BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 307–312 (1996)
ARTICLE NO. 1171

Acyl CoA and Lipid Synthesis from Ketone Bodies by the Extramitochondrial Fraction of Hepatoma Tissue

Leslie Hildebrandt,[1] Terry Spennetta, Ronald Ackerman, Charles Elson, and Earl Shrago[2]

Departments of Medicine and Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

Received June 24, 1996

The compartmentation of the pathway for the synthesis of lipids from ketone bodies was examined in a cell free, post-mitochondrial supernatant fraction of malignant Morris hepatoma 7777 tissue. A fortified supernatant system effectively incorporated radioactive D(−)-3-hydroxybutyrate and acetoacetate into cholesterol and fatty acids, and both ketone bodies were directly converted to their CoA thioesters. Furthermore, a microsome-free (100,000 ×g) cytosolic fraction was also able to acylate 3-hydroxybutyrate to 3-hydroxybutyryl CoA. No previous identification of this enzyme activity has been described. These results which characterize a distinct extramitochondrial pathway for conversion of 3-hydroxybutyrate as well as acetoacetate into lipids also suggest the possibility of a previously undetected enzymatic activity for utilization of this ketone body.

Ketone bodies, important energy yielding substrates, are also preferred precursors for lipid synthesis by lactating mammary gland (1) developing brain (2) and intact hepatoma cells (3,4). The conversion of acetoacetate into lipids can presumably take place in the extramitochondrial compartment of the cell due to the presence of a cytosolic acetoacetyl CoA synthetase (5–7). This enzyme, however, does not utilize D(−)3-hydroxybutyrate as a substrate (6). The initial enzymatic reactions required for the conversion of 3-hydroxybutyrate into cholesterol and fatty acids reportedly occurs in the mitochondrial compartment beginning with its oxidation to acetoacetate by 3-hydroxybutyrate dehydrogenase, and entrance into the TCA cycle via succinyl CoA: acetoacetyl CoA transferase (1). Subsequently, citrate would exit the mitochondria as the key intermediate in the cytosolic formation of acetyl CoA necessary for both cholesterol and fatty acid synthesis (8).

Recently, we observed, (4) as expected, that in isolated intact malignant hepatoma cells, inhibition of citrate conversion to acetyl CoA in the cytosol by (−)-hydroxy citrate severely impaired lipid synthesis from pyruvate (8). However, there was little or no effect of (−)-hydroxycitrate on lipid synthesis with ketone bodies as substrates. Moreover, the results implied that like acetoacetate, 3-hydroxybutyrate might be directly acetylated in the cytosol. In view of these somewhat anomalous findings in the intact cell, it seemed important to compare and document the synthesis of cholesterol and fatty acids as well as formation their acyl CoA esters from acetoacetate and 3-hydroxybutyrate in a cell free, post-mitochondrial supernatant fraction. The results described in this communication identify a complete extramitochondrial pathway for the synthesis of lipids from 3-hydroxybutyrate as well as acetoacetate, and provide evidence for the direct acylation of 3-hydroxybutyrate to 3-hydroxybutyrl CoA.

MATERIALS AND METHODS

Animals and chemicals. Buffalo rats were purchased from Harlan Sprague Dawley Co., Indianapolis, IN. Hepatoma 7777 tumors were originally provided by Dr. H. Pitot, McArdle Laboratory, University of Wisconsin, Madison, WI

[1] Present address: Department of Health, Physical Education, Exercise Science and Nutrition, Northern Arizona University, Flagstaff, AZ, 86011-5095.

[2] To whom correspondence should be addressed. Fax: (608) 262-5860.

0006-291X/96 $18.00

TABLE 1
Incorporation of Radioactive D(−)-3-Hydroxybutyrate, Acetoacetate and Citrate into Cholesterol and Fatty Acids by a Post-mitochondrial Supernatant Fraction from Hepatoma Tissue Fortified with Co-factors[a,b]

	Cholesterol	Fatty Acids
Substrate	nmol/mg protein	
[3-^{14}C]3-hydroxybutyrate	137 ± 5.2	153 ± 19.8
[3-^{14}C]acetoacetate	177 ± 5.2	281 ± 36.9
[1,5^{14}C]citrate	99 ± 2.1	205 ± 36.8

[a] Incubations were carried out for 60 min under conditions described under Materials and Methods.

[b] The results are the average of incubations from 3 animals run in triplicate ± S.D.

and maintained in the hind limb of the rats as previously described (4,9). Radioisotopes were purchased from Amersham Co., Arlington Heights, IL. All other reagents were of the highest grade commercially available.

Preparation of cell-free fractions. Homogenates of hepatomas, host livers and control (non-host) livers were prepared as previously described by Azrolan and Coleman (8). Thirty percent homogenates were made up in 110 mM mannitol, 70 mM sucrose, 100 mM sodium phosphate, 60 mM nicotinamide, 10 mM $MgCl_2$, 5 μg leupeptin/ml and 5 μg pepstatin/ml, pH 7.5. The homogenates were centrifuged at 12,000 ×g for 15 min 4°C to obtain a post mitochondrial supernatant fraction. This fraction was determined to be free of citrate synthetase activity (10) which is a good marker for mitochondrial integrity. Cytosolic fractions were obtained by first preparing a 10% tissue homogenate in the same buffer and centrifuging 100,000 ×g for 1 hour.

Measurement of lipid synthesis and determination of Acyl CoA esters. Three ml of the post mitochondrial or cytosol supernatant fraction was pipetted into a 25 ml flask and supplemented with the following substrates and cofactors (8): 3 mM D(−)-3-hydroxybutyrate, acetoacetate or citrate, 3 μM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 3 μM ATP, 0.25 μM CoA, 1 μM $NADP^+$, 1 μM NAD^+ and 0.5 μM GSH. The flasks were stoppered and for lipid synthesis preincubated for 5 min at 37°C in a metabolic shaker (100 strokes/min) under an atmosphere of 95% oxygen and 5% carbon dioxide. One μCi of D(−)-3-hydroxy[3-^{14}C] butyrate, [3-^{14}C] acetoacetate or [1-5 ^{14}C] citrate was then added and the reaction continued for 60 min. The incorporation of the radiolabel into cholesterol and fatty acids was determined as previously described (4,9). Experimental samples and heat killed blanks were prepared in triplicate. Estimates of the molar incorporation of each substrate in the lipid fractions were determined using the initial specific activities of the substrates in the media. For acyl CoA synthesis, post mitochondrial and cytosolic fractions (3.0 ml) were supplemented with cofactors as above. The reactions were started with the addition of 3.0 mM substrate (acetoacetate, 3-hydroxybutyrate or citrate) and the reactions terminated at timed intervals with the addition of chloroform. The concentration of acyl CoA esters were determined as previously described (4,11).

Determination of protein and statistical analysis. Protein determinations were carried out by the Hartree modification of the Lowry method using bovine serum albumin as a standard (12). Results are expressed as nmol of substrate incorporated into lipid fractions or converted to acyl CoA esters/mg protein. Data were assessed by two way analysis of variance (ANOVA) using SAS general linear models program (13).

RESULTS

The post-mitochondrial supernatant fraction prepared from the hepatomas catalyzed an active conversion of radioactive ketone bodies into cholesterol and fatty acid (Table 1). The supernatant was fortified with substrates and co-factors (8) which permitted some comparison of the results with those obtained in freshly isolated cells (4). Whereas, there were some quantitative differences, both 3-hydroxybutyrate and acetoacetate were similar in precursor efficiency to that of the more common substrates such as citrate, lactate and pyruvate (4). The key finding was that without intervention of mitochondrial enzymes, 3-hydroxybutyrate as well as acetoacetate was an effective substrate for lipid synthesis.

TABLE 2
Comparison of Acyl CoA Synthesis from D(−)-3-Hydroxybutyrate, Acetoacetate and Citrate by Post-mitochondrial Supernatant Fractions from Rat Hepatoma, Host and Control Liver Fortified with Co-factors[a,b]

	Acyl CoA nmol/mg/protein		
Substrate	Hepatoma	Host liver	Control liver
3-Hydroxybutyrate	117.31 ± 5.87	52.75 ± 3.16	14.83 ± 1.10
Acetoacetate	21.37 ± 0.92	35.06 ± 2.91	12.33 ± 0.92
Citrate	14.45 ± 1.59	28.03 ± 3.08	23.37 ± 3.50

[a] Incubations were carried out for 10 min under conditions described under Materials and Methods.
[b] The results are the average ± S.D. from 5 samples.

The direct formation of acyl CoA thioesters during a short incubation of the fortified post mitochondrial supernatant is compared in fractions prepared from hepatomas, host and control livers. The data in Table 2 reflect the sum of acetoacetyl CoA, 3-hydroxybutyryl CoA and acetyl CoA. Under the experimental conditions tested, acetoacetate was similar to citrate as a substrate for acyl CoA synthesis by fractions prepared from hepatomas and livers. The synthesis of acyl CoA esters from 3-hydroxybutyrate was considerably greater in the hepatoma than liver supernatants, and 3-hydroxybutyrate was a much better precursor of acyl CoA esters than either acetoacetate or citrate in the hepatoma fraction.

The synthesis of individual short chain acyl CoA esters by a post mitochondrial fraction of hepatoma tissue is shown in Figure 1. Acetoacetyl CoA is the major ester formed from either 3-hydroxybutyrate or acetoacetate. However, 3-hydroxybutyrate leads to a greater formation of 3-hydroxybutyryl CoA and acetoacetyl CoA than does acetoacetate. The results are consistent with the fact that interconversion of the acyl CoA esters can take place via the cytosolic enzyme, acetoacetyl CoA reductase (14). Citrate can be a precursor of these CoA esters through the combined activity of ATP-citrate lyase, acetoacetyl CoA lyase and acetoacetyl

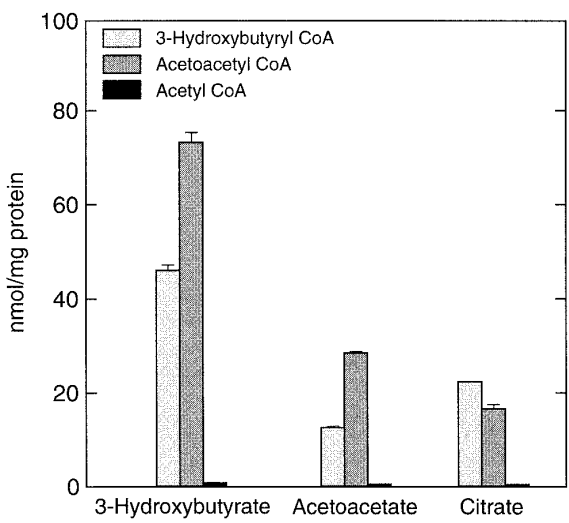

FIG. 1. 3-Hydroxybutyryl CoA, acetoacetyl CoA and acetyl CoA synthesis by post-mitochondrial supernatant fractions prepared from hepatoma tissue, fortified with co-factors and incubated with 3 mM D(−)-3-hydroxybutyrate, acetoacetate or citrate for 10 min. Results are the average ± SD of 3 animals run in triplicate.

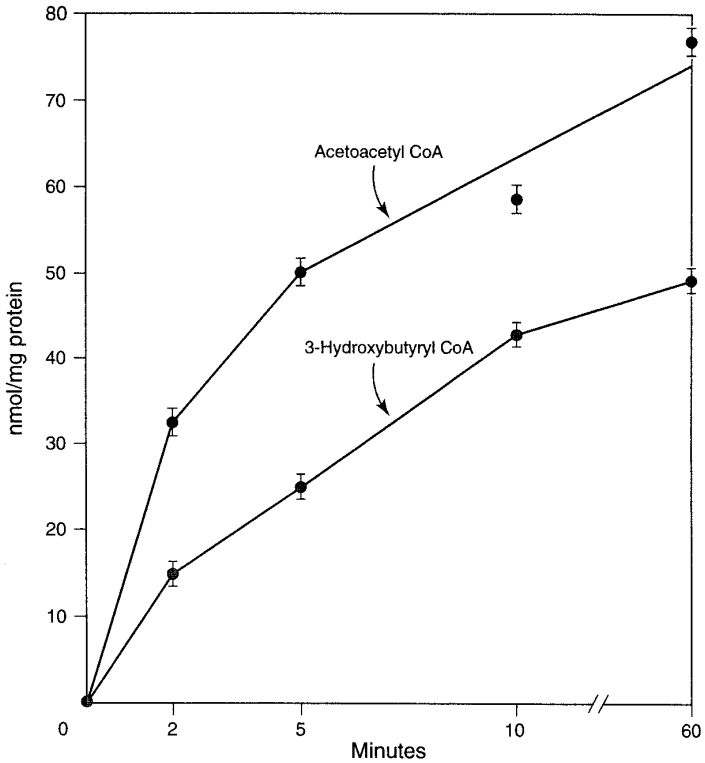

FIG. 2. Time curve of substrate incorporation into 3-hydroxybutyryl CoA and acetoacetyl CoA by post-mitochondrial supernatant fractions prepared from hepatoma tissue and fortified with co-factors. Extracts were incubated with 3 mM D(−)-3-hydroxybutyrate. Results are the average ± SD of 3 animals run in triplicate.

CoA reductase (1). Very little accumulation of acetyl CoA was detected with any of the substrates including citrate.

The time dependent synthesis of 3-hydroxybutyryl CoA and acetoacetyl CoA from 3-hydroxybutyrate by a post-mitochondrial supernatant fraction of hepatoma tissue is shown in Figure 2. There were no acyl CoA esters detected at zero time, thus indicating de novo synthesis. Under the experimental conditions, both 3-hydroxybutyryl CoA and acetoacetyl CoA accumulate linearly for the first few minutes. 3-hydroxybutyryl CoA continues to gradually increase over time, whereas there is a slight decrease in acetoacetyl CoA. As stated, the greater amount of the acetoacetyl CoA (also seen in Figure 1) is likely due to its continual formation from 3-hydroxybutyryl CoA via acetoacetyl CoA reductase (14).

The ability of a cytosol fraction (100,000 ×g) to directly catalyze the synthesis of 3-hydroxybutyryl CoA from 3-hydroxybutyrate is shown in Figure 3. The cytosol prepared from hepatomas in which the microsomes as well as the mitochondria were removed contained an enzymatic activity which synthesized significantly more 3-hydroxybutyryl CoA than did a similar preparation from host liver.

DISCUSSION

The present results, obtained in a cell free system from hepatoma tissue, depicts an extra-mitochondrial pathway for acyl CoA and lipid synthesis from 3-hydroxybutyrate as well as from acetoacetate. They confirm and extend previous findings using intact isolated hepatoma cells where acetoacetate and 3-hydroxybutyrate were observed to be much better precursors

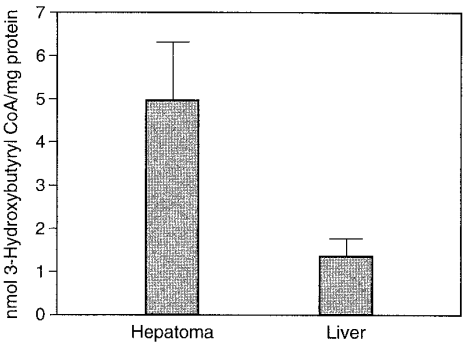

FIG. 3. 3-hydroxybutyryl CoA formation by cytosol fractions prepared from hepatoma (n=5) and host liver (n=7). Fractions fortified with co-factors were incubated for 5 min with 3 mM D(−)-3-hydroxybutyrate. Results represent the average value ± SD.

of cholesterol and fatty acids than other carbon substrates (4). Furthermore, in the previous study, it was shown that inhibition of citrate conversion to acetyl CoA was ineffective in preventing lipid synthesis with either ketone body as a substrate, indicating mitochondrial metabolism via the TCA cycle was not absolutely required. Whereas, the citrate pathway is common to all tissues including tumors which actively synthesize lipids (15), it does not appear to be as important in hepatomas as is the direct utilization of ketone bodies, at least for cholesterol synthesis (4). It is important to appreciate that circulating ketone bodies are elevated in tumor bearing animals (16) and are actively taken up by the tumor tissue (17). Recently, it was reported that acetoacetate was a good substrate for lipid synthesis in hepatoma A5-30D cells (3).

The potential for acetoacetate channeling directly into acetoacetyl CoA and hydroxymethylglutaryl CoA before equilibration with acetyl CoA has been documented in adult liver (5,18), lactating mammary gland (1,19), and developing brain (2). In ruminants the majority of butyrate formed in the rumen is converted to 3-hydroxybutyrate before its appearance in the circulation and further utilization by the lactating mammary gland (20). Hepatomas, and likely other tumors, can now be included in this group of tissues which actively utilize ketone bodies for lipid synthesis.

In liver and mammary gland, a well characterized cytosolic acetoacetyl CoA synthetase is considered to play an important role in the direct synthesis of lipids from acetoacetate (5–7,19). In mammary gland cytosol, a direct reversal of β oxidation for the synthesis of butyryl CoA has been demonstrated (19). The $NADP^+$-linked acetoacetyl CoA reductase enzyme would permit the interconversion of acetoacetyl CoA and 3-hydroxybutyryl CoA (14). Based on previous (4) and present results, the major problem which needs to be addressed is a mechanism to account for the extramitochondrial formation of 3-hydroxybutyryl CoA with 3-hydroxybutyrate as the substrate. Whereas, most reports concentrate on acetoacetate metabolism, we have shown that in isolated hepatoma cells the majority of the 3-hydroxybutyrate converted to lipids appears to take place in the extramitochondrial compartment (4). It has been reported that in liver D-(−) 3-hydroxybutyrate is not a substrate for cytosolic acetoacetyl CoA synthetase (6). An alternative explanation which could not be ruled out using intact cells (4) was that 3-hydroxybutyrate entered the mitochondria, was oxidized to acetoacetate by 3-hydroxybutyrate dehydrogenase and was transported out again for further metabolism by cytosolic acetoacetyl CoA synthetase. This possibility was ruled out in the present study in which it is shown that mitochondria are not required for synthesis of acyl CoA esters and lipids from either ketone body. That contamination of the supernatant by mitochondrial enzymes was

not a factor is noted by the absence of citrate synthetase activity. Although no definitive enzymatic activity has been previously described, one brief report (21) included information on the incorporation of radioactive 3-hydroxybutyrate into fatty acids by a high speed supernatant fraction from rat liver. Taking all factors into consideration, and based particularly on results obtained from this investigation, there does seem to be experimental evidence for a distinct enzyme(s) to directly acylate 3-hydroxybutyrate in the cytosol. The enzymatic acylation of 3-hydroxybutyrate was found to be higher in hepatoma than liver tissue which is consistent with the much greater conversion of 3-hydroxybutyrate to cholesterol and fatty acids in hepatomas than in host or control livers (4). By contrast, acetoacetate serves equally well as substrate in hepatoma and liver. Additional experiments are required to better characterize the proposed enzymatic activity in animal tissues (20) as well as in bacteria which synthesize a polyhydroxybutyrate polymer (22).

ACKNOWLEDGMENT

Research supported by the College of Agriculture and Life Sciences, University of Wisconsin, Madison, Wisconsin.

REFERENCES

1. Robinson, A. M., and Williamson, D. H. (1980) *Physiol Rev.* **60,** 143–187.
2. Webber, R. J., and Edmond, J. (1979) *J. Biol. Chem.* **254,** 3912–3920.
3. Briscoe, D. A., Fiskum, G., Holleran, A. L., and Kelleher, J. K. (1994) *Mol. Cell. Biochem.* **136,** 131–137.
4. Hildebrandt, L. A., Spennetta, T., Elson, C., and Shrago, E. (1995) *Am. J. Physiol.* **269,** C22–C27.
5. Bergstrom, J. D., Wong, G. A., Edwards, P. A., and Edmond, J. (1984) *J. Biol. Chem.* **259,** 14548–14553.
6. Ito, M., Fukui, T., Kamakari, M., Saito, T., and Tomita, K. (1984) *Biochim. Biophys. Acta* **794,** 183–193.
7. Miziorko, H. M., Laib, F. E., and Behnke, L. F. (1990) *J. Biol. Chem.* **265,** 9606–9609.
8. Parlo, R. A., and Coleman, P. S. (1984) *J. Biol. Chem.* **259,** 9997–10003.
9. Mares-Perlman, J. A., and Shrago, E. (1988) *Cancer Res.* **48,** 602–608.
10. Bergmeyer, H. V. (1974) Methods of Enzymatic Analysis, Vol. 1, pp. 443–444, Academic Press, New York.
11. Woldegiorgis, G., Spennetta, G. T., Corkey, B. E., Williamson, J. R., and Shrago, E. (1985) *Anal. Biochem.* **150,** 8–12.
12. Hartree, E. F. (1972) *Anal. Biochem.* **48,** 422–427.
13. Sokal, R. R., and Rohif, F. S. (1981) Biometry, Fireman, New York.
14. Wakil, S. J., and Bressler, R. (1962) *J. Biol. Chem.* **237,** 687–693.
15. Colman, P. S. (1986) *Ann. N.Y. Acad. Sci.* **488,** 451–467.
16. Mares-Perlman, J. A., Francis, A. A. M., and Shrago, E. (1988) *Am. J. Clin. Nutr.* **48,** 50–56.
17. Sauer, L. A., and Dauchy, R. T. (1983) *Cancer Res.* **43,** 3497–3503.
18. Geelen, M. J. H., Lopes-Cardozo, M., and Edmond, J. (1983) *FEBS Lett.* **163,** 269–273.
19. Nandedkar, A. K. N., and Kumar, S. (1969) *Arch. Biochem. Biophys.* **134,** 563–571.
20. Bergman, E. N. (1990) *Physiol. Rev.* **70,** 567–590.
21. Rous, S. (1976) *Life Sci.* **18,** 633–638.
22. Steinbuchel, A., and Schlegel, H. G. (1991) *Mol. Microbiol.* **5,** 535–542.