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Ketone-body metabolism in glioma and neuroblastoma cells

[ketone bodies/3-ketoacid CoA-transferase (3-oxoacid CoA-transferase)/glioma C6 cells/neuroblastoma C1300 cells]

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ABSTRACT **We have examined the metabolism of ketone bodies in neuroblastoma C1300 and glioma C6 cells, two established lines of neural origin. The three ketone body-metabolizing enzymes are present in cells of both lines in the relative proportions normally found in brain (D-3-hydroxybutyrate dehydrogenase < acetoacetyl-CoA thiolase < 3-ketoacid CoA-transferase), the activities of the first two are higher in glioma cells than in neuroblastoma, and that of the third is 2-fold higher in neuroblastoma cells than in glioma cells. The specific activity of 3-ketoacid CoA-transferase (EC 2.8.3.5) in both cell lines increased as the cultures achieved confluence, then decreased. Ketone bodies and especially acetoacetate are preferred substrates for synthesis of neural lipids in cells of both lines. The incorporation of glucose carbon into lipids is significantly reduced in cells of both lines in the presence of ketone bodies. Addition of acetoacetate but not DL-3-hydroxybutyrate to the culture medium resulted in a significant increase in the activity of 3-ketoacid CoA-transferase and also in the rate of acetoacetate oxidation in neuroblastoma cells but not glioma cells. These findings indicate that specific differences exist in the capacity of these two cell lines to metabolize ketone bodies and also that substrate-level regulation of the ketone body-metabolizing pathway exists. These two lines therefore provide a potentially useful system in which the mechanisms of regulation of these enzymes may be examined.**

The importance of acetoacetate (AcAcO) and D-3-hydroxybutyrate (D-3-HB) as fuels for cerebral metabolism and as precursors for lipid synthesis in developing rat brain has been well documented (for review see refs. 1 and 2). Although the metabolism of these ketone bodies by *in vivo* and *in vitro* brain preparations has been investigated, very little is known about their metabolism by neuronal and glial cells (3–5). Because pure preparations of glial and neuronal cells are difficult to obtain and to maintain in culture, established lines of glial and neuronal origin have been widely used as representatives of these cell types (6). Even after transformation, these clonal cell lines retain many of their characteristic metabolic pathways (7, 8).

The present study was, therefore, initiated to investigate possible differences in the metabolism of ketone bodies between two established clonal cell lines, namely glioma C6 and neuroblastoma C1300 (N2a). The results show that there are specific differences between these two cell lines of glial and neuronal origin with respect to their capacities to metabolize ketone bodies and in their regulation by exposure to ketone bodies.

MATERIALS AND METHODS

Cell Culture. Rat glioma C6 and mouse neuroblastoma C1300 (N2a) clones were maintained in Eagle's minimal essential medium containing 4-fold increased concentrations of amino acids and vitamins, and supplemented with 10% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml. The medium also contained 5 mM glucose and 2 mM L-glutamine. The glioma C6 and neuroblastoma C1300 (N2a) cells were plated at 1×10^5 and 1.5×10^5 cells per dish, respectively, in 60-mm-diameter tissue culture dishes in 5 ml of medium, and were grown in a water-jacketed CO_2/air incubator at 37°C. Cells were routinely subcultured with 0.5% trypsin (Difco, 1:250) in phosphate-buffered saline containing 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) at 37°C. For assay of enzyme activities and protein content, the cells were scraped with a rubber policeman in cold buffered sucrose (0.25 M sucrose/10 mM Tris·HCl, pH 7.5/1 mM 2-mercaptoethanol), centrifuged, and resuspended in buffered sucrose.

Enzyme Assays. Cultures were treated with Triton X-100 (final concentration 0.5%), and the activities of acetoacetyl-CoA thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9) (9, 10), and 3-ketoacid CoA-transferase (CoA-transferase; 3-oxoacid CoA transferase, EC 2.8.3.5) (9), in the extracts were measured spectrophotometrically. To measure the activity of D-3-HB dehydrogenase (EC 1.1.1.30) the cell suspension was sonicated for two 15-sec periods, and the enzyme activity was measured spectrophotometrically in a final volume of 0.5 ml (11). A unit of enzyme activity is defined as 1 μmol of substrate utilized or product formed per min at 37°C. Protein was measured by the method of Lowry *et al.* (12) with bovine serum albumin as standard.

Lipid Synthesis. Cells were grown in complete medium supplemented with either AcAcO or DL-3-hydroxybutyrate (DL-3-HB) (where indicated) in 60-mm-diameter tissue culture dishes for the first two consecutive subcultures. The cells were then subcultured (0.9×10^5 cells in 3 ml of the respective media) into sterile 25-ml Erlenmeyer flasks. On the fourth day after subculture, the medium in the flask was removed by aspiration, and 3 ml of complete medium containing one of the three radiolabeled substrates (glucose, AcAcO, or D-3-HB and other additions as indicated) was added. The flask was then sealed with a rubber serum stopper equipped with a hanging polyethylene center well and incubated at 37°C for 6 hr. At the end of the incubation period, 0.5 ml of 36% $HClO_4$ was injected into the flask and 0.3 ml of Hyamine-10X hydroxide was injected into the center well, $^{14}CO_2$ was trapped for 45 min, and radioactivity was determined (13). The medium in the flask was decanted, and the flask was rinsed once with buffered sucrose and the cells were scraped and suspended in 1 ml of buffered sucrose. An aliquot of this suspension was removed for protein determination. From the remainder, the nonsaponifiable lipids and fatty acid fractions were extracted and washed (14), and the

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Abbreviations: CoA-transferase, 3-ketoacid CoA-transferase; AcAcO, acetoacetate; DL-3-HB, DL-3-hydroxybutyrate; D-3-HB, D-3-hydroxybutyrate.

radioactivity was determined. The extracted lipid fractions were free of any significant contamination by radiolabeled substrates.

RESULTS

Activities of Ketone Body-Metabolizing Enzymes in Glioma and Neuroblastoma Cells. Both glioma C6 and neuroblastoma C1300 (N2a) cells grew in logarithmic phase during the first 6 days in culture under the experimental conditions reported, with cell doubling times of approximately 11 and 14 hr, respectively (results not shown). At confluence, the activities of three ketone body-metabolizing enzymes were present in both cell types in the relative proportion (D-3-HB dehydrogenase < acetoacetyl-CoA thiolase < CoA-transferase) (Table 1) in which they are found in rat brain (15, 16). The activities of D-3-HB dehydrogenase and acetoacetyl-CoA thiolase were higher in glioma cells than in neuroblastoma cells, whereas CoA-transferase activity was approximately 2-fold higher in neuroblastoma cells than in glioma cells (Table 1). It is clear from Table 1 that of the three ketone body-metabolizing enzymes, CoA-transferase had the highest activity. We, therefore, measured changes in its specific activity in both cell lines over a 12 day-period (Fig. 1). There was a significant increase in the specific activity of this enzyme in cells of both lines during the first 6–7 days (a logarithmic growth period) in culture. The maximal specific activity of this enzyme, which was reached at about the point of confluence, was approximately 2-fold higher in neuroblastoma cells than in glioma cells (Fig. 1). Thereafter, the specific activity of this enzyme declined gradually over the next 5 days.

Regulation of CoA-Transferase Activity by Ketone Bodies. High concentrations of plasma ketone bodies present in term fetal rats (17, 18), in weaned rats fed a diet high in fat (19), and in fasted weaned rats (20) have been implicated as possible effectors in maintaining the higher levels of ketone body-metabolizing enzymes in developing rat brain. The medium of cultures of glioma and neuroblastoma cells were therefore supplemented with physiological concentrations of AcAcO or DL-3-HB and the effects on growth and CoA-transferase activity were determined. For these experiments the cells were grown for three consecutive subcultures with the same treatment to investigate a chronic effect of added ketone body to the medium on CoA-transferase activity. Because the observed effects were similar in all three subcultures, only the results obtained with the second subculture are reported in Table 2. AcAcO at a concentration of 0.5 mM in the medium stimulated growth and increased the specific activity of CoA-transferase in neuroblastoma cells during the rapid growth period (on day 4). These effects were not seen, however, at confluence (the sixth day of culture). These data indicate that both cell growth and the development of enzyme activity were accelerated without altering the peak values of either (Table 2). The addition of 2.5 mM DL-3-HB (1.25 mM D-3-HB, the physiological isomer) to the medium produced no discernible effects on cell growth and enzyme activity measured on day 4 but caused a significant reduction in these two characteristics in the cells on the sixth day of culture (Table 2). The result of those effects was an increase in the specific activity of CoA-transferase.

Table 1. Activities of ketone body-metabolizing enzymes in glioma and neuroblastoma cells

Enzyme	Specific activity, milliunits/mg protein: Glioma C6	Neuroblastoma C1300 (N2a)
D-3-HB dehydrogenase	6.6 ± 0.3	4.9 ± 0.5
CoA-transferase	117 ± 18	203 ± 10
Acetoacetyl-CoA thiolase	32 ± 1	11 ± 1

The cells were grown in the modified minimal essential medium for 6 days, and enzyme activities were measured. The results are the means ± SEM for six experiments.

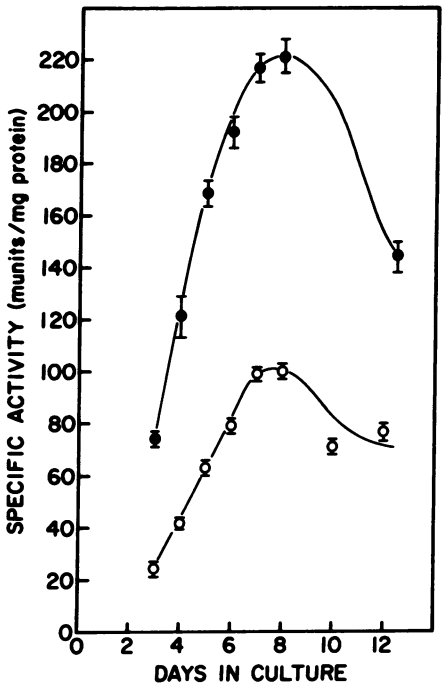

FIG. 1. Specific activity of CoA-transferase in glioma C6 (○) and neuroblastoma C1300 (N2a) (●) cells in culture over a 12 day-period. Cells were grown in the modified minimal essential medium. The results are the means ± SEM for six experiments.

Table 2. Effects of AcAcO and DL-3-HB on growth and CoA-transferase activity in glioma and neuroblastoma cells

Culture conditions: Addition	Time, days	Cell growth, μg protein/dish	Specific activity, milliunits/mg protein
		Neuroblastoma C1300 (N2a)	
None	4	279 ± 9	159 ± 5
AcAcO (0.5 mM)		387 ± 14‡	191 ± 5†
DL-3-HB (2.5 mM)		265 ± 22	197 ± 19
None	6	736 ± 36	217 ± 5
AcAcO (0.5 mM)		745 ± 24	223 ± 4
DL-3-HB (2.5 mM)		631 ± 25*	241 ± 8*
		Glioma C6	
None	4	467 ± 6	78 ± 2
AcAcO (0.5 mM)		440 ± 11*	74 ± 2
DL-3-HB (2.5 mM)		309 ± 7‡	72 ± 2
None	7	1082 ± 20	109 ± 3
AcAcO (0.5 mM)		1099 ± 53	100 ± 3
DL-3-HB (2.5 mM)		983 ± 34	109 ± 3

The cells were grown in modified minimal essential medium (containing 5 mM glucose), supplemented with either AcAcO or DL-3-HB as indicated. Each treatment was continued in the second subculture, and the cells were harvested on day 4 and 6 or 7 as indicated. Similar results were obtained for three successive subcultures, although only the second is shown in the Table. The results are the means ± SEM for six experiments. *P* values, compared to unsupplemented controls: *, <0.05; †, <0.005; ‡, <0.001. Unmarked values were not significantly different from controls ($P > 0.05$).

Table 3. Influence of ketone bodies in the culture medium on the oxidation of and lipid synthesis from labeled glucose, AcAcO, and D-3-HB by neuroblastoma and glioma cells

Line	Addition to medium: During growth (3 days)	Addition to medium: During radiolabeling (6 hr)	nmol of radiolabeled substrate converted to products per mg cellular protein per 6 hr: CO_2	Nonsaponifiable lipids	Fatty acids
		Neuroblastoma C1300 (N2a)			
1	None	[U-^{14}C]Glucose	161 ± 32	1.06 ± 0.17	8.8 ± 2.3
2	None	[U-^{14}C]Glucose + AcAcO + DL-3-HB	99 ± 14	0.14 ± 0.04¶	2.6 ± 1.1†
3	None	[3-^{14}C]AcAcO	169 ± 37	2.83 ± 0.90	29.8 ± 7.3
4	AcAcO	[3-^{14}C]AcAcO	313 ± 18§	7.06 ± 0.46¶	59.9 ± 4.6§
5	None	DL-3-H[3-^{14}C]B*	20 ± 2	0.59 ± 0.12	4.8 ± 1.6
6	DL-3-HB	DL-3-H[3-^{14}C]B*	17 ± 3	1.15 ± 0.15‡	6.4 ± 1.7
		Glioma C6			
7	None	[U-^{14}C]Glucose	175 ± 12	2.41 ± 0.09	17.9 ± 1.6
8	None	[U-^{14}C]Glucose + AcAcO + DL-3-HB	127 ± 5¶	0.51 ± 0.05‖	5.0 ± 0.3‖
9	None	[3-^{14}C]AcAcO	46 ± 6	4.91 ± 0.12	33.1 ± 0.4
10	AcAcO	[3-^{14}C]AcAcO	36 ± 3	3.96 ± 0.37	21.3 ± 2.3‖
11	None	DL-3-H[3-^{14}C]B*	30 ± 1	2.13 ± 0.11	17.4 ± 0.8
12	DL-3-HB	DL-3-H[3-^{14}C]B*	20 ± 1‖	1.40 ± 0.07¶	10.0 ± 0.6‖

Cells were grown in modified minimal essential medium containing 5 mM glucose supplemented with either 0.5 mM AcAcO or 2.5 mM DL-3-HB as indicated. Each treatment was continued for three consecutive subcultures. On the fourth day of the third subculture (which was carried out in Erlenmeyer flasks) the culture medium was replaced with an equal volume of the experimental medium (the culture medium containing 5 mM glucose plus one of the three radiolabeled substrates to give the final concentration indicated). After a 6-hr incubation period, $^{14}CO_2$ was collected and the radioactivity of the lipid fractions was quantitated. The results are the means ± SEM of five or six experiments. *P* values, compared to controls: †, <0.05; ‡, <0.025; §, <0.01; ¶ <0.005; ‖, <0.001.

* D-3-H[3-^{14}C]B was added to the medium containing 2.5 mM DL-3-HB.

In glioma cells, the addition of either 0.5 mM AcAcO or 2.5 mM DL-3-HB to the medium caused a significant delay in both cell growth and accumulation of total activity of the enzyme (Table 2), and therefore, no effect on the specific activity of CoA-transferase was seen on the fourth day. The presence of either ketone body in the medium had no effect on these properties as measured on the seventh day of culture (Table 2). In another series of experiments (not shown) the concentration of AcAcO in the medium of glioma cells was increased to 1 and 5 mM and that of DL-3-HB to 10 mM. The effects on total culture protein and enzyme activities in this experiment (results not shown) were similar to those noted at lower concentrations of these ketone bodies (Table 2).

Lipid Synthesis. To demonstrate a relationship between an increase in CoA-transferase in cells exposed to ketone body in the medium and their capacities to metabolize ketone bodies, a study was carried out to investigate chronic and acute effects of these additions on the metabolism of labeled glucose and ketone bodies by these cells. Neuroblastoma cells oxidized [U-^{14}C]glucose to $^{14}CO_2$ and also incorporated glucose carbon into lipids (Table 3, line 1). In these 6-hr experiments the addition of 0.5 mM AcAcO and 2.5 mM DL-3-HB together in the medium significantly reduced the incorporation of glucose carbon into lipids (lines 1 and 2) but had no significant effect on glucose oxidation. In neuroblastoma cells grown in control medium, the rate of [3-^{14}C]AcAcO oxidation to $^{14}CO_2$ was similar to that observed for the oxidation of glucose (lines 3 and 1). However, the rate of incorporation of AcAcO carbon into the lipid fractions was approximately 3-fold higher compared to incorporation into glucose (lines 3 and 1). When these cells were grown in medium supplemented with 0.5 mM AcAcO for three consecutive subcultures, the oxidation of [3-^{14}C]AcAcO to $^{14}CO_2$ and the incorporation of AcAcO carbon into lipids were enhanced approximately 2-fold (lines 4 and 3). It is noteworthy that in cells grown in control medium the rate of oxidation of 1.25 mM D-3-H[3-^{14}C]B to $^{14}CO_2$ was approximately 1/8th of that of either glucose or AcAcO (compare line 5 with lines 1 and 3). Also, the incorporation of D-3-HB carbon into the lipids was approximately 1/2 that observed for glucose and at most 1/4th of that with AcAcO as substrate. The presence of DL-3-HB in the medium during three consecutive subcultures had very little effect on the metabolism of D-3-H[3-^{14}C]B by neuroblastoma cells (except for the synthesis of nonsaponifiable lipids) (lines 5 and 6).

Glioma C6 cells oxidized [U-^{14}C]glucose to $^{14}CO_2$ at a rate similar to that of neuroblastoma cells. However, the rate of incorporation of glucose carbon into lipids was about 2-fold higher in glioma cells than in neuroblastoma cells (lines 7 and 1). The addition of 0.5 mM AcAcO plus 2.5 mM DL-3-HB to the medium during the experimental period reduced the oxidation of [^{14}C]glucose to $^{14}CO_2$ and the incorporation of glucose carbon into the lipid fraction (lines 7 and 8). Glioma cells oxidized 0.5 mM [3-^{14}C]AcAcO to $^{14}CO_2$ at a rate approximately 25% of that observed for 5 mM [U-^{14}C]glucose (compare line 9 with line 7). However, the rate of incorporation of AcAcO carbon into lipids was approximately 2-fold higher than that observed with [^{14}C]glucose as substrate. The metabolism of [3-^{14}C]AcAcO in glioma cells grown in medium containing 0.5 mM AcAcO for three consecutive subcultures was not significantly affected except for a reduction in the synthesis of fatty acids (lines 9 and 10). The oxidation of D-3-H[3-^{14}C]B by glioma cells was also significantly lower than that of [U-^{14}C]glucose (lines 11 and 7); however, the rate of incorporation of this ketone body carbon into lipids was very similar to that observed with labeled glucose as substrate (lines 11 and 7). Cells grown in the presence of DL-3-HB for three subcultures metabolized labeled D-3-HB at rates significantly lower than those observed for cells grown in unsupplemented medium (lines 11 and 12).

DISCUSSION

The findings presented in this report demonstrate several important differences in the metabolism of ketone bodies between

cell lines of glial and neuronal origin. In previous reports the presence of D-3-HB dehydrogenase in several cell lines (3) and CoA-transferase in tumors of the nervous system (4) has been demonstrated. Our findings extend these observations and show the presence of all three major ketone body-metabolizing enzymes in both neuroblastoma and glioma cell lines. On the basis of enzyme activity their relative abundance in these cells is similar to that in rat brain (15, 16). Furthermore, the levels of CoA-transferase activity in neuroblastoma and glioma cells (203 and 117 milliunits/mg of cellular protein, respectively, at confluence) are in the range observed for this enzyme in adult rat brain [178 ± 5 milliunits/mg of whole brain protein (unpublished observations)]. Close scrutiny of our results, however, shows that there are specific differences in the levels of three ketone body-metabolizing enzymes in these two cell lines, and these differences are consistent with their capacities to metabolize ketone bodies (Table 3).

Although the rates of glucose oxidation were found to be similar in the two cell lines, the rate of lipid synthesis from glucose was approximately 2-fold higher in glioma than in neuroblastoma cells. In general the presence of ketone bodies in the medium caused a reduction in the incorporation of glucose carbon into lipid fractions in both cell lines. A decrease in the oxidation of glucose to CO_2 was observed only in glioma cells (Table 3). Inhibitory effects of ketone bodies on lipid synthesis from glucose were previously observed with cerebral cortex slices from developing rat brain (13). Of the substrates tested for lipid synthesis, AcAcO was preferred in both cell lines. In glioma cells glucose and D-3-HB were equally effective, whereas in neuroblastoma cells D-3-HB was the poorest substrate. However, if one considers the role of glucose as a precursor for lipid synthesis in the presence of unlabeled ketone bodies, the contribution of glucose carbon to lipids diminishes markedly (Table 3). Under physiological conditions such as those prevalent in the blood of suckling rats [5 mM glucose, 0.5 mM AcAcO, and 1 mM D-3-HB (21)], the contribution of ketone body carbon to lipid synthesis is significantly higher than that of glucose. A similar conclusion is also drawn from both *in vivo* (14, 22) and *in vitro* (13, 23) studies of developing rat brain. When these substrates were used, the ratio of nmol of the substrate incorporated in lipid fractions to nmol oxidized to CO_2 was higher in glioma cells than in neuroblastoma cells. For AcAcO, D-3-HB, and glucose these ratios were 0.826, 0.651, and 0.116 in glioma cells and 0.193, 0.270, and 0.061 in neuroblastoma cells, respectively. Recently, Roeder and Tildon (24) reported in an abstract a higher ratio (lipids/CO_2) for AcAcO in the glioma than in the neuroblastoma cells. In their report the ratio varied between 6 and 27.5 for these cell lines. Although a reason for this discrepancy is not known, it is evident from both *in vivo* (14, 21, 22) and *in vitro* (13, 23) studies with rat brain that a relatively greater portion of the ketone body is oxidized compared to its incorporation into cerebral lipids, and hence the calculated ratio should be less than unity.

A preferential use of AcAcO in the cytosolic compartment for neural lipid synthesis was suggested by Buckley and Williamson (25) and subsequently supported by Patel and Owen (26). Although the present study was not designed to investigate the compartmentation of AcAcO metabolism, its high rate of incorporation into lipids is consistent with a direct utilization of AcAcO in the cytosolic compartment as reported in earlier studies (23, 25, 26). It is plausible that the addition of AcAcO to the medium may have increased the activity of cytosolic acetoacetyl-CoA synthetase in cultures of neuroblastoma cells, and hence it could have enhanced the incorporation of AcAcO carbon into neuronal lipids in these cells (Table 3). If this were true, however, it would still not explain the observed increase in the oxidation of labeled AcAcO to CO_2 by neuroblastoma cells (Table 3, line 4). Additionally, in glioma cells, addition of AcAcO had no effect on the activity of CoA-transferase and no increase in either CO_2 formation or lipid synthesis was observed. Taken together, these data suggest that the increase in lipid synthesis is due to an increased flux through the mitochondrial pathway of ketone body oxidation.

Earlier observations have suggested a relationship between the concentrations of ketone bodies in the blood and the level of the ketone body-metabolizing enzymes. For example, prenatally induced ketosis in the fetuses of pregnant rats, and postnatally maintained ketosis in weaned rats by either feeding a diet high in fat or fasting for 48 hr, caused an increased level or sustained high levels of the activity of one of the ketone body-metabolizing enzymes in developing rat brain (18–20). Sokoloff noted that a premature weaning of 10-day-old rats to a diet high in carbohydrate caused a premature fall in the activity of D-3-HB dehydrogenase in the brain, whereas the enzyme activity remained high when 10-day-old pups were weaned onto a synthetic maternal milk diet (see discussion reported in ref. 27). Although these studies clearly suggest a relationship between the concentration of ketone bodies in the blood and the activities of ketone body-metabolizing enzymes in the brain, possible effects of other factor(s) cannot be ruled out in these *in vivo* studies. This ambiguity has been avoided in our experiments, in which established cell lines were used. Our findings lend support to earlier reports and show that AcAcO and not D-3-HB accelerates the increase in the specific activity of CoA-transferase in neuroblastoma cells but not in glioma cells (Table 2). Furthermore, this acceleration in the increase in specific activity of this enzyme is observed during an early phase of growth and does not affect maximal specific activity achieved. Additionally, increasing the concentrations of AcAcO from 0.5 mM to 5 mM in the medium has no additional influence on the activity of CoA-transferase in neuroblastoma cells. This observation explains the lack of enhancement in the postnatal development of this ketone body-metabolizing enzyme in the brain when hyperketosis was induced in suckling rats by feeding the mothers a diet high in fat (19). The mechanism(s) by which AcAcO in the medium accelerates an increase in CoA-transferase activity in neuroblastoma cells are unclear at present, and additional studies will be required to define the changes in the synthesis, degradation, or both of this enzyme. Finally, although caution should be observed in extrapolating our observations with neuroblastoma and glioma cells to their respective cells of origin, our findings do show some important differences in the regulation of metabolism of ketone bodies by two clonal cell lines of glial and neuronal origin.

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