Acute control of fatty acid synthesis by cyclic AMP in the chick liver cell: possible site of inhibition of citrate formation

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Abstract Glucagon and $N,^6O^2$-dibutyryl cyclic adenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) inhibit fatty acid synthesis from acetate by more than 90% and prevent citrate formation in chick hepatocytes metabolizing glucose. With substrates that enter glycolysis at or below triose-phosphates, e.g., fructose, lactate, or pyruvate, Bt_2cAMP has no effect on the citrate level and its inhibitory effect on fatty acid synthesis is substantially reversed. Because acetyl-CoA carboxylase requires a tricarboxylic acid activator for activity, it is proposed that regulation of fatty acid synthesis by Bt_2cAMP is due, in part, to changes in the citrate level. Reduced citrate formation appears to result from a cAMP-induced inhibition of glycolysis. Bt_2cAMP inhibits $^{14}CO_2$ production from [1-^{14}C]-, [6-^{14}C]-, and [U-^{14}C]glucose and has little effect on $^{14}CO_2$ formation from [1-^{14}C]- or [2-^{14}C]pyruvate or from [1-^{14}C]fructose. [^{14}C]Lactate formation from glucose is depressed 50% by Bt_2cAMP. In the presence of an inhibitor of mitochondrial pyruvate transport lactate accumulation is enhanced, but continues to be lowered 50% by Bt_2cAMP. The activity of phosphofructokinase is greatly decreased in Bt_2cAMP-treated cells while the activities of pyruvate kinase and acetyl-CoA carboxylase are unaffected. It appears that decreased glycolytic flux and decreased citrate formation result from depressed phosphofructokinase activity. Fatty acid synthesis from [^{14}C]acetate is partially inhibited by Bt_2cAMP in the presence of fructose, lactate, and pyruvate despite a high citrate level. Incorporation of [^{14}C]fructose, [^{14}C]pyruvate, or [^{14}C]lactate into fatty acids is similarly depressed by Bt_2cAMP. Synthesis of cholesterol from [^{14}C]acetate or [2-^{14}C]pyruvate is unaffected by Bt_2cAMP. These results implicate a second site of inhibition of fatty acid synthesis by Bt_2cAMP that involves the utilization, but not the production, of cytoplasmic acetyl-CoA.—**Clarke, S. D., P. A. Watkins, and M. D. Lane.** Acute control of fatty acid synthesis by cyclic AMP in the chick liver cell: possible site of inhibition of citrate formation. *J. Lipid Res.* 1979. **20:** 974–985.

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Glucagon and cAMP are known to suppress hepatic fatty acid synthesis in the chick and rat; however, the mechanism of this inhibition has not been definitively elucidated (1–5). Recently, we reported (1) that glucagon and Bt_2cAMP drastically reduce both total and cytoplasmic citrate concentration by more than 90% in chick liver cells provided with glucose as primary exogenous substrate. This marked depression in citrate level caused by glucagon or Bt_2cAMP is accompanied by a greater than 90% reduction in fatty acid synthetic rate from [^{14}C]acetate or ^3H_2O.

The site at which fatty acid synthesis is inhibited by cAMP in the chick and rat liver cell is thought to be the acetyl-CoA carboxylase [acetyl-CoA: carbon dioxide ligase (ADP-forming) EC 6.4.1.2]-catalyzed reaction (1–5). Since catalysis by the carboxylase has an absolute requirement for citrate as allosteric activator (6–10), inhibition of fatty acid synthesis by glucagon or Bt_2cAMP in chick liver cells was attributed to reduced cytoplasmic citrate level (1, 9).

In contrast to the effect of glucagon or Bt_2cAMP on the citrate level in the chick liver cell, these agents have little effect on citrate level in the rat liver cell (2, 5, 10). Thus a significant species difference exists between chick and rat with regard to the action of cAMP or glucagon on hepatocyte citrate concentration and its possible effect on acetyl-CoA carboxylase activity.

The specific site(s) at which glucagon or cAMP acts to depress cellular citrate level in the chick liver cell has not been identified. Glucagon and cAMP treatment of rat liver cells has been reported to reduce

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; Bt_2cAMP, N^6O^2-dibutyryl cAMP; MOPS, morpholinopropane sulfonate.

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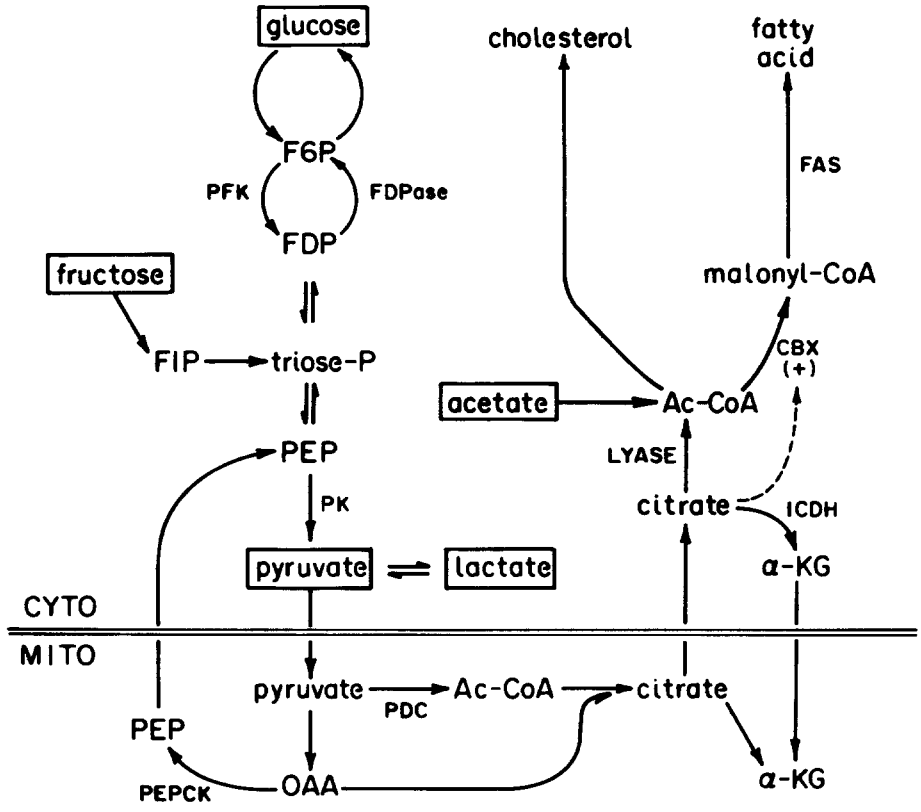
glucose and dihydroxyacetone flux to lactate while stimulating the rate of dihydroxyacetone and fructose conversion to glucose (11–14). This action of cAMP on rat liver cells has also been associated with a depression in the catalytic efficiencies of pyruvate kinase and phosphofructokinase and with an increase in fructose-1,6-diphosphatase activity (15). A reduced glucose flux to lactate (or pyruvate) might account for the reduced cytoplasmic citrate level and, hence, for diminished acetyl-CoA carboxylase activity and lowered rate of fatty acid synthesis in chick hepatocytes treated with glucagon or Bt_2cAMP (see **Scheme 1**). However, an inhibitory effect of these agents on glycolytic flux, or on pyruvate kinase and phosphofructokinase activities has not been demonstrated in the chick.

In this report we present evidence that the block in citrate formation caused by Bt_2cAMP or glucagon treatment of chick liver cells is at least partially due to a reduced flux of glucose to pyruvate. Furthermore, the depressed rate of hepatocyte glycolysis is associated with a diminished phosphofructokinase activity. Our results indicate that while a major fraction of the inhibitory effect of cAMP on fatty acid synthesis is explained by changes in citrate concentration, Bt_2cAMP exerts a secondary inhibitory effect on acetyl-CoA utilization for fatty acid synthesis which is independent of cellular citrate levels. The site of this cAMP effect on fatty acid synthesis remains unknown.

EXPERIMENTAL METHODS

Materials

The hepatocyte incubation medium consisted of Eagle's basal medium with Earle's salts and 0.22% $NaHCO_3$, but without amino acids and phenol red as previously described (16). This medium was supplemented with glucose to produce a total concentration of 25 mM except where indicated. Porcine glucagon was a gift of Dr. Walter N. Shaw, Eli Lilly and Company (Indianapolis, IN). N^6,O^2-Dibutyryl adenosine-3′,5′-cyclic phosphate monosodium dihy-



Scheme 1. Metabolic pathways from glucose to fatty acids and cholesterol. Unbroken lines indicate pathways; the broken line indicates allosteric activation by citrate. Enzyme abbreviations are: PFK, phosphofructokinase; FDPase, fructose diphosphatase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; PDC, pyruvate dehydrogenase complex; LYASE, ATP: citrate lyase; ICDH, isocitrate dehydrogenase; CBX, acetyl-CoA carboxylase; FAS, fatty acid synthetase. CYTO refers to the cytoplasmic compartment and MITO to the mitochondrial compartment.

drate (Bt_2cAMP) was purchased from Calbiochem (San Diego, CA). DNAase I and selected lots of Type III collagenase were obtained from Worthington Biochemical Corporation (Freehold, NJ). [U-^{14}C]Glucose, [1-^{14}C]glucose, [6-^{14}C]glucose, and [1-^{14}C]fructose were purchased from Amersham Corporation (Arlington Heights, IL). [U-^{14}C]Fructose, [1-^{14}C]- and [2-^{14}C]pyruvate, [U-^{14}C]lactate, [1-^{14}C]acetate, and ^3H_2O were purchased from New England Nuclear Corporation (Boston, MA). NCS-tissue solubilizer was purchased from Amersham Corporation. All enzymes and substrates were purchased from Boehringer-Mannheim (Indianapolis, IN). Dowex resins were obtained from Bio-Rad Laboratories (Richmond, CA). Cyano-4-hydroxycinnamate was obtained from Aldrich (Metuchen, NJ).

Isolation and incubation of chick liver cells

Leghorn chicks (10–15 days old, mixed sex) were fed a high-carbohydrate, low-fat diet ad libitum for at least 48 hr prior to being killed. Hepatocytes were prepared by external digestion with collagenase as previously described (16). The cells were filtered through 100-mesh silk screen to remove aggregated Kupffer cells and then were washed twice in phosphate (7 mM)-buffered hepatocyte incubation medium (pH 7.3). Cell viability estimated by trypan blue exclusion was 85–95%. The hepatocyte isolation procedure required about 2 hr.

Prior to incubation, the medium was equilibrated with 10% CO_2–90% air at 37°C. One milliliter of cell suspension containing $40–50 \times 10^6$ cells (10–13 mg cell dry weight) was mixed with 2 ml of hepatocyte incubation medium in a 25-ml polypropylene Erlenmeyer flask and incubated under 10% CO_2–90% air at 37°C in a gyratory bath (80 oscillations per min). Serum was not present during incubation. Incubations were of 60 min duration unless otherwise specified. Except where indicated, 5 mM sodium acetate was present in all incubations. Bt_2cAMP was added at a level of 0.1 mM. In experiments where fructose was a variable addition, hepatocytes were washed and suspended in glucose-free phosphate-buffered medium.

$^{14}CO_2$ formation and fatty acid and cholesterol synthesis

[U-^{14}C]-, [1-^{14}C]-, and [6-^{14}C]glucose and [U-^{14}C]- and [1-^{14}C]fructose were added at a level of 2–3 μCi per flask. [1-^{14}C]Acetate, [U-^{14}C]lactate, [1-^{14}C]- and [2-^{14}C]pyruvate were added at 2.25 μCi per flask and ^3H_2O at a level of 1.0 mCi per ml of medium. Conditions of the respective experiments are described in the tables. To measure the rate of formation of $^{14}CO_2$, reaction flasks were capped with serum stoppers fitted with suspended polyethylene wells. After incubation, cells were quenched with 6% $HClO_4$. Alkaline NCS tissue solubilizer (0.25 ml) was then added to each polyethylene well and the flasks were incubated for 2 hr at 37°C. $^{14}CO_2$ trapped in the well was counted in toluene scintillator. The rate of formation of $^{14}CO_2$ was linear for approximately 2 hr.

For measurement of radioisotope incorporation into total fatty acids and cholesterol, an aliquot of the incubation medium plus cells was added to tubes containing 1.0 ml of 70% KOH (17). The volume was brought to 5 ml with ethanol and the mixture held at 100°C for 2 hr. Nonsaponifiable lipids were extracted into petroleum ether and used for cholesterol isolation as described below. The aqueous phase was acidified by addition of 2 ml of concentrated HCl. Fatty acids were extracted into petroleum ether and the extract was backwashed with three 5-ml volumes of 5% acetic acid (for [^{14}C]acetate) or 0.5 M glucose (for [^{14}C]glucose) or 0.5 M fructose (for [^{14}C]fructose). Extracts were taken to dryness and the residues were dissolved in a toluene-based scintillator fluid and counted.

The extract containing nonsaponifiable lipids was washed twice with 0.1 M NaOH and twice with 5% acetic acid and taken to dryness under N_2. Cholesterol was isolated as the digitonide by a modification of the method of Sperry and Webb (18) and Crawford (19). The residue was dissolved in 2 ml of acetone–absolute ethanol 1:1. Two drops of glacial acetic acid and 0.5 ml of 1% cholesterol in absolute ethanol were added, and cholesterol was precipitated by the addition of 5 ml of 0.5% digitonin in 70% ethanol. After standing at room temperature for 24 hr the suspension was centrifuged at 27,000 *g* for 10 min. The pellet was washed once with 5 ml of acetone–diethyl ether 1:1 and once with 4 ml of diethyl ether, after which it was taken to dryness, dissolved in 1 ml of methanol, and counted in the toluene scintillator fluid.

Lactate isolation and lactate and citrate determination

Following incubation, cells plus medium were added to 0.5 ml of ice-cold 70% perchloric acid. After centrifugation to remove the denatured protein, an aliquot of the supernate was brought to 50 mM potassium phosphate and then neutralized on ice with KOH. The $KClO_4$ precipitate was removed by centrifugation and the supernate was used for lactate and citrate analyses.

The lactate dehydrogenase and glutamate–pyruvate

transaminase-coupled fluorometric assay for lactate was used as described by Lowry and Passoneau (20). Cyano-4-hydroxycinnamate did not interfere with lactate analysis. Citrate concentrations were determined in the neutralized perchloric acid fraction by the fluorometric enzyme assay described by Williamson and Corkey (21).

To isolate [^{14}C]lactate, 1.0 ml of the neutralized perchloric acid extract was applied to tandem columns (0.7 × 4 cm) of Dowex-Ag 50 W-X4-H^+ form (top) and Dowex-Ag 1-X4-Acetate form (bottom). Lactate and neutral compounds are eluted from the Dowex-Ag 50 column with water; lactate is retained by the Dowex-Ag 1 column, while the neutral compounds are eluted with 50 ml of water. Lactate was then eluted from the Dowex-Ag 1 column with 40 ml of 1 N acetic acid. This procedure does not release pyruvate[4] or phosphorylated intermediates (12). Recovery of lactate by enzymatic or radiochemical analysis was 90–95%.

For experiments in which lactate measurements were made, glucose was not present in the phosphate-buffered medium used to wash and suspend the hepatocytes. This precaution was necessary to minimize lactate formation by the cells before initiating the reaction.

Phosphofructokinase assay

Phosphofructokinase activity was measured in the supernatant fraction of disrupted chick liver cells previously incubated in the presence or the absence of Bt_2cAMP or glucagon. Since chick liver phosphofructokinase is cold-sensitive (22), all procedures were carried out at room temperature.

The chick hepatocyte suspensions were lightly centrifuged to separate cells from incubation media. To the cell pellet was added 3.0 ml of buffer (pH 7.5) containing potassium phosphate, 2.0 mM; EDTA, 1.0 mM; and dithiothreitol, 1.0 mM. The cells were disrupted by hand homogenization (50 strokes) using a close-fitting glass homogenizer. Potassium morpholinopropane sulfonate (MOPS, pH 7.5) was immediately added to the disrupted cells to produce a final concentration of 30 mM. Cell debris was removed by centrifugation for 2 min in an Eppendorf microfuge and the supernatant was assayed immediately for phosphofructokinase activity.

Phosphofructokinase activity was determined by a modification of the procedure of Kono and Uyeda (22). The assay mixture contained (total volume, 1.0 ml) Tris-Cl, pH 7.5, 50 mM; EDTA, 1 mM; $MgCl_2$, 4 mM; dithiothreitol, 2.5 mM; NADH, 0.35 mM; ATP 0.5 mM; fructose-6-phosphate, as indicated; bovine serum albumin, 0.01%; aldolase, 0.5 unit; triose phosphate isomerase, 2 units; and α-glycerophosphate dehydrogenase, 0.5 unit. Before use, coupling enzymes were diluted with 0.1% bovine serum albumin and dialyzed overnight against 50 mM Tris-Cl, pH 7.5 containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol. The reaction was initiated by adding cell homogenate. One unit of enzyme catalyzes the phosphorylation of 1 μmol of fructose-6-phosphate per min at 37°C.

Pyruvate kinase assay

Pyruvate kinase assays were conducted either on supernates of digitonin-disrupted cells by the method of Feliu, Hue, and Hers (23) or on cell homogenates by the method of Ljungstrom and Ehman (24). In Experiment 1 (Table 3) the digitonin release procedure was a modification of the technique of Zurrendonk and Tager (25). Cell suspensions were mixed with sucrose, 0.25 M; MOPS, 17 mM (pH 7.5); EDTA, 2.5 mM; dithiothreitol, 5 mM; NaF, 50 mM; and digitonin, 0.8 mg per ml. After vortex mixing for 1 min, cell debris was removed by centrifugation for 1 min in a Beckman Microfuge B. In Experiment 2, (Table 3) cell-free homogenates were also prepared by hand homogenization with cold 2 mM potassium phosphate (pH 7.5) and 0.17 mM dithiothreitol. After 50 strokes the composition of the homogenate was immediately changed to 50 mM phosphate (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol. One unit of pyruvate kinase catalyzes the formation of 1 μmol of pyruvate per min at 30°C.

RESULTS

Effect of Bt_2cAMP on the glycolytic pathway

Chick hepatocytes metabolizing glucose and acetate as sole exogenous substrates exhibit greatly depressed (>90%) cytoplasmic and mitochondrial citrate concentrations and rates of fatty acid synthesis upon exposure to glucagon or Bt_2cAMP (Table 5, ref 1). This decline in cellular citrate level and concomitant drop in fatty acid synthesis appears to be due, in part, to reduced glucose flux to lactate/pyruvate; several lines of evidence that support this proposal are described below.

Bt_2cAMP exerts a differential inhibitory effect on the oxidation of [^{14}C]glucose, [^{14}C]fructose, and [^{14}C]-pyruvate to $^{14}CO_2$. The rate of oxidation of glucose to $^{14}CO_2$ is markedly inhibited by Bt_2cAMP whether

[4] Clarke, S. D., P. A. Watkins, and M. D. Lane. Unpublished results.

[U-^{14}C]-, [1-^{14}C]-, or [6-^{14}C]glucose is the labeled substrate (**Table 1**). However, the degree of inhibition by Bt_2cAMP depends upon the location of the ^{14}C label in glucose. As will be discussed later, the flux of labeled glucose to fatty acids is depressed more than 95% by Bt_2cAMP.

The rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose exceeded that from [6-^{14}C]glucose, a result that would be expected were the hexose monophosphate shunt operative in chick hepatocytes. However, the ratio of rates of $^{14}CO_2$ production from [1-^{14}C]glucose relative to [6-^{14}C]glucose was 1.24 which indicates a low activity of this pathway in chick liver cells compared to that in rat liver or adipose tissue. In the latter two cases the ratio was 2–5 and 10–19, respectively (26, 27). Moreover, the ratio of rates of $^{14}CO_2$ formation from [1-^{14}C]glucose relative to [6-^{14}C]glucose actually rose slightly to 1.50 in the presence of Bt_2cAMP; nevertheless, the absolute rates of $^{14}CO_2$ formation from [1-^{14}C]- and [6-^{14}C]glucose were inhibited by Bt_2cAMP to about the same extent. Since Bt_2cAMP inhibits fatty acid synthesis from glucose by more than 95%, the flux of glucose through the hexose monophosphate shunt in chick hepatocytes appears to be independent of the rate of fatty acid synthesis. The continued flow of glucose through the hexose monophosphate shunt in the presence of Bt_2cAMP accounts for the somewhat less pronounced reduction in $^{14}CO_2$ formation from [1-^{14}C]glucose, than from [6-^{14}C]glucose, in the presence of the cyclic nucleotide (Table 1).

The rate of formation of $^{14}CO_2$ from [1-^{14}C]- and [2-^{14}C]pyruvate, as well as from [1-^{14}C]fructose, was not greatly affected by the exposure of chick hepatocytes to Bt_2cAMP. Fructose enters glycolysis at the level of triose phosphate and consequently bypasses two potential regulatory enzymatic steps in the glycolytic pathway, i.e., the phosphorylation of glucose and fructose-6-phosphate (Scheme 1). The greater inhibitory effect of Bt_2cAMP on the rate of $^{14}CO_2$ formation from [^{14}C]glucose compared to [^{14}C]fructose or [^{14}C]pyruvate is indicative of a site of inhibition above the level of triose phosphate in the glycolytic pathway. As discussed below, dilution of the specific activity of the labeled hexose phosphate pool by Bt_2cAMP-activated glycogenolysis is insufficient to explain the inhibitory effect on glucose oxidation to $^{14}CO_2$. It is interesting that the rate of formation of $^{14}CO_2$ from [2-^{14}C]pyruvate is not affected by the addition of 5 mM unlabeled acetate (Table 1). This shows that the formation of acetyl-CoA from unlabeled acetate in the mitochondrion does not occur at a rate sufficient to dilute the intramitochondrial labeled acetyl-CoA pool.

Consistent with the foregoing results, which indicate that Bt_2cAMP may inhibit glycolysis, was the finding (**Table 2**) that the cyclic nucleotide decreased the rate of lactate formation from glucose. Like $^{14}CO_2$ formation from [^{14}C]glucose (Table 1), the rate of lactate formation, measured both enzymatically and by [^{14}C]lactate formation, is inhibited about 50% by Bt_2cAMP (Table 2). In the presence of cyano-4-

TABLE 1. Effect of Bt_2cAMP on rate of ^{14}C-labeled glucose, fructose, and pyruvate oxidation to $^{14}CO_2$

Experiment	Labeled Substrate	Added Acetate	Rate of Incorporation into $^{14}CO_2$ by Cells Treated with: No Addition	Bt_2cAMP
			nmol/min/mg cell dry wt	
I	[U-^{14}C]Glucose	–	2.1	0.7 (33%)
		+	2.4	1.0 (42%)
II	[1-^{14}C]Glucose	+	0.56	0.36 (64%)
	[6-^{14}C]Glucose	+	0.45	0.24 (53%)
	[1-^{14}C]Fructose	+	0.80	0.70 (88%)
III	[1-^{14}C]Pyruvate	–	18.3	15.7 (86%)
		+	16.9	13.1 (78%)
	[2-^{14}C]Pyruvate	–	7.8	6.2 (79%)
		+	7.5	6.3 (84%)

Chick liver cells were incubated with the ^{14}C-labeled substrate for 60 min and ^{14}C activity incorporated into $^{14}CO_2$ was determined as described in Experimental Procedure. Glucose (25 mM) was present in all incubations except when fructose (25 mM) was the labeled substrate. Pyruvate and acetate were added as sodium salts where indicated at a level of 5 mM. Each data point represents the average of duplicate flasks of cells (the range was <5% of the mean in all cases). Values in parentheses are the Bt_2cAMP values expressed as percent of control.

TABLE 2. Effect of Bt_2cAMP on lactate formation from glucose

Experiment	Additions	Lactate Formed: Total	Lactate Formed: ^{14}C Activity Incorporated	Lactate Formed: Specific Activity
		nmol/min/mg cell dry wt	*cpm/min/mg cell dry wt*	*cpm/nmol*
I	None	15	2700	180
	Bt_2cAMP	8.0 (53%)	1200 (44%)	150
II	None	12	1800	150
	Bt_2cAMP	7.0 (58%)	1100 (63%)	160
III	Cyano-4-hydroxycinnamate	85		
	Cyano-4-hydroxycinnamate + Bt_2cAMP	39 (46%)		

Chick liver cells were incubated with [U-^{14}C]glucose (25 mM) for 30 min (Experiment II) or 45 min (Experiments I and III) as described in Experimental Procedure. The lactate formed (cells plus medium) was isolated chromatographically and the total quantity formed and the ^{14}C activity incorporated into lactate were determined. Initial lactate concentration was <0.5 nmol per mg of cell dry weight and lactate formation was linear for at least 45 min. Sodium acetate (5 mM) was present in all incubations and 0.1 mM Bt_2cAMP and 2 mM cyano-4-hydroxycinnamate as indicated. Values in parentheses are percentages of controls. Each data point represents the average of triplicate flasks. Standard deviations were <10% of the mean in all cases.

hydroxycinnamate (2 mM), a potent inhibitor of mitochondrial pyruvate transport (28), lactate accumulation is greatly increased in both the presence and the absence of Bt_2cAMP. Indicative of the block of mitochondrial pyruvate uptake was the 99% inhibition of rate of [U-^{14}C]glucose incorporation into fatty acids in the presence of the inhibitor. Under conditions where pyruvate uptake is blocked by cyano-4-hydroxycinnamate, Bt_2cAMP inhibits lactate formation with glucose as substrate by approximately 50% (Table 2). The effect of Bt_2cAMP on lactate formation in the presence of cyano-4-hydroxycinnamate points to a specific inhibition of the glycolytic pathway, rather than to an activating effect of Bt_2cAMP on mitochondrial utilization of pyruvate, e.g., for gluconeogenesis.[5] Moreover, Bt_2cAMP has little effect on the specific activity of lactate formed from [U-^{14}C]glucose (Table 2). Therefore, inhibition by Bt_2cAMP of the incorporation of labeled glucose into lactate is not the result of dilution of the specific activity of the hexose phosphate pool caused by accelerated glycogenolysis.

Effect of glucagon and Bt_2cAMP on phosphofructokinase and pyruvate kinase activity

Inhibition by Bt_2cAMP of lactate (or pyruvate) formation from glucose at a site above the triose phosphate level of glycolysis appears to be caused by a decrease in phosphofructokinase activity (**Fig. 1**). Exposure of chick hepatocytes to glucagon or Bt_2cAMP caused a marked decrease of phosphofructokinase activity assayed in the absence of 5′-AMP, an allosteric activator of the enzyme (Fig. 1). Inhibition was greatest at limiting substrate concentration, 67–90% inhibition occurring at 0.2–0.4 mM fructose-6-phosphate. At a saturating level of 10 mM fructose-6-phosphate, inhibition by glucagon was largely re-

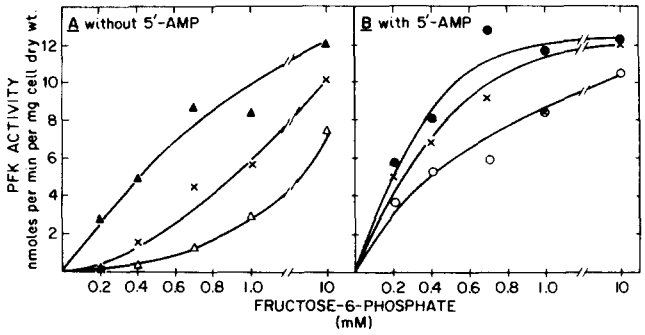


Fig. 1. Effect of glucagon and Bt_2cAMP treatment of chick liver cells on phosphofructokinase activity. Chick liver cells were incubated 30 min in medium containing 25 mM glucose and glucagon (5 μg per ml) or Bt_2cAMP (0.1 mM) as indicated. Cell-free extracts were prepared and assayed for phosphofructokinase activity as described in Experimental Procedure. Data points are the mean of two experiments and the range between experiments was less than 10% of the mean. 5′-AMP refers to adenosine-5′-phosphate and was added to the phosphofructokinase assay reaction mixture where indicated to yield a final concentration of 5 mM. Solid symbols (▲, ●) refer to extracts from untreated control cells, open symbols (△, ○) to extracts from cells treated with Bt_2cAMP, and ×'s to extracts from cells treated with glucagon.

[5] Bt_2cAMP has no effect on the rate of [^{14}C]glucose formation from 5 mM [1-^{14}C]labeled lactate or pyruvate in chick hepatocytes under identical conditions in the presence or absence of 25 mM unlabeled glucose (Watkins, P. A., and M. D. Lane. Unpublished results).

TABLE 3. Effect of Bt_2cAMP treatment of chick liver cells on pyruvate kinase activity

Experiment	Enzyme	Substrate Concentration	Enzyme Activity of Cells Treated with: No Additions	Enzyme Activity of Cells Treated with: Bt_2cAMP
		mM	*mU/mg cell dry wt*	
I	Pyruvate kinase	0.05 PEP	8	8
		1.0 PEP	15	18
II	Pyruvate kinase	0.1 PEP	6	(5)[a]
		0.5 PEP	10	(10)[a]

[a] Cells treated with glucagon (5 μg/ml) rather than Bt_2cAMP.

Chick liver cells were incubated for 60 min in a medium containing 25 mM glucose and 5 mM acetate in the absence or presence of 0.1 mM Bt_2cAMP. Cell-free extracts were prepared and assayed for pyruvate kinase activity as described in Experimental Procedure. The data points represent the averaged results from duplicate or triplicate flasks. The range in the case of duplicates and the standard deviation in the case of triplicates was <10% of the mean in all cases. F6P and PEP refer to D-fructose-6-phosphate and phosphenolpyruvate, respectively.

versed and inhibition by Bt_2cAMP was significantly reduced. Bt_2cAMP reduced phosphofructokinase activity more effectively than did glucagon. This is probably because Bt_2cAMP bypasses the cell surface glucagon receptor which sustains some damage during the collagenase treatment of the cells. Neither Bt_2cAMP nor cAMP added immediately after cell disruption affects phosphofructokinase activity (results not shown).

5′-AMP, which reduced the level of fructose-6-phosphate needed to achieve maximal velocity, also significantly reversed the inhibitory effects of glucagon and Bt_2cAMP (Fig. 1). The fact that both substrate, fructose-6-phosphate, and activator, 5′-AMP, overcame inhibition without affecting V_{max} suggests that glucagon and Bt_2cAMP decrease catalytic efficiency by raising the K_m for fructose-6-phosphate.

Under conditions where phosphofructokinase activity from chick hepatocytes treated with glucagon or Bt_2cAMP was greatly depressed, pyruvate kinase activity was not affected (**Table 3,** ref. 29). This is in contrast to the well-documented cAMP-dependent phosphorylation and inactivation of rat liver pyruvate kinase (12, 23, 29).

The degree of impairment of glycolytic flux elicited by Bt_2cAMP treatment of chick hepatocytes (Tables 1 and 2) correlates closely with the reduction in phosphofructokinase activity (Fig. 1). This plus the failure of Bt_2cAMP or glucagon to affect pyruvate kinase, PEP-carboxykinase, or pyruvate dehydrogenase (Table 1) activities implicates the fructose-6-phosphate to fructose-1,6-diphosphate reaction as the site of inhibition of glycolysis by cAMP in the chick liver cell.

Effect of Bt_2cAMP and metabolizable substrate on cellular citrate level and fatty acid synthesis from various labeled precursors

Bt_2cAMP drastically inhibited fatty acid synthesis from ^3H_2O and [1-^{14}C]acetate when glucose was the primary metabolizable substrate (**Table 4**). The ability of Bt_2cAMP to impair glucose flux through glycolysis by reducing the catalytic efficiency of phosphofructokinase in chick hepatocytes may account for the Bt_2cAMP-induced suppression of citrate accumulation and inhibition of [U-^{14}C]glucose flow to fatty acids (Table 4). Citrate, a feed-forward allosteric activator of avian liver acetyl-CoA carboxylase (Scheme 1), is required to maintain the enzyme in the catalytically-active polymeric state (8, 9). Cytoplasmic citrate concentrations in chick liver cells maintained on glucose as primary substrate and exposed to Bt_2cAMP were sufficiently low to prevent polymerization of acetyl-CoA carboxylase and, hence, activation of the enzyme (8, 9). This explains, in part at least, the failure of Bt_2cAMP-treated hepatocytes to incorporate [1-^{14}C]acetate or ^3H_2O (plus unlabeled acetate) into fatty acids at normal rates (Table 4).

Chick hepatocytes incubated without added glucose and in the absence of Bt_2cAMP retain the capacity to synthesize citrate and incorporate [1-^{14}C]acetate into fatty acids (Table 4). Presumably, this reflects the utilization of endogenous glycogen as a precursor of glycolytic intermediates and citrate. Citrate level and fatty acid synthetic rate were far lower, however, than when the cell medium contained 25 mM glucose (Table 4). Bt_2cAMP effectively blocked citrate accumulation when exogenous glucose or only endogenous glycogen were substrates (Table 4). Moreover, Bt_2cAMP inhibited fatty acid synthesis from [U-^{14}C]glucose, [1-^{14}C]acetate, or ^3H_2O by 90–95% (Table 4).

In contrast, the addition of substrates, e.g., fructose, pyruvate, or lactate, that enter glycolysis down-stream from fructose-1,6-diphosphate totally overcomes the inhibition of citrate accumulation by Bt_2cAMP and substantially reverses the inhibition of fatty acid synthesis from [1-^{14}C]acetate (Table 4). A reduction in glycolytic flux due to the inhibition of phosphofructokinase by Bt_2cAMP would account for both the reduction in cellular citrate levels and the rate of fatty acid synthesis with substrates that enter glycolysis above fructose-6-phosphate and a partial reversal of this reduction with substrates that enter below fructose-1,6-diphosphate.

There appears to be a second site of inhibition

TABLE 4. Effect of Bt_2cAMP and metabolizable substrate on cellular citrate level and fatty acid synthesis from various labeled precursors

Substrate and ^{14}C- or ^{3}H-Label	Added Acetate	Citrate Level		Fatty Acid Synthesis	
		Control	+Bt_2cAMP	Control	+Bt_2cAMP
		nmol/mg cell dry wt		*nmol/min/mg cell dry wt*	
None					
[1-^{14}C]acetate	+	0.60	0.16	0.44	0.07
Glucose					
[U-^{14}C]glucose	+	1.3	0	0.21	0.01
[1-^{14}C]acetate	+	2.9	0.38	2.2	0.14
$^{3}H_2O$	+	ND[a]	ND	5.0	0.05
Fructose					
[U-^{14}C]fructose	+	1.9	2.4	0.59	0.24
[1-^{14}C]acetate	+	3.2	4.8	3.83	1.63
Pyruvate + glucose					
[2-^{14}C]pyruvate	−	2.0	2.2	1.20	0.79
[2-^{14}C]pyruvate	+	4.5	5.6	0.64	0.39
$^{3}H_2O$	+	ND	ND	7.8	3.1
[1-^{14}C]acetate	+	4.1	6.0	2.2	1.5
Lactate + glucose					
[U-^{14}C]lactate	−	ND	ND	4.3	1.9
[U-^{14}C]lactate	+	ND	ND	1.9	0.80
[1-^{14}C]acetate	+	4.6	5.9	2.3	1.6

[a] Not determined.

Chick liver cells were incubated for 60 min with the ^{14}C-labeled substrate and unlabeled substrate indicated. Cellular citrate and the rate of incorporation of ^{14}C or ^{3}H into fatty acid were determined as described in Experimental Procedure; ^{14}C or ^{3}H incorporation rates were linear for approximately 2 hr. Bt_2cAMP was added at a level of 0.1 mM; glucose and fructose were added at a level of 25 mM; pyruvate, lactate, and acetate were added as their sodium salts at a level of 5 mM. Each value is the average of duplicate or triplicate flasks where the range or standard deviation, respectively, was <10% of the mean in all cases. Values for ^{14}C or ^{3}H incorporated into fatty acids were corrected for radioactivity contaminating fatty acid extracts of cell suspensions at zero time. These corrections were <5% of total radioactivity incorporated into extractable fatty acids.

of fatty acid synthesis by Bt_2cAMP, however, which lies beyond cytoplasmic acetyl-CoA in the fatty acid synthetic pathway and which is independent of cellular citrate concentration. This is indicated by the observation (Table 4) that fatty acid synthesis from [1-^{14}C]acetate in the presence of unlabeled fructose, pyruvate, or lactate is still partially inhibited (~50%) by Bt_2cAMP despite the complete restoration of cellular citrate concentration. In agreement is the fact that the rate of incorporation of [^{14}C]fructose, [^{14}C]pyruvate, or [^{14}C]lactate into fatty acids is also inhibited approximately 50% by Bt_2cAMP (Table 4). That Bt_2cAMP acts specifically on fatty acid synthesis at a point beyond cytoplasmic acetyl-CoA is shown by the failure of the cyclic nucleotide to inhibit incorporation of [2-^{14}C]pyruvate or [1-^{14}C]acetate into cholesterol (**Table 5;** also see Scheme 1). Under these conditions, the incorporation of [2-^{14}C]pyruvate into fatty acids is inhibited by 40–50% and [1-^{14}C]acetate incorporation is inhibited by more than 90% (Table 5). Moreover, because [1-^{14}C]acetate incorporation into cholesterol is only slightly decreased by Bt_2cAMP while incorporation into fatty acids is almost totally suppressed, it is likely that acetate activation by cytoplasmic thiokinase is not impaired by the cyclic nucleotide. It should be noted that both hepatic fatty acid and cholesterol synthesis utilize the same cytoplasmic acetyl-CoA pool (See Scheme 1 and ref 30).

The reduced rates of incorporation of [^{14}C]fructose, [^{14}C]pyruvate, or [^{14}C]lactate into fatty acids caused by Bt_2cAMP cannot be accounted for by isotope dilution due to accelerated glycogenolysis, since $^{3}H_2O$ incorporation in the presence of 5 mM pyruvate is also depressed by 50% (Table 4). Although the presence of unlabeled acetate (5 mM) appeared to dilute the cytoplasmic pool of labeled acetyl-CoA, as indicated by the reduced incorporation of [^{14}C]pyruvate and [^{14}C]lactate into fatty acids, the magnitude of the inhibitory effect of Bt_2cAMP remained the same (Tables 4 and 5).

Since acetyl-CoA carboxylase is known to be inhibited by fatty acyl-CoA derivatives (31), the possibility was considered that Bt_2cAMP might cause an elevated cytoplasmic level of long-chain acyl-CoA. It was found, however, that exposure of chick hepato-

TABLE 5. Effect of Bt_2cAMP on cholesterol and fatty acid synthesis from [1-^{14}C]acetate and [2-^{14}C]pyruvate

Experiment	Labeled Substrate	Additions	^{14}C-Activity Incorporated into	
			Cholesterol	Fatty Acids
			nmol/min/mg cell dry wt	
I	[1-^{14}C]Acetate	None	0.10	1.9
		Bt_2cAMP	0.077	0.17
II	[1-^{14}C]Acetate	None	0.028	2.2
		Bt_2cAMP	0.020	0.14
		Pyruvate	0.078	2.3
		Bt_2cAMP + pyruvate	0.090	1.5
III	[2-^{14}C]Pyruvate	None	0.14	1.1
		Bt_2cAMP	0.16	0.62
		Acetate	0.051	0.55
		Bt_2cAMP + acetate	0.055	0.34

Chick liver cells were incubated for 60 min with the ^{14}C-labeled substrate indicated in the presence or absence of 0.1 mM Bt_2cAMP. The rate of incorporation of ^{14}C activity into fatty acids and cholesterol was determined as described in Experimental Procedure. All incubations contained 25 mM glucose; acetate and pyruvate were added as their sodium salts at a level of 5 mM. Each value is the average of duplicate or triplicate flasks where the range or standard deviation, respectively, was <10% of the mean in all cases.

cytes to 0.1 mM Bt_2cAMP for 60 min, which inhibits fatty acid synthesis from [^{14}C]acetate by more than 90%, causes no significant change in cytoplasmic long-chain fatty acyl-CoA concentration. Control and Bt_2cAMP-treated cells contained 0.078 nmol and 0.062 nmol of cytoplasmic long-chain fatty acyl-CoA per mg of cell dry weight, respectively. Hence, inhibition of fatty acid synthesis from acetyl-CoA by Bt_2cAMP cannot be ascribed to a fatty acyl-CoA-mediated effect on acetyl-CoA carboxylase activity. To date, it has not been possible to identify the secondary inhibitory site of Bt_2cAMP which is independent of cellular citrate concentration.

DISCUSSION

When chick hepatocytes metabolizing glucose and acetate as sole exogenous substrates are treated with Bt_2cAMP (or glucagon), the level of cellular citrate and the rate of incorporation of [U-^{14}C]glucose, [1-^{14}C]acetate, or ^3H_2O into fatty acids decrease concomitantly by over 90% (Tables 4 and 5; ref 1). Most of the citrate (~75%) within the chick liver cell is found in the cytoplasmic compartment where acetyl-CoA carboxylase is localized (1). The carboxylase isolated from chick liver exists in either a catalytically inactive protomeric form or an active polymeric filamentous form (see Reaction 1 below; ref 6–8, 10).

$$\text{1) Protomeric form (“inactive”)} \underset{-\text{ citrate}}{\overset{+\text{ citrate}}{\rightleftharpoons}} \text{Polymeric form (“active”)}$$

Citrate, the obligate allosteric activator of the enzyme, is required for both catalysis and polymerization. Therefore, it has been proposed that the state of the protomer–polymer equilibrium, hence the rate of fatty acid synthesis, is determined by cytoplasmic citrate concentration (Reaction 1; ref 6–10). Thus, the ability of cAMP to modulate cellular citrate level when glucose is the primary carbohydrate source could be an important mechanism for regulating fatty acid synthesis in the chick liver cell (Table 4; ref 1). Until this report, however, the site at which Bt_2cAMP inhibits citrate formation from glucose in chick hepatocytes was unknown.

Several lines of evidence indicate that glucose flux to citrate in chick hepatocytes is inhibited by cAMP at a site in the glycolytic pathway, apparently at the phosphofructokinase-catalyzed reaction. It is evident that the step limited by Bt_2cAMP, i.e., the formation of fructose diphosphate from fructose-6-phosphate, can be bypassed with fructose, pyruvate, or lactate, which enter glycolysis at or down-stream from triose phosphate (Tables 1 and 4). Thus, fructose, pyruvate, or lactate largely overcome the inhibition by Bt_2cAMP of citrate formation, of CO_2 production from glucose (Tables 1 and 4), and of incorporation of [^{14}C]acetate into fatty acids (Table 4; ref 24). Moreover, the rate of formation of lactate (Table 2) from [^{14}C]glucose is reduced by 50–60%. Furthermore, in the presence of cyano-4-hydroxycinnamate (an inhibitor of mitochondrial pyruvate transport), Bt_2cAMP still inhibits the rate of lactate accumulation by 55%; like

Bt_2cAMP (Table 4), cyano-4-hydroxycinnamate almost totally blocks fatty acid synthesis from [^{14}C]glucose (results not shown). This observation indicates that the effect of Bt_2cAMP on lactate accumulation is not due to accelerated utilization of lactate for gluconeogenesis.[5]

The isotopic labeling experiments and those on citrate and lactate accumulation, which indicate a Bt_2cAMP-induced inhibition of glycolysis between glucose and triose phosphate, are supported by the observation that cell-free extracts of glucagon or cyclic nucleotide-treated chick liver cells exhibit a pronounced decrease in the catalytic efficiency of phosphofructokinase (Fig. 1). Although Bt_2cAMP treatment of the hepatocytes greatly reduced phosphofructokinase activity at low substrate concentrations, the presence of the positive effector, 5′-AMP, or of saturating substrate levels (fructose-6-phosphate) tended to overcome the inhibition (Fig. 1).

In contrast to the response of rat liver to glucagon or cAMP treatment, the cAMP-induced block in glucose flux to lactate in chick liver cells is not associated with a change in kinetic behavior of pyruvate kinase (Table 3, ref 29). Apparently the K-type pyruvate kinase of chick liver is less sensitive to allosteric effects than the L-type of rat liver (32). Therefore, the impairment in phosphofructokinase activity by glucagon or Bt_2cAMP treatment of chick liver cells appears to be largely responsible for the diminished flux of glucose through glycolysis to lactate and pyruvate.

The mechanism by which Bt_2cAMP impairs the catalytic efficiency of phosphofructokinase in the chick liver cell is unknown. Soling and associates (33, 34) proposed that rat liver phosphofructokinase may exist in two forms, i.e., as an active phosphorylated tetramer or as an inactive nonphosphorylated monomer. Furthermore, transition between the two states appeared to be dependent upon nutritional state and to be mediated by an ATP-dependent kinase and a Mg^{2+}-dependent phosphatase. We have observed that phosphofructokinase activity in chick liver extracts is quickly abolished by prior incubation with $MgCl_2$ and that this inhibition is reversed by ATP. The significance of this finding remains unclear, as we have been unable to demonstrate that prior treatment of cells with Bt_2cAMP either accelerates the action of $MgCl_2$ or attenuates reversal by ATP.

In this connection the proposed interaction between phosphofructokinase and fructose diphosphatase (35, 36) may be relevant. Accelerated "substrate cycling" at the level of

$$\text{fructose-6-phosphate} \underset{\text{FDPase}}{\overset{\text{PFK}}{\rightleftharpoons}} \text{fructose-1,6-diphosphate}$$

has been suggested in rat liver cells treated with glucagon or cAMP (11). It is unclear, however, whether such cycling is due to reduced phosphofructokinase activity, accelerated fructose diphosphatase activity, or both. Fructose diphosphatase is capable of reducing the apparent activity of phosphofructokinase by removing tightly bound allosteric effector, i.e., fructose-1,6-diphosphate, from phosphofructokinase (35). Taunton et al. (15) reported that perfusion of rat liver with glucagon not only leads to a decline in phosphofructokinase activity, but also to an increase in fructose diphosphatase activity. In light of these findings, it will be necessary to determine whether cAMP affects phosphofructokinase activity in the chick liver cell through a direct or an indirect mechanism.

Inhibition of phosphofructokinase activity by Bt_2cAMP and the consequent reduction in glucose flux through glycolysis could explain the large depression in citrate formation from glucose and the accompanying inhibition of fatty acid synthesis when glucose is substrate. Compelling evidence for the involvement of citrate in the formation and maintenance of the polymeric filamentous form of acetyl-CoA carboxylase in the intact chick liver cell has recently been obtained (9). Filaments generated by the exposure of purified avian carboxylase to citrate are 70–100 Å in width by up to 5000 Å in length, whereas protomers that 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 (25), causes the release of carboxylase at a rate inversely related to cellular citrate concentration. When chick liver cells are exposed to Bt_2cAMP, which lowers intracellular citrate levels, the rate of carboxylase release is greatly accelerated (9). These and other findings (6–9) indicate that carboxylase filaments are present in the intact chick liver cell when the cytoplasmic citrate level is high and undergo depolymerization when the citrate level falls.

The effect of glucagon and Bt_2cAMP on cellular citrate level in the rat liver cell appears to differ from that in the chick liver cell. The large decrease in total cellular citrate level caused by glucagon and Bt_2cAMP, which accompany decreased fatty acid synthesis from [^{14}C]acetate and ^3H_2O in chick liver cells, is not ob-

served in rat hepatocytes (2, 5, 10). Although the basis for this apparent discrepancy has not been resolved, two possible explanations should be considered: *1*) while it is evident (ref 1 and this report) that cytoplasmic citrate level is drastically reduced by either glucagon or Bt_2cAMP in the chick liver cell, it is not known whether the same is true in the rat liver cell since only total, and not cytoplasmic, citrate levels have been measured (2, 5, 10); or *2*) while it is true that the glycolytic pathway is inhibited by cAMP in both rat and chicken liver cells (11–14 and this report), the impact of this inhibition on mitochondrial oxaloacetate levels might be expected to differ because of the mitochondrial localization of PEP carboxykinase (PEPCK) in chick liver versus the cytoplasmic localization of this enzyme in rat liver. A high mitochondrial PEPCK activity in the chick hepatocyte would tend to deplete mitochondrial oxaloacetate if the glucose to pyruvate flux is compromised; a lowered mitochondrial oxaloacetate level would be expected to decrease the rate of citrate formation.

The results presented here indicate that a second site of inhibition of fatty acid synthesis by Bt_2cAMP exists in the utilization of cytoplasmic acetyl-CoA per se. Despite the fact that Bt_2cAMP does not prevent citrate formation from fructose, lactate, or pyruvate, it continues to partially inhibit the incorporation of these substrates, as well as that of ^3H_2O and [1-^{14}C]-acetate into fatty acids (Table 4). Under these conditions the effect of cAMP on citrate and fatty acid synthesis is similar to that found with rat hepatocytes (2, 37). However, this inhibition is less extensive than the nearly complete block in fatty acid synthesis by Bt_2cAMP with glucose as substrate.

The specific location of the second site of Bt_2cAMP inhibition of fatty acid synthesis remains obscure. Preliminary investigations[5] indicate that the production of NADPH via hexose monophosphate shunt (Table 1) or the catalytic efficiency of NADP-isocitrate dehydrogenase and malic enzyme are not significantly affected by the cyclic nucleotide. Furthermore, prior treatment of cells with Bt_2cAMP does not impair the activity of avian acetyl-CoA carboxylase (1), alter the extent of phosphorylation or dephosphorylation of the carboxylase (40),[6] or raise the concentration of cytoplasmic long-chain acyl-CoA derivatives. We recently reported (1) that glucagon, which inhibits fatty acid synthesis in the chick liver cell to the same extent as Bt_2cAMP, causes a 25% decrease in the esterification of [^{14}C]palmitate to acylglycerols; under these conditions, acylglycerol formation from [^{14}C]-acetate is inhibited more than 95% by the hormone. Similarly, glucagon stimulates the incorporation of [^{14}C]acetate into ketones (acetoacetate plus β-hydroxybutyrate) by 25–30% (41). These results are consistent with a glucagon-induced stimulation of β-oxidation in the chick liver cell and would partially account for the secondary inhibitory effect of Bt_2cAMP on fatty acid synthesis.

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[6] We have been unable to confirm earlier reports (38, 39) that acetyl-CoA carboxylase is inhibited by a cAMP-dependent protein kinase-catalyzed phosphorylation.

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