Fatty acid oxidation and esterification in isolated rat hepatocytes: regulation by dibutyryl adenosine 3′,5′-cyclic monophosphate

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Abstract Isolated rat hepatocytes rapidly utilized [^{14}C]palmitate and, in particular, synthesized large amounts of neutral lipids from palmitate. Incorporation into cellular lipids occurred at a linear rate proportional to the medium concentration of fatty acids. Oxidation of [^{14}C]palmitate to CO_2 increased with time and was much slower than palmitate esterification. Since [^{14}C]acetate and [^{14}C]glucose were oxidized to CO_2 at a linear rate, the lag in fatty acid oxidation to CO_2 did not involve enzymatic steps subsequent to acetate formation. The relative contribution of palmitate to esterification and to CO_2 formation depended upon the molar ratio of palmitate to albumin (ν) and the length of incubation. Dibutyryl cyclic AMP (1 mM) reduced the oxidation of palmitate and acetate to CO_2 by about 50 and 90%, respectively, but did not alter palmitate esterification. However, equivalent concentrations of sodium butyrate produced similar decreases in CO_2 formation. Dibutyryl cyclic AMP (1 mM) also stimulated palmitate oxidation to water-soluble products, principally ketone bodies, by 50–100%. Sodium butyrate exerted no effect, while monobutyryl cyclic AMP and cyclic AMP both stimulated this pathway significantly. These results indicate that both ν and dibutyryl cyclic AMP regulate the metabolism of fatty acids by isolated hepatocytes and suggest that hormonal stimulation of adenyl cyclase controls hepatic lipid metabolism.

Supplementary key words acetate oxidation · fatty acid concentrations · butyrate · glucose oxidation

The pathways and enzymes of hepatic fatty acid metabolism are well characterized, but little is known about their regulation. One factor that significantly affects fatty acid metabolism is the molar ratio (ν) of fatty acid to albumin. In Ehrlich ascites tumor cells, ν, rather than the concentration of fatty acids, was shown to be the major determinant of fatty acid uptake (1). This ratio also determined the amount of fatty acid converted to lipids, CO_2, and ketones by perfused liver (2, 3) and by isolated liver cells (4). In addition, the concentration of glucose in the incubation medium influenced fatty acid metabolism in tumor cells (5). In liver slices, both triglyceride synthesis and CO_2 production from fatty acids proceed at a linear rate (6). In contrast, an initial lag in fatty acid oxidation was found in mechanically isolated liver cells (7) and in tumor cells (8).

Several studies have suggested that 3′,5′-cyclic AMP may alter hepatic lipid metabolism. Dibutyryl cyclic AMP significantly increased ketone body production by perfused rat liver (3) and liver homogenates (9). Stimulation of a hepatic lipase by glucagon, an effect presumably mediated through cyclic AMP, was reported (9), but its quantitative importance has been questioned (2). More recently, in vivo administration of cyclic AMP to rats was shown to produce a chronic reduction in the level of hepatic enzymes involved in fatty acid synthesis (10). In addition, cyclic AMP decreased the incorporation of [^{14}C]acetate into both fatty acids and cholesterol in isolated liver cells and liver slices (11–14).

The present report confirms the early delay in fatty acid oxidation by isolated rat liver cells and demonstrates that alterations in ν produce significant changes in the relative magnitude of fatty acid oxidation and esterification. Although dibutyryl cyclic AMP reduced the oxidation of [^{14}C]palmitate and [^{14}C]acetate to CO_2, the significance of this finding is diminished by the similar effects of sodium butyrate. However, our results indicate that dibutyryl cyclic AMP significantly enhances the oxidation of [^{14}C]palmitate to water-soluble products, principally ketone bodies, and that this effect is not duplicated by sodium butyrate.

MATERIALS AND METHODS

Chemicals

Collagenase and hyaluronidase (type I) were purchased from Worthington Biochemical Corp., Freehold, N.J., and Sigma Chemical Co., St. Louis, Mo., respectively.

[1-^{14}C]Palmitic acid (55.2 mCi/mmole), sodium [1-^{14}C]acetate (2.0 mCi/mmole), [U-^{14}C]glucose (3.0 mCi/mmole), [U-^{14}C]leucine (304 mCi/mmole), and POPOP (*p*-bix[2-(5-phenyloxazolyl)]-benzene) were obtained from New England Nuclear, Boston, Mass. Hydroxide of Hyamine and PPO (2,5-diphenyloxazole) were products of Packard Instrument Co., Downers Grove, Ill. Butyric acid (sodium salt) was supplied by J. T. Baker Chemical Co. (Phillipsburg, N.J.). Adenosine 5′-phosphate (sodium salt), N^6-monobutyryl 3′,5′-cyclic AMP (sodium salt), and theophylline were purchased from Calbiochem (San Diego, Calif.). N^6-2′-*O*-Dibutyryl 3′,5′-cyclic AMP (sodium salt) was obtained from Plenum Scientific Research, Inc., Hackensack, N.J. Bovine serum albumin, containing less than 1 μg of fatty acid/g, was supplied by Miles Laboratories, Elkhart, Ind. Sodium penicillin G was purchased from E. R. Squibb & Sons, New York, and streptomycin sulfate from Pfizer Laboratories, New York. Silica gel G, prepared for thin-layer chromatography according to Stahl, was obtained from Brinkmann Instruments, Inc., Westbury, N.Y.

Cells

Male Sprague-Dawley albino rats, fed ad lib., weighing 175–250 g, were anesthetized with ether, and a 21-gauge scalp vein needle was inserted into the isolated portal vein. The method of Capuzzi, Rothman, and Margolis (11) was used to isolate hepatocytes after perfusion of the liver with 30 ml of a solution that contained collagenase (120 units/ml) and hyaluronidase (0.10%). Cells were counted in a hemocytometer. Viability, determined by supravital staining with toluidene blue, ranged from 75 to 90%. Only those cells that excluded dye were included in the cell counts for each incubation flask; final hepatocyte concentrations ranged from 1.5 to 5.0 × 10^5 cells/ml.

Incubation conditions

Incubations in 25-ml Erlenmeyer flasks were begun by the addition of 0.45–1.5 × 10^6 cells, in 0.5 ml of Ca^{2+}–Hanks buffer at pH 7.4 with 5 mM glucose, to 2.5 ml of the same solution, previously warmed to 37°C, which also contained sodium penicillin G (4 mg), streptomycin sulfate (0.1 mg), and labeled substrates as indicated. Flasks were incubated at 37°C in a shaking water bath at approximately 100 oscillations/min.

Solutions of albumin were heated to 50°C and added to the sodium salts of labeled and unlabeled palmitic acid to form the fatty acid–albumin complex. Albumin solutions were gently stirred until clear, and the pH was then adjusted to 7.4. A stock solution of labeled palmitate was prepared with a molar ratio (ν) of [^{14}C]palmitate to albumin of 1.9. The stock solution of unlabeled palmitate–albumin had a molar ratio of 4.1. The desired molar ratio of fatty acid to albumin in each incubation flask was obtained by combining the appropriate amounts of these two solutions with additional fatty acid-poor albumin. The specific activity was determined by measuring the radioactivity in an aliquot of the final mixture in which the fatty acid content had previously been calculated. Triglycerides were measured by an automated, fluorometric method (15).

Isolation of labeled lipids

At the end of the incubation period, flasks were placed on ice and their contents were transferred to 12-ml conical centrifuge tubes that contained an excess of unlabeled substrate. Tubes were centrifuged for 5 min at 3000 *g*. The supernates were removed and saved. After the cellular pellet was resuspended in 0.5 ml of water, 5 ml of isopropanol–heptane 3:1 was added and the tubes were vigorously shaken and allowed to stand for 1 hr (16). Heptane (3 ml) and water (2 ml) were added to form a two-phase system. The bottom phase was discarded and the upper (heptane) phase was washed twice with 50% ethanolic KOH (17). An aliquot of the washed upper phase was counted as described below.

Lipid classes were separated by thin-layer chromatography on silica gel G plates developed in heptane–diethyl ether–glacial acetic acid 70:20:1. An aliquot of the washed heptane phase was dried under air and dissolved in 2 drops of chloroform, which were applied to the silicic acid plate. This was repeated twice to achieve maximal recovery. Final recoveries ranged from 75 to 85%. All lipid classes were identified with the use of standards that were run simultaneously. Plates were sprayed with rhodamine 6G; fractions were scraped into a scintillation vial and counted in 10 ml of toluene scintillation fluid.

To determine the release of labeled esterified lipids from hepatocytes, the incubation medium was lyophilized and then extracted and washed with ethanolic KOH as described above. Fatty acids in the incubation medium were extracted according to the method of Dole (16). The release of water-soluble material was measured after protein in the incubation medium was precipitated by the addition of 2 ml of 30% perchloric acid and the pH was adjusted to 8.5 with 20% KOH. The precipitated protein and insoluble perchlorates were removed by centrifugation, and radioactivity was determined in an aliquot of the supernatant solution.

Collection of labeled CO_2

At the end of the incubation period, CO_2 was released from the medium and cells by the addition of 0.5 ml of 6 N H_2SO_4. The flasks were incubated for an additional 20 min at 37°C to collect CO_2. In experiments with labeled palmitate, $^{14}CO_2$ was collected in plastic wells that contained 0.2 ml of hydroxide of Hyamine. The wells were removed and radioactivity was counted as described below. In experiments with [^{14}C]acetate, $^{14}CO_2$ was collected in plastic wells that contained 0.2 ml of 6 N

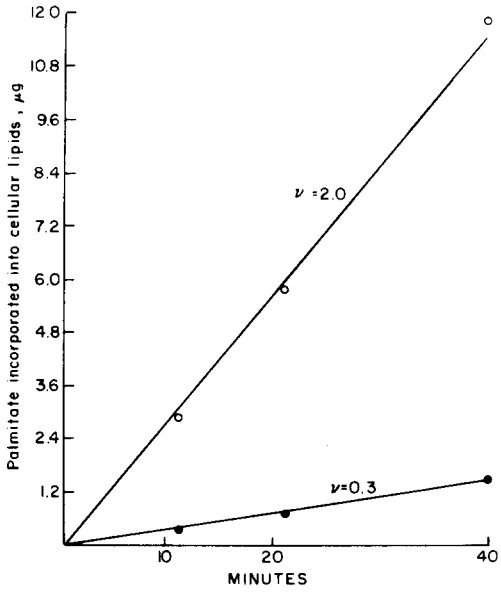

Fig. 1. Time course of palmitate incorporation into cellular lipids at two molar ratios (ν) of palmitate to albumin. Cells (1.7×10^5/ml) were incubated as described in Materials and Methods in 3 ml of Ca^{2+}–Hanks buffer that contained glucose (5 mM). Flasks with the indicated values of were prepared with the following additions: for $\nu = 0.3$, 8 μl of a stock solution ($\nu = 4.1$) containing 10.2 μg of unlabeled palmitate and 0.64 mg of albumin, 25 μl of a stock solution ($\nu = 1.9$) containing 13.0 μg of [^{14}C]palmitate and 1.9 mg of albumin, and 17.5 mg of albumin; for $\nu = 2.0$, 80 μl of a stock solution ($\nu = 4.1$) containing 102 μg of unlabeled palmitate and 6.4 mg of albumin, 100 μl of a stock solution ($\nu = 1.9$) containing 52 μg of [^{14}C]palmitate and 7.6 mg of albumin, and 6.0 mg of albumin. Thus, each flask contained 20 mg of albumin. Each point is the average of two duplicate flasks from a single experiment that is representative of three separate experiments.

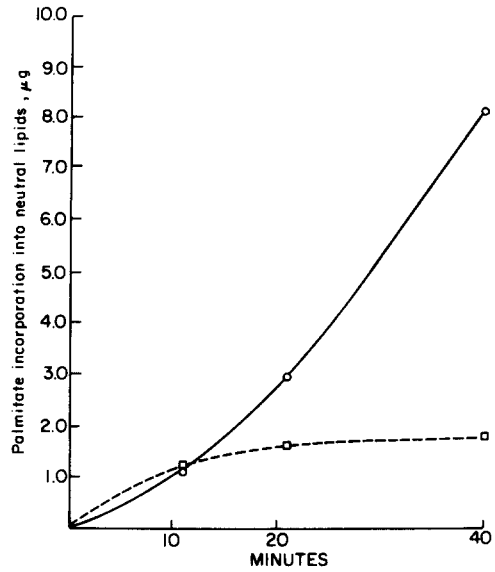

Fig. 2. Time course of palmitate incorporation into cellular diglycerides and triglycerides. Incubations were carried out at a molar ratio of 2.0 as described in the legend to Fig. 1. Lipid fractions were separated by thin-layer chromatography as indicated in Materials and Methods. Triglycerides, o——o; diglycerides, □- - -□.

NaOH. The NaOH was quantitatively transferred with a tuberculin syringe to a 12-ml conical centrifuge tube. Each well was also washed with an additional 0.2 ml of 6 N NaOH, which was added to the centrifuge tube. Labeled CO_2 was precipitated as $BaCO_3$ by the addition of 3 ml of 1 M $BaCl_2$. After centrifugation for 5 min at 3000 *g*, the supernatant solutions were discarded, and the precipitates were washed with 3 ml of 0.15 M sodium acetate. The tubes were recentrifuged as above and the supernates were discarded. Tubes were tightly closed with a rubber cap that held a center well containing hydroxide of Hyamine. After the removal of 5 cm^3 of air, 1 ml of 70% $HClO_4$ was injected, and the contents of each tube was gently agitated with a Vortex mixer at a low speed to avoid contamination of the Hyamine. After the tubes were incubated for 20 min at 37°C, the wells were removed, wiped, and placed into counting vials. Alternatively in later experiments, the method of Siperstein and Fagan (18) was used to collect $^{14}CO_2$ from [^{14}C]acetate oxidation. Both methods allow complete collection of $^{14}CO_2$ and provide zero-time flasks with approximately 1% of the radioactivity in incubated flasks.

TABLE 1. Distribution of [^{14}C]palmitate among lipid classes

Lipid Class	$\nu = 0.3$	$\nu = 2.0$[a]
Phospholipids	6 ± 1	2 ± 1
Diglycerides[b]	28 ± 1	18 ± 1
Triglycerides	60 ± 2	72 ± 2
Cholesterol esters	3 ± 1	3 ± 0

Lipid classes were separated by thin-layer chromatography as described in Materials and Methods. Each value is the average of duplicate determinations with the average error indicated. Similar data were obtained in three separate experiments.

[a] The percentages do not total 100 because a small amount of label was recovered in an unidentified component that chromatographed between cholesterol esters and triglyceride. It probably was an isopropyl ester formed during the extraction procedure.

[b] In the solvent system described in Materials and Methods, cholesterol was not completely separated from diglycerides. When this separation was achieved by development of plates in diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2 (20), less than 1% of the radioactivity was recovered in the cholesterol fraction.

Determination of radioactivity

Labeled lipids were transferred to counting vials in heptane, dried under air, and counted in 10 ml of toluene scintillation fluid containing 4 g of PPO and 100 mg of POPOP per liter. Labeled CO_2, collected in 0.2 ml of hydroxide of Hyamine, was transferred to counting vials containing 10 ml of Bray's scintillation fluid (19). Counts in $^{14}CO_2$ were corrected by use of an internal standard. Radioactivity in water-soluble material was determined by dissolving 0.3 ml of deproteinized supernate in Bray's scintillation fluid. Quenching was corrected with the use of an external standard. Discs impregnated with labeled protein were transferred to vials and counted in 10 ml of toluene scintillation fluid containing 4 g of PPO and 100

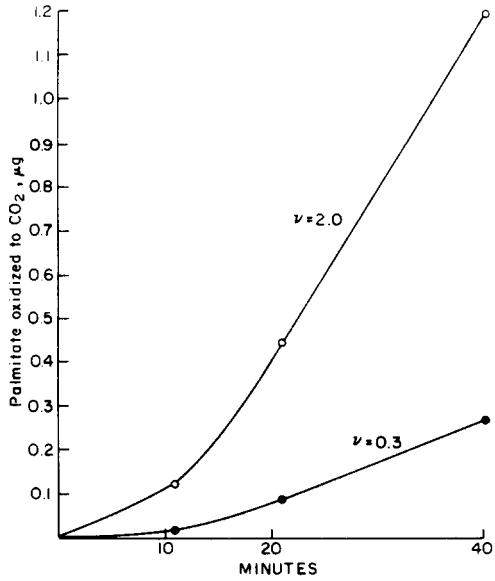

Fig. 3. Time course of palmitate oxidation to CO_2 at molar ratios of 0.3 and 2.0. Cell numbers and incubation conditions were identical with those given in the legend to Fig. 1. $^{14}CO_2$ was collected and radioactivity determined as described in Materials and Methods.

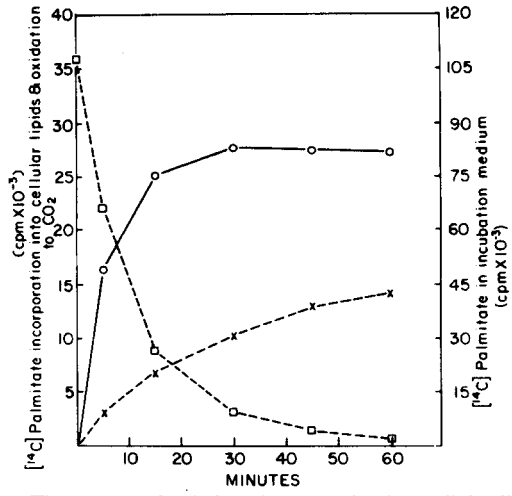

Fig. 4. Time course of palmitate incorporation into cellular lipids, oxidation to CO_2, and disappearance from the incubation medium under conditions when substrate is rate limiting. Cells (2.1 × 10^5/ml) were incubated in 3 ml of Ca^{2+}_i–Hanks buffer that contained 5 mM glucose, 2.6 μg of [^{14}C]palmitate (108,000 cpm), and 0.4 mg of albumin. Palmitate/albumin molar ratio (ν) equaled 1.9. ^{14}C-labeled cellular lipids, o—o; $^{14}CO_2$, ×----×; [^{14}C]palmitate in incubation medium, □---□.

mg of POPOP per liter. All counting was done in a Beckman model L2-50 liquid scintillation counter with an efficiency of 90% for ^{14}C.

RESULTS

Incorporation of [^{14}C]palmitate into cellular lipids

[^{14}C]Palmitate was incorporated into a variety of cellular lipids (Table 1). At both high and low values of ν, about 85% of the incorporated label was in glycerides, predominantly triglycerides. As ν was increased, relatively more label was recovered in the triglyceride fraction. Phospholipids and cholesterol esters each constituted a small fraction of the newly formed lipids, but some losses of phospholipids undoubtedly occurred in the extraction and washing procedures. In two separate experiments, extraction of the incubation medium revealed no release of labeled neutral lipids by the cells during the incubation period.

Palmitate was incorporated into total cellular lipids at a linear rate (Fig. 1). During longer incubations the time course was linear for at least 2 hr. In contrast, a nonlinear pattern of label accumulation was observed for individual lipids (Fig. 2). Incorporation into triglycerides accelerated with time, whereas diglyceride accumulation reached a plateau after 20 min. A similar time course of incorporation was observed with ν = 0.3. The increasing rate of triglyceride synthesis may reflect the continued esterification of labeled diglyceride precursors.

Fig. 1 also illustrates the direct relationship between the concentration of palmitate in the incubation medium and its incorporation into cellular lipids. Thus, when the molar ratio of fatty acid to albumin was raised from 0.3 to 2.0 (with albumin concentration constant), palmitate esterification increased eightfold.

With a fatty acid concentration of 51 μg/ml in the incubation medium (ν = 2.0), 500,000 cells incorporated 11.8 μg of palmitate into cellular lipids in 40 min (Fig. 1). The amount of fatty acid incorporated into triglycerides (8.1 μg) is about 6% of the initial triglyceride content (140 μg) of this number of cells, indicating the capacity of isolated liver cells to synthesize large amounts of neutral lipids from fatty acids.

Oxidation of [^{14}C]palmitate to CO_2

Comparison of Figs. 1 and 3 shows that the oxidation of palmitate to CO_2 occurred at a much slower rate than its esterification. In contrast to the linear rate of palmitate esterification, the rate of palmitate oxidation increased throughout the 40-min incubation period (Fig. 3). Acceleration, however, was greatest during the initial 20 min. When compared with the first 10 min of incubation, CO_2 production increased by 450% and 350% in the next 10 min at the low and high molar ratios (ν), respectively. In longer incubations, [^{14}C]palmitate was oxidized to CO_2 at a linear rate during the second and third hours. The dissociation between the rates of palmitate oxidation and esterification became most evident when a limiting amount of label was added to the incubation medium. As shown in Fig. 4, CO_2 production continued for 30 min after the

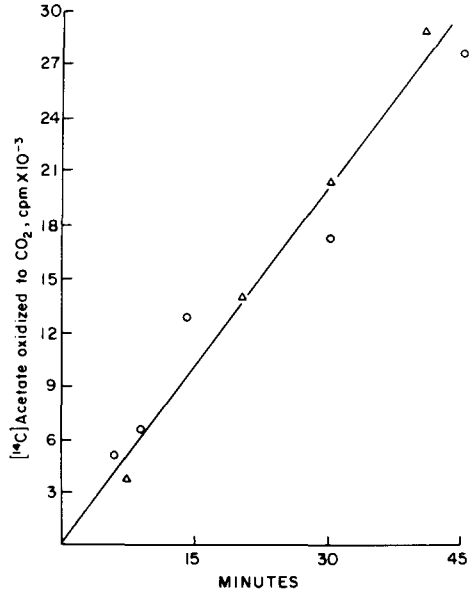

Fig. 5. Time course of [^{14}C]acetate oxidation to $^{14}CO_2$. Cells (5 × 10^5/ml) were incubated in 3 ml of Ca^{2+}–Hanks buffer that contained 5 mM glucose and [^{14}C]acetate (2.8 × 10^6 cpm). Each point is the average of duplicate flasks from two separate experiments. Values for the first experiment are depicted as circles, those from the second as triangles.

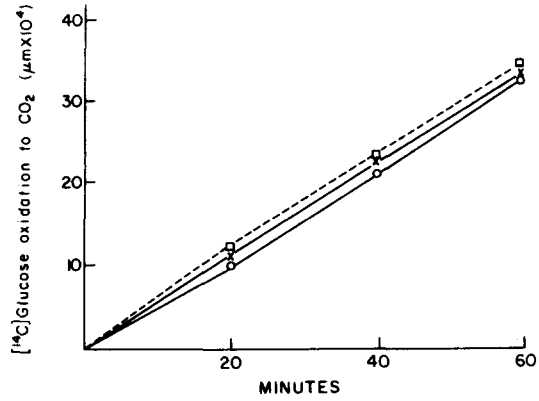

Fig. 6. Effect of fatty acid concentration on time course of [^{14}C]glucose oxidation to $^{14}CO_2$. Cells were incubated in 3 ml of Ca^{2+}–Hanks buffer that contained 20 mg of albumin with [^{14}C]glucose (0.1 mM) alone, ○——○; [^{14}C]glucose (0.1 mM) with 0.0256 mg of unlabeled palmitate, ×----×; or [^{14}C]glucose (0.1 mM) with 0.256 mg of unlabeled palmitate, □- - -□. Each point is the average of duplicate flasks.

amount of label in cellular lipids had reached a plateau. More significantly, CO_2 production at 30 min had increased by 240% over its level at 5 min, while during the same interval cellular labeled lipid had increased by only 70%. A similar lag in fatty acid oxidation to CO_2 was attributed by Sauer, Mahadevan, and Erfle (7) to a delay in the labeling of tricarboxylic acid cycle intermediates by [^{14}C]acetyl CoA. In order to evaluate this possibility, the oxidation rates of [^{14}C]acetate and [^{14}C]glucose to CO_2 were investigated. It is evident from Figs. 5 and 6 that both acetate and glucose were oxidized at a linear rate.

Temperature markedly altered palmitate oxidation to CO_2 compared with its esterification (Table 2). In experiments done prior to instituting the procedure of prewarming the incubation medium to 37°C before addition of the cells (see Materials and Methods), an even more rapid acceleration of CO_2 production occurred. It is therefore critical to control temperature in order to properly evaluate the early time course of palmitate oxidation. This temperature dependence may reflect the activation of some intermediate, possibly a rate-limiting step in the oxidation of long-chain fatty acid. In contrast, the reduction in palmitate esterification at the lower temperature is more compatible with the overall reduction in cellular metabolism that results from a decrease of 16°C in the temperature of the incubation medium. Oxidation of [^{14}C]acetate was also less sensitive than that of [^{14}C]palmitate; a similar reduction in the incubation temperature produced only a fourfold depression in CO_2 formation from acetate.

The relative contributions of oxidation and esterification to palmitate metabolism depended upon both the molar ratio of palmitate to albumin and the time of incubation. When $\nu = 0.3$, the ratio of palmitate esterification to palmitate oxidation to CO_2 decreased from 20 at 10 min to 5.4 at 40 min. With $\nu = 2.0$, these values were 24 and 10 at 20 and 40 min, respectively. Thus, acceleration in the rate of palmitate oxidation to CO_2 was evident at both molar ratios but was more pronounced at the lower value. This finding suggests a greater accumulation of products of incomplete oxidation of palmitate at the higher molar ratio. Support for this hypothesis was obtained by com-

TABLE 2. Effect of temperature on [^{14}C]palmitate oxidation to CO_2 and incorporation into cellular lipids

Incubation Time	Incorporation into Cellular Lipids			Oxidation to $^{14}CO_2$		
	Temperature		Relative Incorporation,	Temperature		Relative Oxidation,
	21°C	37°C	37°C/21°C	21°C	37°C	37°C/21°C
min	*cpm*			*cpm*		
20	7,111	23,015	3.2	183	4,547	24.8
40	13,211	38,335	2.9	574	13,699	23.9

Cells (4.0 × 10^5/ml) were incubated in 3 ml of Ca^{2+}–Hanks buffer with 5 mM glucose, 17.3 mg of albumin, 12 μg of [^{14}C]palmitate (5.4 × 10^5 cpm), and 11 μg of unlabeled palmitate at two different temperatures. Each value is the mean of duplicate flasks from two separate experiments.

parison of water-soluble products (of incomplete palmitate oxidation) accumulating in the incubation medium when different molar ratios of fatty acid to albumin were added. When $\nu = 0.3$, 0.16 μg of palmitate was converted to water-soluble products in 10 min; after 40 min of incubation the label in water-soluble material had increased approximately fivefold to 0.85 μg. However, when ν was raised to 2.0, the amount of palmitate metabolized to water-soluble material was 0.43 μg in 10 min and 6.5 μg in 40 min, a 15-fold increase. As with [^{14}C]palmitate oxidation to $^{14}CO_2$, it is apparent that ^{14}C-labeled water-soluble products also accumulated at an accelerating rate at both the low and high molar ratios of fatty acid to albumin.

Finally, the relative quantitative significance of each pathway can be judged by noting the fraction of the metabolized substrate converted to either cellular lipid, CO_2, or water-soluble products by the end of the incubation. At $\nu = 0.3$, 1.5 μg of palmitate, or 56% of the total metabolized, was present in cellular lipid, 0.3 μg (11%) had been oxidized to CO_2, and 0.9 μg (33%) was converted to water-soluble products. At $\nu = 2.0$, 11.8 μg of palmitate, or 61% of the total metabolized, was present in cellular lipid, 1.2 μg (6%) had been oxidized to CO_2, and 6.5 μg (33%) was converted to water-soluble products.

Effects of glucose concentration on the metabolism of [^{14}C]palmitate

To determine the effect of glucose metabolism on cellular utilization of fatty acids, hepatocytes were incubated with labeled palmitate and various concentrations of glucose. The presence of glucose, up to 10 mM, in the incubation medium did not alter the rate of palmitate esterification or oxidation. Conversely, the oxidation of glucose to CO_2 was unaffected by the addition of albumin (20 mg) or albumin plus high concentrations of unlabeled palmitate (314 meq/l) to the incubation medium (Fig. 6).

Effects of dibutyryl cyclic AMP on the metabolism of [^{14}C]palmitate and [^{14}C]acetate

As shown in Table 3, dibutyryl cyclic AMP reduced the conversion of labeled palmitate to CO_2 and enhanced the formation of water-soluble products from [^{14}C]palmitate, but did not affect the incorporation of palmitate into cellular lipids. Since the 2′-*O*-butyryl group of dibutyryl cyclic AMP can be cleaved in cell-free buffers (21) and after entry into cells (22, 23), it is possible that the observed inhibition of palmitate oxidation is not an effect of the cyclic nucleotide but results from the release of free butyrate and its subsequent metabolic effects. Two approaches were employed to determine whether the cleaved butyrate or the nucleotide itself was responsible for the inhibition of palmitate oxidation. First, similar concentrations of sodium butyrate and dibutyryl cyclic AMP were compared for their reduction of palmitate oxidation to CO_2 and stimulation of its conversion to water-soluble material. Similar experiments were carried out to determine the effects of sodium butyrate on the oxidation of [^{14}C]acetate to CO_2, which was previously shown to be exquisitely sensitive to inhibition by dibutyryl cyclic AMP. Secondly, other cyclic nucleotide preparations, including N^6-monobutyryl cyclic AMP and cyclic AMP, were tested to determine whether they produced effects similar to their dibutyryl counterpart. Since both of these compounds are more susceptible to phosphodiesterase attack and penetrate the hepatocyte less readily than the dibutyryl molecule (22, 24), it was expected that their effects might be proportionately less. However, if the actions of the three nucleotides were parallel, it would suggest that the observed results were produced by the cyclic AMP portion of the molecule rather than by released butyrate.

As shown in Table 4, dibutyryl cyclic AMP was less potent than sodium butyrate in reducing palmitate oxida-

TABLE 4. Comparison of the effects of dibutyryl 3′,5′-cyclic AMP and sodium butyrate on [^{14}C]palmitate oxidation to CO_2

	$^{14}CO_2$	Reduction
	cpm	%
Control	7930 ± 500	
Dibutyryl cyclic AMP (0.1 mM)	7693 ± 333	3
Dibutyryl cyclic AMP (1 mM)	5658 ± 90	29
Sodium butyrate (2 mM)	4960 ± 58	38
Sodium butyrate (1 mM)	5225 ± 225	34
Sodium butyrate (0.5 mM)	5520 ± 55	31
Sodium butyrate (0.1 mM)	6507 ± 100	18

Cells were incubated for 1 hr in 3 ml of Ca^{2+}–Hanks buffer containing 5 mM glucose, 30 mg of albumin, 40 μg of [^{14}C]palmitate (1.03×10^6 cpm), and 25 μg of unlabeled palmitate. Each value is the average of duplicate flasks with the average error indicated. Similar data were obtained in four separate experiments.

TABLE 3. Effects of dibutyryl 3′,5′-cyclic AMP on [^{14}C]palmitate metabolism

	Oxidation to $^{14}CO_2$			Conversion to ^{14}C-labeled Water-soluble Products			Incorporation into Cellular Lipids	
Time	Control	Dibutyryl Cyclic AMP	Inhibition	Control	Dibutyryl Cyclic AMP	Stimulation	Control	Dibutyryl Cyclic AMP
min	*cpm*	*cpm*	%	*cpm*	*cpm*	%	*cpm*	*cpm*
8	1,058 ± 0	967 ± 37	9	10,198 ± 1,193	18,542[a]	82	20,329 ± 433	21,462 ± 223
17	4,960 ± 192	3,947 ± 265	21	19,435 ± 1,997	35,036 ± 1,185	81	38,092 ± 30	38,372 ± 173

Cells (6.6×10^5/ml) were incubated in 3 ml of Ca^{2+}–Hanks solution containing 5 mM glucose, 1 mM dibutyryl cyclic AMP, 24 mg of albumin, 15.5 μg of unlabeled palmitate, and 20.8 μg of [^{14}C]palmitate (8.6×10^5 cpm). Each value is the average of duplicate flasks with the average error indicated. Similar data were obtained in six separate experiments.

[a] Not done in duplicate.

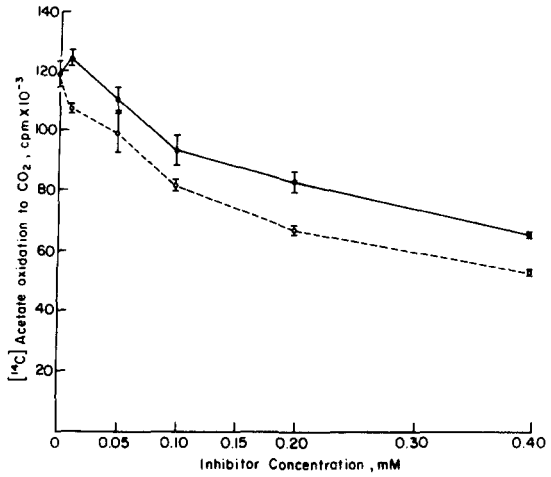

Fig. 7. Dose-response curves for the effects of dibutyryl cyclic AMP and sodium butyrate on the oxidation of [^{14}C]acetate to CO_2. Cells were incubated for 1 hr in 3 ml of Ca^{2+}–Hanks buffer that contained 5 mM glucose, [^{14}C]acetate (8.4 × 10^6 cpm), and unlabeled acetate (7.3 mM). Each point is the average of duplicate flasks; the range is represented by the solid bar. Similar data were obtained in four separate experiments. ●——●, dibutyryl cyclic AMP; ○——○, sodium butyrate.

tion to CO_2. Sodium butyrate produced a significant inhibition even at 0.1 mM, although dibutyryl cyclic AMP had no effect at this concentration. As shown in Table 5, N^6-monobutyryl cyclic AMP, cyclic AMP, and 5′-AMP each stimulated palmitate oxidation to CO_2. The data suggest that the effect observed after addition of dibutyryl cyclic AMP was not produced by the cyclic AMP portion of the molecule. It may have been secondary to the release of free butyrate and its subsequent metabolic effects. However, no firm conclusion can be drawn without the demonstration that this process actually occurs in isolated hepatocytes.

Fig. 7 demonstrates that the dose-response curves of dibutyryl cyclic AMP and sodium butyrate for inhibition of [^{14}C]acetate oxidation to CO_2 are quite similar. As with palmitate oxidation, the other nucleotides had divergent effects (Table 5). N^6-Monobutyryl cyclic AMP had no effect while both cyclic AMP and 5′-AMP stimulated acetate oxidation slightly. In other experiments, N^6-monobutyryl cyclic AMP failed to alter acetate oxidation even at higher concentrations. Similarly, stimulation by cyclic AMP and 5′-AMP did not increase at higher concentrations. Theophylline (1 mM) inhibited both palmitate and acetate oxidation consistently, but the effect was small.

In contrast to palmitate and acetate oxidation to CO_2, the stimulatory action of dibutyryl cyclic AMP on palmitate oxidation to water-soluble material was completely dissociated from the effects of sodium butyrate (Table 6). It was also significant that equal concentrations of N^6-monobutyryl cyclic AMP and cyclic AMP had similar effects, though smaller in magnitude, to those of the dibutyryl nucleotide. In three separate experiments, theophylline stimulated this pathway from 2 to 11%. Sodium butyrate and 5′-AMP either had no effect or were slightly inhibitory.

TABLE 5. Effects of other nucleotides and theophylline on the oxidation of [^{14}C]palmitate and [^{14}C]acetate to $^{14}CO_2$

Additions	Oxidation of [^{14}C]-Palmitate to $^{14}CO_2$		Oxidation of [^{14}C]-Acetate to $^{14}CO_2$	
	cpm	*% change*	*cpm*	*% change*
None	1,163 ± 60		70,410 ± 3960	
N^6-Monobutyryl cyclic AMP (1 mM)	1,730 ± 80	+49	69,330 ± 1080	−2
Cyclic AMP (1 mM)	1,625 ± 45	+40	90,563 ± 555	+29
5′-AMP (1 mM)	1,685 ± 10	+45	79,690 ± 1828	+13
Theophylline (1 mM)	983 ± 5	−16	52,543 ± 775	−25

Cells were incubated for 1 hr in 3 ml of Ca^{2+}–Hanks buffer containing 5 mM glucose and either [^{14}C]acetate (2.8 × 10^6 cpm) or 40 μg of [^{14}C]-palmitate (1.03 × 10^6 cpm), 125 μg of unlabeled palmitate, and 30 mg of albumin. Each value is the mean of duplicate flasks with the average error indicated. Similar data were obtained in four separate experiments.

In an attempt to determine whether sodium butyrate reduced [^{14}C]acetate oxidation principally through dilution of labeled substrate, a constant amount of sodium butyrate (0.4 mM) was added to incubation mixtures containing three different concentrations of [^{14}C]acetate. At the lowest concentration of [^{14}C]acetate (0.18 mM), sodium butyrate (0.4 mM) reduced substrate oxidation to $^{14}CO_2$ by 61%. When the concentration of [^{14}C]acetate was raised sixfold to 1.2 mM, [^{14}C]acetate oxidation to CO_2 was still reduced by 62%. Even when the concentration of substrate was increased approximately 60-fold to 10.2 mM, sodium butyrate (0.4 mM) still produced a 52% reduction in [^{14}C]acetate oxidation. If sodium butyrate acted primarily through dilution of the pool of labeled acetate, little or no reduction of acetate oxidation should occur at the higher concentrations of labeled substrate.

Finally, each of the three cyclic nucleotides consistently stimulated protein synthesis (3–17%), as measured by [^{14}C]leucine incorporation (25). This contrasts with an earlier report that showed significant inhibition of protein synthesis in liver homogenates by cyclic AMP (12).

DISCUSSION

The rapid oxidation and esterification of [^{14}C]palmitate by isolated liver cells reflects the important role of fatty acids in hepatic metabolism. Fatty acids serve as the major energy source for the liver (26). Moreover, oxidation of fatty acids stimulates gluconeogenesis and provides substrate for ketogenesis (27). Fatty acids are esterified to form phospholipids, an important structural component of both cellular membranes and serum lipoproteins, and triglycerides, which may be stored in the liver or secreted

TABLE 6. Effect of various nucleotides and sodium butyrate on the oxidation of [^{14}C]palmitate to ^{14}C-labeled water-soluble material

Additions	[^{14}C]Palmitate Oxidized to $^{14}CO_2$	Stimulation
	cpm	%
None	9,306 ± 305	
Cyclic AMP (1 mM)	10,602 ± 108	14
N^6-Monobutyryl cyclic AMP (1 mM)	13,986 ± 225	50
Dibutyryl cyclic AMP (0.1 mM)	15,315 ± 30	65
Dibutyryl cyclic AMP (1.0 mM)	16,062 ± 264	73
Sodium butyrate (0.1 mM)	9,507 ± 345	2
Sodium butyrate (1.0 mM)	9,600 ± 180	6
5′-AMP (1.0 mM)	6,105 ± 336	−34
Theophylline (1.0 mM)	9,510 ± 456	2

Cells were incubated for 1 hr in 3 ml of Ca^{2+}–Hanks buffer containing 5 mM glucose, 23 mg of albumin, 15 μg of [^{14}C]palmitate (4.2×10^5 cpm), and 12.5 μg of unlabeled palmitate. Each value is the average of duplicate flasks with the average error indicated. Similar data were obtained in three separate experiments.

into the blood as part of the very low density lipoproteins.

Although the overall utilization of palmitate in these experiments was directly proportional to the molar ratio of fatty acid to albumin (ν), the amount of palmitate metabolized by each pathway in the cell varied with the value of ν. As ν was raised, there was a relatively greater increase in esterification of fatty acids than in their oxidation to CO_2. However, the formation of other water-soluble products of palmitate oxidation increased in proportion to the rise in palmitate esterification at the higher molar ratio. In other studies with hepatocytes isolated by enzymatic methods, Ontko (4) found a distribution of label from [^{14}C]palmitate into cellular lipids, CO_2, and ketone bodies that is similar to the results shown in Table 3. He also reported that a larger proportion of the palmitate was converted into acetoacetate and β-hydroxybutyrate as the substrate concentration was raised. It has been proposed that an alteration in metabolic pathways may change the metabolism of palmitate at high substrate concentrations. Thus, tricarboxylic acid cycle activity slows as β oxidation accelerates (28). This may result from an increase in the ratio of NADH to NAD, with subsequent conversion of oxaloacetate to malate. However, Ontko (4) has shown that the reduction in Krebs cycle activity at high substrate concentrations was too small to account for the increase in ketone bodies. Instead, he attributed most of the increase in ketone body formation to enhanced β oxidation. Mayes and Felts (26) suggested that liver cells control fatty acid oxidation to maintain a constant level of energy production. Thus, when the substrate load was raised, hepatocytes shuttled a larger proportion of the fatty acid into incomplete oxidation products, mainly ketone bodies. In addition to these effects of substrate concentration, the products formed from labeled palmitate depend upon the type of liver preparation employed. For example, mechanically isolated hepatocytes (7) esterified fatty acids at a significantly lower rate than did our cells harvested by enzymatic techniques.

The glucose concentration of the incubation medium affected neither fatty acid oxidation nor esterification. This finding differs from the results in Ehrlich ascites tumor cells where addition of glucose to the incubation medium spared the oxidation of fatty acids but stimulated their esterification into cellular lipids (5). Moreover, isolated hepatocytes esterified a greater proportion of the fatty acids than did the tumor cells. One possible explanation for these differences is the availability of endogenous glycogen within the hepatocyte to provide the glucose required for synthesis of the glycerol portion of triglycerides and phospholipids. Alternatively, reutilization of glyceride glycerol released by turnover of intracellular triglycerides may serve as a source of glycerophosphate for synthesis of triglycerides and phospholipids by liver cells.

Fatty acid esterification was linear during 40 min of incubation if the fatty acid concentration in the incubation medium remained fairly constant. In contrast, palmitate oxidation to CO_2 accelerated throughout the entire incubation period when substrate levels remained constant. A similar early lag in palmitate oxidation was observed by Spector, Steinberg, and Tanaka (8) in tumor cells and by Sauer et al. (7) in hepatocytes isolated with a tissue press. The latter workers attributed the lag to a delay in the labeling of intermediates of the tricarboxylic acid cycle. However, the results of the present studies seem incompatible with this explanation because both [^{14}C]acetate and [^{14}C]glucose were oxidized to CO_2 at a linear rate. Moreover, although Sauer et al. (7) demonstrated that the level of radioactivity in tricarboxylic acid cycle intermediates reached a plateau after an initial lag period, this sequence may have resulted from a simultaneous depletion of the substrate pool. In contrast, our results demonstrate that the incomplete products of palmitate oxidation (water-soluble material) also accumulate at an accelerating rate. Fatty acid oxidation was so rapid in their experiments that the concentration of radioactive palmitate had fallen considerably by 15 min and all of the substrate had been utilized by 30 min.

Several alternate explanations can be suggested for the lag in palmitate oxidation to CO_2. First, exogenous fatty acids may be rapidly esterified to form an intracellular pool from which fatty acyl residues are later cleaved to enter the mitochondria. Alternatively, both an intracellular pool of labeled esterified lipids and labeled palmitate from the incubation medium may provide residues for oxidation. To account for the accelerating production of CO_2 from palmitate, radioactivity in the intermediate pool should increase with time but at a decelerating rate. The diglyceride pool meets this requirement because the rate of increase of label in this lipid class declines with time. In contrast, triglyceride radioactivity increased throughout

the incubation period. Further support for an intermediate pool is provided by the continued production of labeled CO_2 in the absence of any further lipid synthesis under conditions when [^{14}C]palmitate was present in limiting amounts. However, the possibility exists that a pool of intracellular [^{14}C]palmitate that had been either compartmentalized or partially metabolized and was no longer available for esterification was still providing substrate for $^{14}CO_2$ production.

Endocrine factors play a major role in the control of hepatic lipid metabolism. Several lines of evidence indicate that glucagon and epinephrine, which raise cyclic AMP levels in the liver, stimulate ketogenesis and gluconeogenesis (3, 29, 30). In vivo studies are complicated by the fact that concomitant stimulation of adipose tissue lipase by these hormones also increases the amount of fatty acid transported to the liver (31, 32). Recently, Heimberg, Weinstein, and Kohout (3) showed that glucagon and dibutyryl cyclic AMP stimulate ketone body formation in the perfused liver, although the effect was diminished at relatively high levels of added free fatty acids. However, other workers (30) have concluded that direct stimulation of hepatic ketogenesis by glucagon is of little quantitative significance. Although it is difficult to determine the relative effects of hormones and fatty acid concentrations on hepatic ketogenesis, the present studies suggest that each plays an important role. When the molar ratio of fatty acid to albumin was raised, palmitate esterification and partial oxidation to water-soluble material rose out of proportion to the increase in CO_2 production. Dibutyryl cyclic AMP also produced profound effects on fatty acid metabolism. Earlier studies demonstrated that dibutyryl cyclic AMP and cyclic AMP inhibited hepatic incorporation of [^{14}C]acetate into both fatty acids and cholesterol (11–13). The present report indicates that dibutyryl cyclic AMP, N^6-monobutyryl cyclic AMP, and cyclic AMP all enhance the oxidation of palmitate to water-soluble products. Most effective is the dibutyryl derivative, which apparently diffuses across liver cell membranes most readily and is least susceptible to enzymatic degradation (22–24). Furthermore, neither sodium butyrate nor 5′-AMP, degradation products of dibutyryl cyclic AMP, significantly affected palmitate conversion to water-soluble material. Both of these results suggest that the observed stimulation reflects an action of the cyclic nucleotide. Approximately 60–70% of the water-soluble material is composed of ketone bodies, and preliminary experiments indicate that both dibutyryl and N^6-monobutyryl cyclic AMP enhance ketone body formation from palmitate by 50–100%. [1]

Dibutyryl cyclic AMP also significantly reduced palmitate and acetate oxidation to CO_2. However, the physiological significance of these findings is obscured by the fact that sodium butyrate produced similar effects. The results also indicate that butyrate does not exert its inhibitory effect solely through dilution of the pool of labeled substrate. Instead, it appears that butyrate has a direct inhibitory effect on acetate oxidation. Although competition with acetate for activation by a specific thiokinase might explain the inhibition by butyrate, this mechanism probably could not account for the significant inhibition of palmitate oxidation. These results emphasize the need to control any experiment involving the dibutyryl derivative of cyclic AMP with equivalent concentrations of sodium butyrate (33).

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