, implicating citrate as activator of the carboxylase in the intact cell has been lacking.

Inhibition of fatty acid synthesis resulting from a cAMP-mediated decrease in cellular citrate concentration 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 1B). As illustrated in Table 4A, glucagon or Bt_2cAMP treatment of liver cell suspensions in a medium containing glucose as the sole carbon source decreases cellular citrate concentration and fatty acid synthetic rate to about the same extent.[44] The addition of pyruvate, which markedly increases the cellular citrate level, causes a corresponding rise 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 is largely prevented, as is the effect of these agents in reducing cellular citrate concentration (Table 4A). Lactate has a similar effect to that of pyruvate (results not shown).

To assess the dependence of fatty acid synthesis upon the citrate level, the kinetics of [^{14}C]acetate incorporation into fatty acids and changes in the cellular citrate concentration were compared.[44] Hepatocytes were used immediately after isolation, since such cells have a low citrate content. As illustrated in Figure 11A, both [^{14}C]acetate incorporation into fatty acids and the accumulation of cellular citrate exhibit similar kinetic patterns with comparable lag periods. A constant rate of fatty acid synthesis was

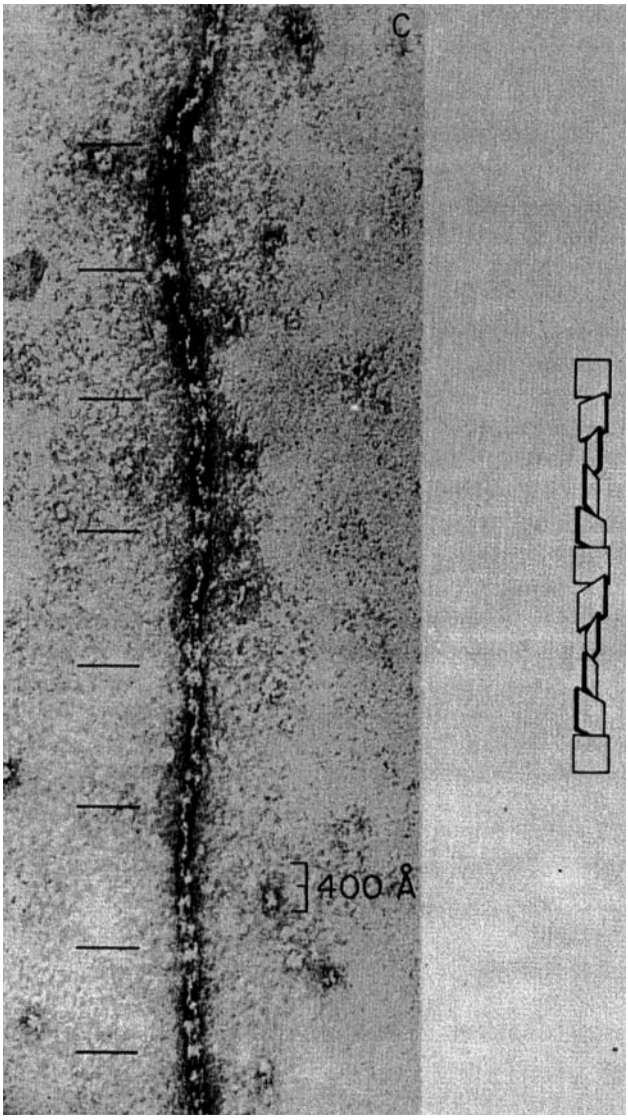

FIGURE 10. Filamentous form of chicken liver acetyl-CoA carboxylase in the presence of citrate. The enzyme was applied to carbon support films and stained with uranyl acetate. The filament has a twisted appearance with indentations along the filament axis suggestive of a helical structure. The characteristic wide-narrow-wide pattern and a longitudinal periodicity of 120 to 140 Å is best explained by the twisting of a flat helical filament composed of protomeric units, 130 Å in length (diagram). (From Lane, M. D., Polakis, S. E., and Moss, J., *Curr. Top. Cell. Regul.*, 8, 139, 1974. With permission.)

achieved only after 30 min, presumably when sufficient citrate had accumulated to maximally activate acetyl-CoA carboxylase. Glucagon prevents the rise of both the citrate level and the fatty acid synthetic rate. If glucagon is added after the citrate concentration has risen to a level which supports maximal fatty acid synthesis, both citrate concentration and fatty acid synthetic rate fall abruptly (Figure 11B). Thus, it appears that the rate of fatty acid synthesis from acetyl-CoA is dependent upon cellular citrate concentration.

TABLE 4

Effect of Glucagon and Dibutyryl-cAMP on Cellular Citrate Levels and Fatty Acid Synthesis in Chicken Liver Cells

A. Total Cellular Citrate and Fatty Acid Synthesis

Experiment	Treatment	Cellular citrate (nmol/mg of cell dry weight)	[1-^{14}C]Acetate incorporated into fatty acids (nmol/min/mg of cell dry weight)
1	None	1.37	0.65
	Bt_2cAMP	0.12	0.05
2	None	1.13	0.87
	Glucagon	0.21	0.10
3	Pyruvate	4.11	2.28
	Pyruvate + Bt_2 cAMP	6.93	1.52
4	Pyruvate	6.71	1.57
	Pyruvate + glucagon	7.91	1.20

B. Intracellular Distribution of Citrate

Experiment	Treatment	Cytoplasm (nmol/mg of cell dry weight)	Mitochondria (nmol/mg of cell dry weight)	Recovery (%)
1	None	1.15 (83%)	0.24 (17%)	≥100
	Bt_2cAMP	0.12 (71%)	0.05 (29%)	≥100
2	None	0.93 (84%)	0.18 (16%)	97
	Glucagon	0.17 (81%)	0.04 (19%)	≥100
3	Pyruvate	3.06 (80%)	0.77 (20%)	94
	Pyruvate + Bt_2cAMP	5.66 (80%)	1.44 (20%)	≥100
4	Pyruvate	4.74 (72%)	1.86 (28%)	98
	Pyruvate + glucagon	6.18 (73%)	2.30 (27%)	≥100

Note: Liver cell suspensions were incubated for 1 hr with 5 m*M* [1-^{14}C]acetate and appropriate additions. Sodium pyruvate was added at a level of 5 m*M*. After removal of an aliquot to assess [^{14}C]acetate incorporation into fatty acids, suspensions were either (1) mixed with 6% $HClO_4$ and assayed for total cellular citrate or (2) subjected to the digitonin disruption rapid-stop technique, and the resultant fractions assayed for citrate. Numbers in parentheses are percentages of total citrate (cytoplasmic plus mitochondrial) in the cell compartment.

From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.

De novo fatty acid synthesis is strictly a cytoplasmic process.[22] Therefore, it was important to determine whether the citrate concentration in this cell compartment and the 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. Thus, a modification of the digitonin disruption-rapid-stop technique[18,45] was employed, as illustrated in Figure 12. Cells are incubated and then exposed briefly to 0.08% digitonin, which disrupts the plasma and outer mitochondrial membranes. Benzene tricarboxylic acid, an inhibitor of mitochondrial citrate transport, and an ATP trapping system (hexokinase and glucose) to block

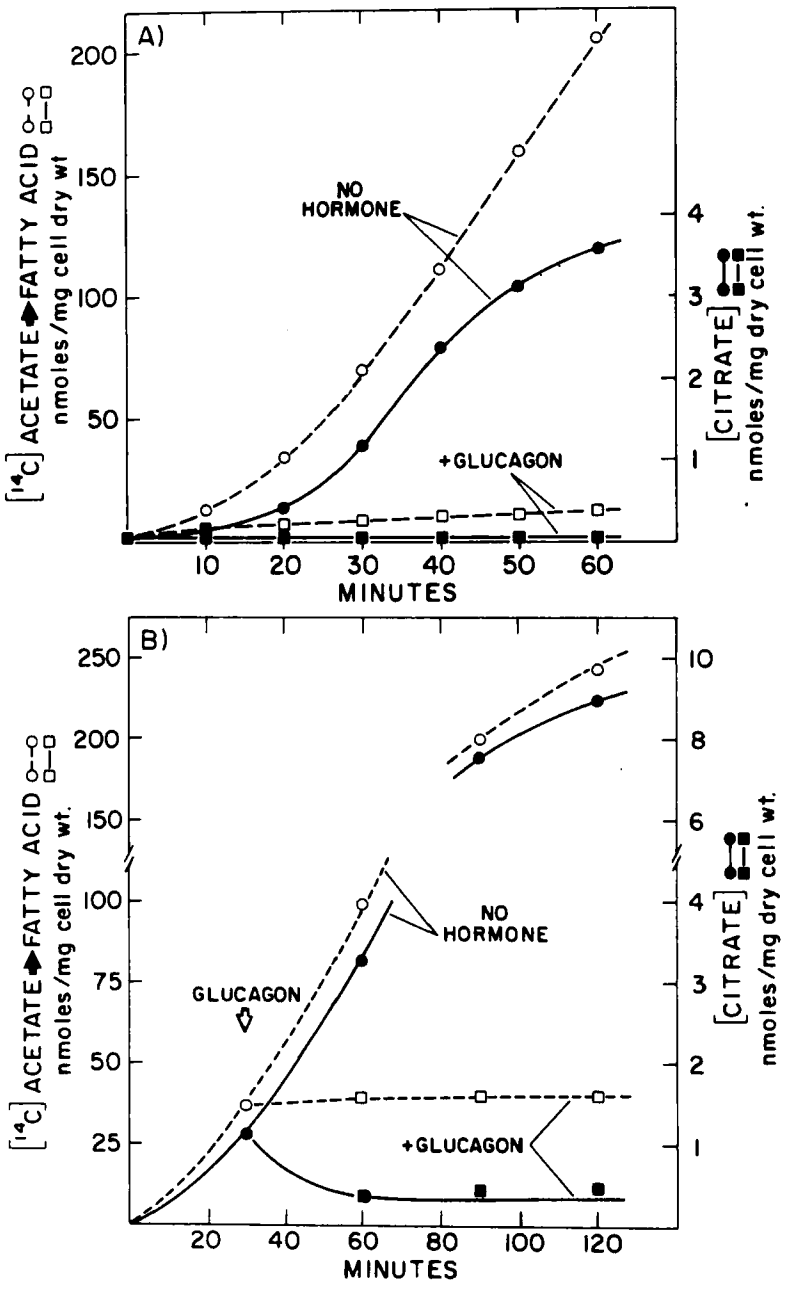

FIGURE 11. Effect of glucagon on cellular citrate level and on [^{14}C]acetate incorporation into fatty acids by hepatocyte suspensions. (A) Freshly prepared hepatocytes were incubated with 5m*M* [1-^{14}C]acetate-containing medium in the presence or absence of glucagon. (B) Freshly prepared hepatocytes were incubated with 10 m*M* [1-^{14}C]acetate-containing medium for 30 min, at which time glucagon was added to half of the flasks. At the times indicated in A and B, [^{14}C]acetate incorporated into fatty acids of cells plus medium and total cellular citrate levels were determined. (From Watkins, P. A., Tarlow, D. M., and Lane, M. D., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.

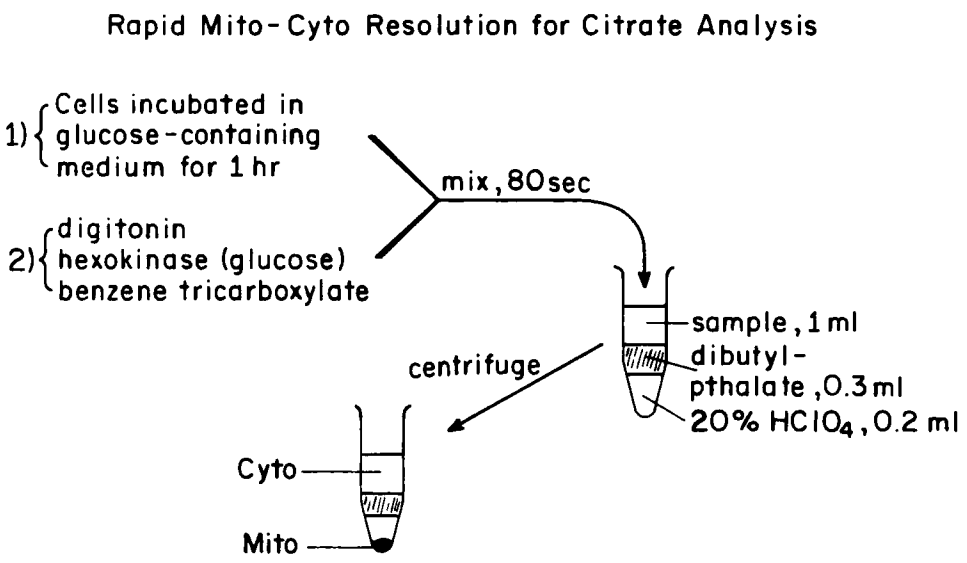

FIGURE 12. Procedure for rapid mitochondria (mito)-cytoplasm (cyto) resolution for citrate analysis.

cytoplasmic ATP-citrate lyase are also present during cell lysis. Within 10 to 20 sec, intracellular components become exposed to benzene tricarboxylic acid and the ATP trapping system; under these conditions, cytoplasmic citrate levels were found to remain constant for at least 5 min.[44] Mitochondria, and other particulate components are then rapidly sedimented (<20 sec) through a layer of dibutylphthalate into 20% $HClO_4$ after which the supernate ("cytoplasm") is immediately quenched with perchloric acid. Under these conditions, but without perchloric acid, 92% of the mitochondrial marker, citrate synthase, is recovered in the pellet and 92% of the cytoplasmic marker, lactate dehydrogenase, in the supernate.[44] Moreover, the recovery of citrate in the mitochondrial and cytoplasmic fractions equals that of cells quenched immediately with perchloric acid.[13]

Using this procedure, we found that citrate is localized primarily in the cytoplasm of chick liver cells. After a 1-hr incubation of the hepatocytes with glucose or glucose and pyruvate as carbon sources, about 75% of the cellular citrate is found in the cytoplasm (Table 4B). As with total cellular citrate concentration, the level of citrate in the cytoplasm at the site of acetyl-CoA carboxylase action is drastically lowered by glucagon or Bt_2cAMP with glucose as the primary carbon source. When pyruvate is present, the effects of these agents on both the cytoplasmic citrate level and fatty acid synthesis are prevented.

Together with previous studies[14,20,22,26,27] showing that tricarboxylic acid activator is absolutely required for catalysis by isolated avian liver acetyl-CoA carboxylase, these results implicate citrate as a genuine activator of fatty acid synthesis in the intact liver cell. Thus, citrate serves dual functions in the cytoplasm, both as precursor of fatty acids and cholesterol and as feed-forward activator of acetyl-CoA carboxylase (see Figure 9).

MECHANISM BY WHICH cAMP LOWERS THE CYTOPLASMIC CITRATE LEVEL

The results presented above indicate that glucagon, acting via cAMP, inhibits fatty acid synthesis by blocking the formation of citrate, an essential activator of acetyl-CoA carboxylase. Since unlabeled glucose was the only carbon source (other than

[^{14}C]acetate) in these experiments, it is likely that the site of inhibition lies on the pathway from glucose to citrate. The fact that pyruvate or lactate enter midway through the pathway and overcome the glucagon- (or Bt_2cAMP-) induced reduction of the citrate level and inhibition of [^{14}C]acetate incorporation into fatty acids indicates a cAMP-mediated block between glucose and pyruvate (Figure 9). Recent investigations in our laboratory[6] show that glycolysis (measured as [^{14}C]glucose incorporation into pyruvate plus lactate) in the absence or presence of cyano-4-hydroxycinnamate, an inhibitor of mitochondrial pyruvate uptake, is inhibited by Bt_2cAMP. We have also found[6] that the phosphofructokinase activity of chick liver cells treated with Bt_2cAMP is markedly decreased, and the extent of this decrease accounts for the inhibition of glycolysis by the cyclic nucleotide. Pyruvate kinase, an enzyme inhibited by cAMP-mediated phosphorylation in rat liver,[11,25,31] is not affected by Bt_2cAMP in chick liver cells. Thus, it appears that the block in carbon flux from glucose to cytoplasmic citrate in the chick liver cell is due to inhibition of the glycolytic pathway at the step catalyzed by phosphofructokinase (see Figure 9).

DIBUTYRYL cAMP-INDUCED DEPOLYMERIZATION OF ACETYL-CoA CARBOXYLASE FILAMENTS IN THE INTACT LIVER CELL

Although it has been established[14,20,23,26,27] that isolated acetyl-CoA carboxylase exists in a polymeric filamentous form in the presence of citrate, evidence for the existence of carboxylase filaments in the intact cell has not been reported. Using an indirect approach, we now have evidence[25] for the occurrence of the filamentous form of the enzyme in the intact chick liver cell in culture. Digitonin, which perforates the plasma membrane, rendering it immediately permeable to cytosolic enzymes, causes release of acetyl-CoA carboxylase at a rate inversely related to cellular citrate concentration and the apparent state of polymerization of the enzyme. Under these conditions, mitochondria and other organelles remain entrapped within the digitonin-treated cell ghosts.[18,45] Soluble mitochondrial matrix components also remain entrapped because the inner mitochondrial membrane is resistant to digitonin treatment. Figure 13 shows that lactate dehydrogenase, a cytosolic enzyme, is quantitatively released from chick liver cells in monolayer culture within 2 min after exposure to a buffered digitonin solution. Moreover, citrate synthase, a mitochondrial enzyme,[7] and aryl sulfatase, a lysosomal enzyme,[2] are not liberated to a significant extent, even following prolonged exposure to digitonin. Prior treatment of cultures with 0.1 m*M* Bt_2cAMP has no effect on the release of these enzymes.

Because of the large differences in size and Stokes radius of the polymeric, filamentous, and protomeric forms of acetyl-CoA carboxylase,[14,16,20,23,26] their rates of release from digitonin-treated cells should differ substantially. It was assumed that the position of the protomer-polymer equilibrium would be shifted in the intact hepatocyte, as it is in vitro,[16,26] by altering the cytoplasmic citrate concentration. As described above, exposure of chick liver cell suspensions to Bt_2cAMP causes a 90 to 95% drop in both the cytoplasmic citrate concentration and the fatty acid synthetic rate. Under identical conditions, treatment of chick liver cell monolayers with Bt_2cAMP has no effect on acetyl-CoA carboxylase activity per se in cell-free extracts prepared by homogenization.

Figure 14 shows that brief exposure of liver cells to Bt_2cAMP greatly accelerates the rate of carboxylase release into the digitonin supernate. This effect appears to result from a shift in the position of the protomer-polymer equilibrium toward protomer, since citrate added to the digitonin disruption medium markedly slowed the Bt_2cAMP-stimulated rate of carboxylase release (Figure 14). Citrate, which promotes polymerization of the isolated enzyme, cannot permeate the plasma membrane until it is per-

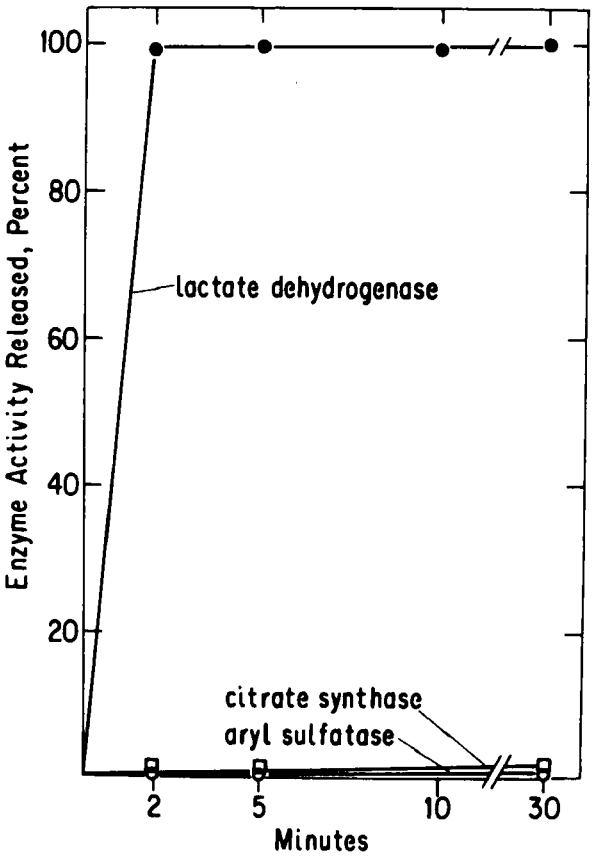

FIGURE 13. Kinetics of release of cytosolic, mitochondrial, and lysosomal marker enzymes from digitonin-treated liver cell monolayers. Chick liver cell monolayers after 48 hr in culture were incubated with buffered digitonin solution for the time indicated, were processed, and the percentage of cellular lactate dehydrogenase (170 munits per culture dish), citrate synthase (35 munits per culture dish), and aryl sulfatase (10 munits per culture dish) released into the supernate determined. Percent enzyme activity released refers to the percentage of total enzyme activity (cell homogenate plus supernate) released into the supernate. (From Meredith, M. J. and Lane, M. D., *J. Biol. Chem.*, in press, 1978. With permission.)

forated by digitonin.[44] The addition to the digitonin disruption medium of malonyl-CoA, an agent which promotes rapid depolymerization of the isolated carboxylase,[15,16,26] greatly accelerated the rate of carboxylase release from cell monolayer (Figure 14). Thus, the rate of carboxylase release from the perforated liver cell can be influenced by exogenous citrate or malonyl-CoA, which rapidly enter the cell and alter the state of polymerization of the enzyme.

The polymeric state of acetyl-CoA carboxylase during its release from digitonin-treated cells was assessed by an independent method relying upon the differential susceptibility of the protomeric and polymeric forms to inactivation by avidin. Avidin binds irreversibly to the biotinyl prosthetic group of most biotin enzymes.[22] However, in the case of acetyl-CoA carboxylase, the protomeric form is rapidly inactivated, while the polymeric filamentous form is resistant to inactivation.[26,33] Apparently, the biotinyl prosthetic group becomes sterically hindered at the active site in the polymeric form

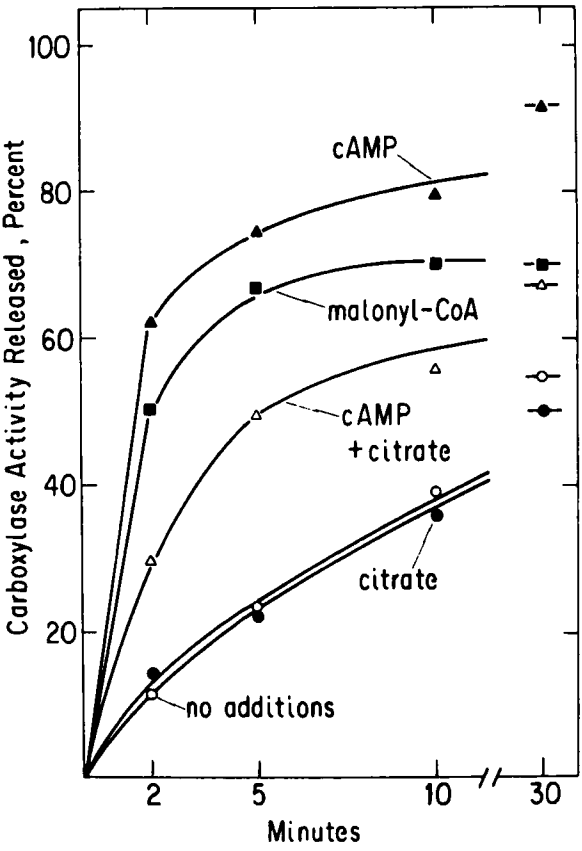

FIGURE 14. Effect of conditions that alter the polymeric state of acetyl-CoA carboxylase on its rate of release from digitonin-treated liver cell monolayers. Chick liver cell monolayers after 48 hr in culture were incubated with buffered digitonin solution for the time indicated, were processed, and the percentage of cellular acetyl-CoA carboxylase activity (10 munits per plate) released into the supernate determined. Variable treatments as indicated included Bt_2cAMP; 15 min preincubation of monolayers with 0.1 m*M* Bt_2cAMP in standard culture medium and/or the presence of 5 m*M* citrate or 0.1 m*M* malonyl-CoA in the buffered digitonin release solution. (From Meredith, M. J., and Lane, M. D., *J. Biol. Chem.*, in press, 1978. With permission.)

and, therefore, inaccessible to avidin.[26,33,37] Avidin, being a small spherical molecule,[37] rapidly enters the digitonin-perforated cell and inactivates carboxylase in the "protomeric" form before its release.[25] Conditions that promote depolymerization and rapid release of carboxylase, i.e., prior exposure of cells to Bt_2cAMP and the presence of malonyl-CoA, also promote inactivation by avidin of both carboxylase released and carboxylase not yet released[25] (results not shown). This pattern of susceptibility to avidin indicates that the carboxylase is largely in the protomeric state under these conditions. When the high intracellular citrate concentration is maintained during digitonin treatment,[25] the enzyme becomes completely resistant to inactivation by avidin, and the rate of release is slowed. This result is consistent with the hypothesis that the rapidly released species of the carboxylase is protomeric in nature. These findings indicate that carboxylase filaments exist in the intact chick liver cell when the cytoplasmic citrate level is high and undergo depolymerization when citrate levels fall.

RECAPITULATION

An important factor in the survival of living organisms is their ability to regulate the storage and mobilization of metabolizable energy. Mechanisms have evolved for diverting the metabolic flux toward energy storage when caloric intake exceeds immediate need. In higher animals, surplus metabolizable energy is converted into and stored primarily as triglyceride. The storage and mobilization of reserve fuels, notably triglyceride and glycogen, are under hormonal control in vivo.

To investigate these mechanisms under the controlled conditions of cell culture, a nonproliferating chick liver cell system was developed which yields cell monolayers with morphological and lipogenic properties characteristic of the physiological-nutritional state of the donor animal. Fatty acid synthesis occurs in culture at in vivo rates and responds-to hormones, e.g., insulin and glucagon, which affect this process in vivo. Cells derived from fed chicks maintain high rates of fatty acid synthesis for several days when insulin is present and lose this capacity slowly ($t_{1/2}$ = 22 hr) when insulin is removed. Glucagon or 3′,5′-cyclic AMP derivatives cause an immediate cessation of fatty acid synthesis. Like glycogenesis, therefore, *de novo* lipogenesis appears to be acutely regulated by hormones whose actions are mediated by cAMP.

The focal point of the cAMP-mediated inhibition of fatty acid synthesis appears to be cytoplasmic acetyl-CoA carboxylase. This enzyme catalyzes the first committed step of fatty acid synthesis beyond the acetyl-CoA branchpoint and is, therefore, an appropriate site for regulation. Our investigations show that the homogeneous carboxylase from chick liver exists in either a catalytically inactive protomeric form or an active polymeric filamentous form (see Reaction 1). Citrate, the required allosteric activator of the enzyme, is required for both catalysis and polymerization. Thus, it is proposed that the state of the protomer-polymer equilibrium, hence the rate of fatty acid synthesis, is determined by the cellular citrate concentration.

Both glucagon and Bt_2cAMP cause an immediate and marked decrease in the cytoplasmic citrate concentration of chick liver cells. The close correlation between the fatty acid synthetic rate and the cellular citrate level supports the proposal that citrate acts as feed-forward activator of acetyl-CoA carboxylase. Several lines of evidence indicate that cAMP mediates the inhibition of a step in the metabolic pathway between glucose and citrate. The site of inhibition appears to be at the phosphofructokinase-catalyzed conversion of fructose-6-phosphate to fructose-1,6-diphosphate.

Compelling evidence for the existence of acetyl-CoA carboxylase filaments in the intact liver cell has been obtained. Filaments generated by the exposure of purified carboxylase to citrate are 70 to 100 Å in width by up to 5000 Å in length, whereas the protomers which comprise the filaments have a maximum dimension of 130 Å; thus, a large difference in the Stokes radii of the two forms exists. Digitonin, which perforates the plasma membrane rendering it immediately permeable to cytoplasmic enzymes, causes the release of carboxylase at a rate inversely related to the cellular citrate concentration. When cells are exposed to Bt_2cAMP, which lowers intracellular citrate levels, the rate of carboxylase release is greatly accelerated. These and other findings indicate that carboxylase filaments are present in the intact liver cell when the cytoplasmic citrate level is high and undergo depolymerization when citrate level falls. Thus, it appears that the position of the protomer-polymer equilibrium, hence acetyl-CoA carboxylase activity and fatty acid synthetic rate, are determined, at least in part, by the cytoplasmic citrate level of the chick liver cell.

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REFERENCES

1. **Bennett, V. and Cuatrecasas, P.,** Mechanism of activation of adenylate cyclase by *Vibrio cholerae* enterotoxin, *J. Membr. Biol.*, 22, 29, 1975.
2. **Bergmeyer, H. U., Gawehn, K., and Grassl, M.,** Enzymes as biochemical reagents, in *Methods of Enzymatic Analysis*, Vol. 1, Bergmeyer, H. U., Ed., Academic Press, New York, 1974, 435.
3. **Carlson, C. A. and Kim, K.-H.,** Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation and dephosphorylation, *J. Biol. Chem.*, 248, 378, 1973.
4. **Carlson, C. A. and Kim, K.-H.,** Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation and dephosphorylation, *Arch. Biochem. Biophys.*, 164, 478, 1974.
5. **Carlson, C. A. and Kim., K.-H.,** Differential effects of metabolites on the active and inactive forms of hepatic acetyl CoA carboxylase, *Arch. Biochem. Biophys.*, 164, 490, 1974.
6. **Clarke, S. D., Watkins, P. A., and Lane, M. D.,** Dibutyryl-cAMP inhibition of lipogenesis in chick hepatocytes, *Fed. Proc. Abstr.*, 37, 329, 1978; *J. Lipid Res.*, in press, 1979.
7. **Clinkenbeard, K. D., Reed, W. D., Mooney, R. A., and Lane, M. D.,** Intracellular localization of the 3-hydroxy-3-methylglutaryl-CoA cycle enzymes in liver: separate cytoplasmic and mitochondrial 3-hydroxy-3-methylglutaryl-CoA generating systems for cholesterogenesis and ketogenesis, *J. Biol. Chem.*, 250, 3108, 1975.
8. **Diem, K. and Lentner, C.,** Insulin, in *Scientific Tables*, 7th ed., Ciba-Geigy, Basel, Switzerland, 1971, 734.
9. **Diem, K. and Lentner, C.,** Glucagon, in *Scientific Tables*, 7th ed., Ciba-Geigy, Basel, Switzerland, 1971, 737.
10. **Exton, J. H., Robison, G. A., Sutherland, E. W., and Park, C. R.,** Studies on the role of adenosine 3′,5′-monophosphate in the hepatic actions of glucagon and catecholamines, *J. Biol. Chem.*, 246, 6166, 1971.
11. **Feliu, J. E., Hue, L., and Hers, H-G.,** Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2762, 1976.
12. **Galton, D. J.,** Lipogenesis in human adipose tissue, *J. Lipid Res.*, 9, 19, 1968.
13. **Goodridge, A. G.,** Molecular basis for the dietary and hormonal regulation of *de novo* fatty acid synthesis, *PAABS Rev.*, 5, 483, 1976.
14. **Gregolin, C., Ryder, E., Kleinschmidt, A. K., Warner, R. C., and Lane, M. D.,** Molecular characteristics of liver acetyl-CoA carboxylase, *Proc. Natl. Acad. Sci. U.S.A.*, 56, 148, 1966.
15. **Gregolin, C., Ryder, E., Warner, R. C., Kleinschmidt, A. K., Chang, H-C., and Lane, M. D.,** Liver acetyl-CoA carboxylase. II. Further molecular characterization, *J. Biol. Chem.*, 243, 4236, 1968.
16. **Gregolin, C., Ryder, E., Warner, R. C., Kleinschmidt, A. K., and Lane, M. D.,** Liver acetyl CoA carboxylase: the dissociation-reassociation process and its relation to catalytic activity, *Proc. Natl. Acad. Sci. U.S.A.*, 56, 1751, 1966.
17. **Guchhait, R. B., Zwergel, E. E., and Lane, M. D.,** Acetyl-CoA carboxylase. Subunit structure of the protomeric form of the avian liver enzyme. *J. Biol. Chem.*, 249, 4776, 1974.
18. **Hoek, J. B. and Tager, J. M.,** The oxidoreduction state of free NAD(P) and mass-action ratio of total nicotinamide nucleotides in isolated rat-liver mitochondria, *Biochim. Biophys. Acta*, 325, 197, 1973.
19. **Jefferson, L. S., Exton, J. H., Butcher, R. W., Sutherland, E. W., and Park, C. R.,** Role of adenosine 3′,5′-monophosphate in the effects of insulin and anti-insulin serum on liver metabolism, *J. Biol. Chem.*, 243, 1031, 1968.
20. **Kleinschmidt, A. K., Moss, J., and Lane, M. D.,** Acetyl-CoA carboxylase: filamentous nature of the animal enzymes, *Science*, 166, 1276, 1969.
21. **Lane, M. D. and Moss, J.,** Regulation of fatty acid synthesis in animal tissues, in *Metabolic Pathways: Metabolic Regulation* Vol. 5, Vogel, H. J., Ed., Academic Press, New York, 1971, chap. 2 and 23.
22. **Lane, M. D., Moss, J., and Polakis, S. E.,** Acetyl coenzyme A carboxylase, in *Current Topics in Cellular Regulation*, Vol. 8, Horecker, B. L. and Stadtman, E. R., Eds., Academic Press, New York, 1974, 139.

23. **Lane, M. D., Polakis, S. E., and Moss, J.,** Acetyl coenzyme A carboxylase, in *Subunit Enzymes, Biochemistry and Functions*, Vol. 2, Ebner, K. E., Ed., Marcel Dekker, New York, 1975, 181.
24. **Lee, K-W. and Kim, K.-H.,** Regulation of rat liver acetyl-CoA carboxylase: evidence for interconversion between active and inactive forms of enzyme by phosphorylation and dephosphorylation, *J. Biol. Chem.*, 252, 1748, 1977.
25. **Meredith, M. J. and Lane, M. D.,** Acetyl-CoA carboxylase: evidence for polymeric filament to protomer transition in the intact avian liver cell, *J. Biol. Chem.*, 253, 3381, 1978.
26. **Moss, J. and Lane, M. D.,** Acetyl CoA carboxylase. III. Further studies on the relation of catalytic activity to polymeric state, *J. Biol. Chem.*, 247, 4944, 1972.
27. **Moss, J., Yamagishi, M., Kleinschmidt, A. K., and Lane, M. D.,** Acetyl-CoA carboxylase: purification and properties of the bovine adipose tissue enzyme, *Biochemistry*, 11, 3779, 1972.
28. **O'Hea, E. K. and Leveille, G. A.,** Lipid biosynthesis and transport in the domestic chick (*Gallus domesticus*), *Comp. Biochem. Physiol.*, 30, 149, 1969.
29. **Pohl, S. L., Birnbaumer, L., and Rodbell, M.,** The glucagon-sensitive adenylate cyclase system in plasma membranes of rat liver, *J. Biol. Chem.*, 246, 1849, 1971.
30. **Qureshi, A. A., Jenik, R. A., Kim, M., Lornitzo, F. A., and Porter, J. W.,** Separation of two active forms (Holo-*a* and Holo-*b*) of pigeon liver fatty acid synthetase and their interconversion by phosphorylation and dephosphorylation, *Biochem. Biophys. Res. Commun.*, 66, 344, 1975.
31. **Riou, J. P., Claus, T. H., and Pilkis, S. J.,** Control of pyruvate kinase activity by glucagon in isolated hepatocytes, *Biochem. Biophys. Res. Commun.*, 73, 591, 1976.
32. **Robison, G. A., Butcher, E. W., and Sutherland, E. W.,** *Cyclic AMP*, Academic Press, New York, 1975, 64.
33. **Ryder, E., Gregolin, C., Chang, H.-C., and Lane, M. D.,** Liver acetyl-CoA carboxylase: insight into the mechanism of activation by tricarboxylic acids and acetyl-CoA, *Proc. Natl. Acad. Sci. U.S.A.*, 57, 1455, 1967.
34. **Shrago, E., Glenon, J. A., and Gordon, E. S.,** Comparative aspects of lipogenesis in mammalian tissues, *Metabol. Clin. Exp.*, 20, 54, 1971.
35. **Shrago, E. and Spennetta, T.,** The carbon pathway for lipogenesis in isolated adipocytes from rat, guinea pig, and human adipose tissue, *Am. J. Clin. Nutr.*, 29, 540, 1976.
36. **Shrago, E., Spennetta, T., and Gordon, E.,** Fatty acid synthesis in human adipose tissue, *J. Biol. Chem.*, 244, 2761, 1969.
37. **Stoll, E., Ryder, E., Edwards, J. B., and Lane, M. D.,** Liver acetyl-CoA carboxylase: activation of model partial reactions by tricarboxylic acids, *Proc. Natl. Acad. Sci., U.S.A.*, 60, 986, 1968.
38. **Tarlow, D. M., Meredith, M. J., Watkins, P. A., and Lâné, M. D.,** Effect of cAMP on insulin-induced anetyl-CoA carboxylase synthesis, *Fed. Proc. Abstr.*, 37, 1308, 1978.
39. **Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., and Lane, M. D.,** Lipogenesis and the synthesis and secretion of very low density lipoprotein by avian liver cells in nonproliferating monolayer culture: hormonal effects, *J. Cell Biol.*, 73, 332, 1977.
40. **Titanji, V. P. K., Zetterqvišt, O., and Engstrom, L.,** Regulation *in vitro* of rat liver pyruvate kinase by phosphorlyation-dephosphorylation reactions, catalyzed by cyclic-AMP dependent protein kinases and a histone phosphatase, *Biochim. Biophys. Acta*, 422, 98, 1976.
41. **Unger, R. H.,** Glucagon physiology and pathophysiology, *New Engl. J. Med.*, 285, 443, 1971.
42. **Volpe, J. J. and Vagelos, P. R.,** Mechanisms and regulation of biosynthesis of saturated fatty acids, *Physiol. Rev.*, 56, 339, 1976.
43. **Wadke, M., Brunengraber, H., Lowenstein, J., Dolhun, J. J., and Arsenault, G. P.,** Fatty acid synthesis by the liver perfused with deuterated and tritiated water, *Biochemistry*, 12, 2619, 1973.
44. **Watkins, P. A., Tarlow, D. M., and Lane, M. D.,** Mechanism for acute control of fatty acid synthesis by glucagon and 3′:5′-cyclic AMP in the liver cell, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1497, 1977.
45. **Zuurendonk, P. F. and Tager, J. M.,** Rapid separation of particulate components and soluble cytoplasm of isolated rat-liver cells, *Biochem. Biophys. Acta*, 333, 393, 1974.