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ACETOACETATE AND GLUCOSE AS SUBSTRATES FOR LIPID SYNTHESIS BY RAT BRAIN OLIGODENDROCYTES AND ASTROCYTES IN SERUM-FREE CULTURE

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We have compared glucose and acetoacetate as precursors for lipogenesis and cholesterogenesis by oligodendrocytes and astrocytes, using mixed glial cultures enriched in oligodendrocytes. In order to differentiate between metabolic processes in oligodendrocytes and those in astrocytes, the other major cell type present in the mixed culture, we carried out parallel incubations with cultures from which the oligodendrocytes had been removed by treatment with anti-galactocerebroside serum and guinea-pig complement. The following results were obtained: 1. Both oligodendrocytes and astrocytes in culture actively utilize acetoacetate as a precursor for lipogenesis and cholesterogenesis. 2. In both cell types, the incorporation of acetoacetate into fatty acids and cholesterol exceeds that of glucose by a factor of 5–10 when the precursors are present at concentrations of 1 mM and higher. 3. Glucose stimulates acetoacetate incorporation into fatty acids and cholesterol, whereas acetoacetate reduces the entry of glucose into these lipids. This suggests that glucose is necessary for NADPH generation, but that otherwise the two precursors contribute to the same acetyl-CoA pool. 4. Both with acetoacetate and with glucose as precursor, oligodendrocytes are more active in cholesterol synthesis than astrocytes. 5. Using incorporation of ^3H_2O as an indicator for total lipid synthesis, we estimated that acetoacetate contributes one third of the acetyl groups and glucose one twentieth when saturating concentrations of both substrates are present.

Introduction

Oligodendrocytes synthesize and maintain myelin in the central nervous system. In view of the composition of myelin [1], oligodendrocytes must have the potential of synthesizing very large quantities of lipids. This potential is particularly expressed in the period that myelination is most active, which is, in the suckling rat, the third postnatal week. It has been estimated that in this period oligodendrocytes daily synthesize an amount of lipid equivalent to 3-times the weight of their cell body [2].

Hepatic ketogenesis is very active in suckling rats [3], which is mainly due to the high fat content of rat milk. This leads to high concentrations (1–2 mM) of acetoacetate and D-3-hydroxybutyrate in the blood of the pups [3–5]. Studies in vivo have shown that these ketone bodies can be used by the brain for lipogenesis and cholesterogenesis (Refs. 6–10; for reviews see Refs. 3 and 11). The enzymes that convert acetoacetate to acetoacetyl-CoA (acetoacetyl-CoA synthetase, EC 6.2.1.-) and to acetyl-CoA (acetyl-CoA acetyltransferase, EC 2.3.1.9) are present in the cytosol of brain cells [12–14]. The existence of these cytosolic enzymes explains why acetoacetate and glucose are not metabolically equivalent as donors of acetyl groups for the synthesis of fatty acids and cholesterol

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene.

[9,10,14]. Experiments with brain slices [15] and whole brain homogenates [16] have shown that ketone bodies are better lipogenic precursors than glucose, particularly for the synthesis of cholesterol. However, it should be emphasized that these studies have been carried out with whole brain preparations and that the results do not necessarily apply to oligodendrocytes. Pleasure et al. [17] reported a 20-fold enrichment of cytosolic acetoacetyl-CoA synthetase in bovine oligodendrocytes, when compared to whole brain. This led to the suggestion that during the suckling period ketone bodies may be more important as lipid precursors for oligodendrocytes than for other cell types in the brain.

In the present study we have investigated the contributions of glucose and acetoacetate to lipogenesis and cholesterogenesis in a primary culture system of oligodendrocytes and astrocytes maintained in a serum-free medium [18]. The metabolic processes occurring in oligodendrocytes and astrocytes could be distinguished by developing a method for complement-mediated cytolysis of the oligodendrocyte population in these mixed glial cultures. The following questions were addressed. (i) Do oligodendrocytes and astrocytes both utilize ketone bodies for lipid synthesis? (ii) Are there quantitative differences in this respect between these two glial cell types? (iii) What are the relative contributions of glucose and acetoacetate to lipogenesis and cholesterogenesis in both cell types (iv) Is the preferential use of acetoacetate for the synthesis of cholesterol also expressed in glial cells maintained in culture?

Materials and Methods

Animals. Wistar rats, 13 days pregnant, were obtained from the Central Institute for the Breeding of Laboratory Animals (Zeist, The Netherlands). Pups were used for the isolation of oligodendrocytes at 7 days of age.

Cell cultures. Cells were isolated and maintained in culture as described previously [18] with the following modifications: the cells were seeded at $6 \cdot 10^4$ cells per cm^2 in poly(L-lysine)-coated tissue culture flasks (25 cm^2, Nunc, Denmark) in 5 ml of Dulbecco's Modified Eagle's Medium with 10% newborn calf serum. After 24 h the medium was replaced by 5 ml of a serum-free medium [18], with final concentrations of 5 mM glucose, 1 mM pyruvate and 1 mM glutamine. After 48 h in vitro, 10^{-5} M cytosine 1-β-D-arabinofuranoside was added to inhibit the proliferation of astrocytes. The medium was replaced by fresh medium after 5 days in vitro. The cultures were used for experiments after 7 days in vitro. At this stage the cultures contained 60–75% oligodendrocytes as estimated from galactocerebroside or myelin basic protein immunofluorescence [18] and sensitivity to anti-galactocerebroside and complement-mediated cytolysis (see below). At least 90% of the other cells present in the cultures were astrocytes, as estimated from glial fibrillary acidic protein immunofluorescence [18].

Complement-mediated cytolysis of oligodendrocytes. Antibodies against galactocerebrosides were raised in rabbits as described by Bologa et al. [19]. Guinea-pig blood was obtained by cardiac puncture. Serum was obtained by centrifugation and stored at $-20\,^{\circ}C$. The complement system remained active for at least 1 month. To remove oligodendrocytes, cultures were incubated with heat-inactivated (30 min, 56 °C) anti-galactocerebroside serum and guinea-pig serum for one h. The cultures were then washed with culture medium and were maintained in fresh medium for 12–24 h. Optimal concentrations of anti-galactocerebroside serum and guinea-pig serum were tested for each batch. Addition of 100–200 μl of each serum was usually sufficient to lyse completely the population of oligodendrocytes.

Incubations with radioactive precursors. After 7 days the cultures were incubated with various radioactive precursors in 2 ml of serum-free medium. Parallel incubations were carried out with cultures from which the oligodendrocytes had been removed by treatment with anti-galactocerebroside serum and guinea-pig serum. After incubation the cultures were placed on ice. Subsequently, the medium was removed and the cultures were washed twice with ice-cold Hanks balanced salts solution and the cells were harvested with a rubber policeman. An aliquot of lipid isolated from rat brain was added as carrier and total lipids were isolated according to Bligh and Dyer [20]. The incorporation of label into different lipid classes was analysed by thin-layer chromatography [21]. The

lipid spots were visualized by exposure of the thin-layer plates to iodine vapour, then scraped off and extracted [20]. The extracts were then counted for radioactivity in xylene/0.3% PPO/0.02% POPOP. The incorporation of radioactivity into fatty acids and cholesterol was determined as described earlier [10]. Radioactivity measured was corrected for quenching using the channels ratio method. All data are presented as pmol or dpm per culture rather than per mg protein. After treatment with complement, membrane fragments remained attached to the bottom of the flasks and the protein content of the lysed cultures was therefore not representative for metabolically active astrocytes. Untreated cultures contained approx. 100 μg of protein [22].

Radiochemicals. D-[6-^{3}H]Galactose, $Na_2^{35}SO_4$, ^3H_2O, D-[U-^{14}C]glucose and ethyl [3-^{14}C] acetoacetate were bought from Amersham International U.K., or from New England Nuclear, Boston, MA. Ethyl [3-^{14}C]acetoacetate was hydrolysed as described before [9].

Chemicals. Potassium acetoacetate was prepared by hydrolysis of ethylacetoacetate (BDH, Poole, U.K.) in 1 M KOH for 1 h at 37°C followed by neutralization and freeze-drying to remove the ethanol. Culture media were prepared from their components (Sigma, St. Louis, MO) because of the high glucose content of commercially available media (5–25 mM). Newborn calf serum was obtained from Flow (Irvine, U.K.).

Results

Elimination of oligodendrocytes from glial cultures, using anti-galactocerebroside serum and guinea-pig complement

Incubation of oligodendrocyte-enriched cultures with anti-galactocerebroside serum and guinea-pig serum caused a destruction of the oligodendrocytes while astrocytes remained unaffected. Typical morphological changes that were observed by phase-contrast microscopy during this incubation were: fragmentation of the oligodendrocyte processes, then condensation of the nucleus to a very small volume, and extensive swelling of the cell body, followed by cytolysis. The first effects were visible within 10 min of incubation and after 1 h more than 95% of the oligodendrocytes were lysed. After washing and a 12–24 h recuperation period in serum-free medium, only few intact oligodendrocytes could be detected (less than 1% of the original population). We also tested the efficiency of this procedure by incubating treated and untreated cultures with D-[6-^{3}H]galactose and $Na_2^{35}SO_4$ and analysing the incorporation of these substrates into galactocerebrosides, sulfatides, phospholipids and neutral lipids. Table I shows that the incorporation of D-[6-^{3}H]galactose into cerebrosides and sulfatides was lowered drastically after elimination of oligodendrocytes from the cultures ($< 5\%$ and $< 10\%$ of controls, respectively). The incorporation of

TABLE I

INCORPORATION OF D-[6-^{3}H]GALACTOSE AND $Na_2^{35}SO_4$ INTO LIPIDS BY OLIGODENDROCYTE-ENRICHED CULTURES BEFORE AND AFTER TREATMENT WITH ANTI-GALACTOCEREBROSIDE SERUM AND COMPLEMENT

Cultures were incubated with serum-free medium (untreated) or with anti-galactocerebroside serum and guinea-pig serum (treated). After washing and a 24 h recuperation period, the cultures were incubated in 2 ml of serum-free medium with 10 μCi D-[6-^{3}H]galactose (119 Ci/mol) or with 20 μCi $Na_2^{35}SO_4$ (30 Ci/mol) for 24 h. Incorporation of the labeled precursors into lipids is presented in dpm per culture (means of duplicates). n.d., not detectable.

	Incorporation of [6-^{3}H]galactose		Incorporation of $Na_2^{35}SO_4$	
	Untreated cultures	Treated cultures	Untreated cultures	Treated cultures
Cerebrosides	7136	290	60	n.d.
Sulfatides	767	66	351	n.d.
Neutral lipids	3568	709	n.d.	n.d.
Phospholipids	6207	4291	n.d.	n.d.

[^{35}S]sulfate into sulfatides was no longer detectable after this treatment. On the other hand, incorporation of galactose into neutral lipids and phospholipids was less affected, indicating that the astrocytes did contribute to the synthesis of these lipid classes. Treatment of cultures with anti-galactocerebroside serum alone, or in combination with heat-inactivated guinea-pig serum (30 min, 56 °C), had no effect on the incorporation of [6-^{3}H]galactose and [^{35}S]sulfate (data not shown). Treatment of cultures with guinea-pig serum alone induced damage to oligodendrocytes and reduced the incorporation of [6-^{3}H]galactose and [^{35}S]sulfate by approx. 50%. Complement can attack myelin even in the absence of specific antibodies against myelin, probably because myelin activates the complement cascade via the alternative pathway [23]. We assume that the effect of complement on oligodendrocytes is also due to such a mechanism because the plasma membrane of oligodendrocytes is continuous with myelin in vivo.

Acetoacetate and glucose as precursors for the synthesis of fatty acids and cholesterol

To compare the utilization of acetoacetate and of glucose as lipid precursors, we incubated cultures with D-[U-^{14}C]glucose or with [3-^{14}C]acetoacetate at various concentrations of these substrates. Parallel incubations were carried out with cultures from which oligodendrocytes had been removed.

Fig. 1 shows a time-course experiment carried out with 0.75 mM D-[U-^{14}C]glucose and 1 mM [3-^{14}C]acetoacetate. At these concentrations both [U-^{14}C]glucose and [3-^{14}C]acetoacetate were incorporated linearly up to 4 h. Both in oligodendrocytes and in astrocytes the incorporation of [3-^{14}C]acetoacetate into fatty acids and into cholesterol was approx. 5-times higher than the incorporation of [U-^{14}C]glucose.

We found that increasing acetoacetate concentrations reduced the incorporation of [U-^{14}C]glucose into fatty acids and cholesterol but that increasing glucose concentrations stimulated the incorporation of [3-^{14}C]acetoacetate (Fig. 2). This stimulation of the incorporation of [3-^{14}C]acetoacetate by glucose is probably due to the fact that glucose is necessary for the generation of

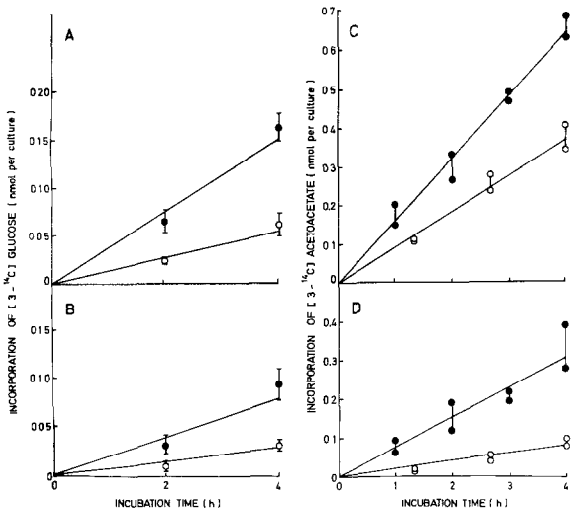

Fig. 1. Incorporation of [U-^{14}C]glucose and [3-^{14}C]acetoacetate into lipids: contribution of astrocytes and of oligodendrocytes. Panels A and C show the incorporation of ^{14}C into fatty acids and panels B and D that into cholesterol. Oligodendrocyte-enriched glial cultures (●) were compared with cultures (○) from which oligodendrocytes had been removed by preincubation with anti-galactocerebroside serum and guinea-pig serum. Cultures were incubated in 2 ml of serum-free medium with 0.75 mM glucose and 1.0 mM acetoacetate and either 5 μCi D-[U-^{14}C]glucose (3.3 Ci/mol) (panels A and B) or 5 μCi [3-^{14}C]acetoacetate (2.5 Ci/mol) (panels C and D).

NADPH for lipid synthesis. Other experiments with still higher substrate concentrations (> 1 mM; data not shown) demonstrated that the incorporation rates did not increase any further and that the incorporation of label from [3-^{14}C]acetoacetate into lipids remained 5–10-times higher than from [U-^{14}C]glucose.

The smaller contribution by astrocytes to total lipid synthesis in the experiment shown in Fig. 2 as compared to Fig. 1 can be explained by a lower percentage of astrocytes (30 vs. 40%, respectively).

From the data of Fig. 2 we have calculated, for both substrates, the ratio between incorporation into cholesterol and into fatty acids (Table II). This ratio indicates the distribution of the labeled substrate between the synthesis of cholesterol and of fatty acids. Ratios obtained from incubations with various substrate concentrations (Fig. 2) did not differ significantly. The ratios were about 2-fold higher for oligodendrocytes than for astrocytes, indicating a relatively high rate of

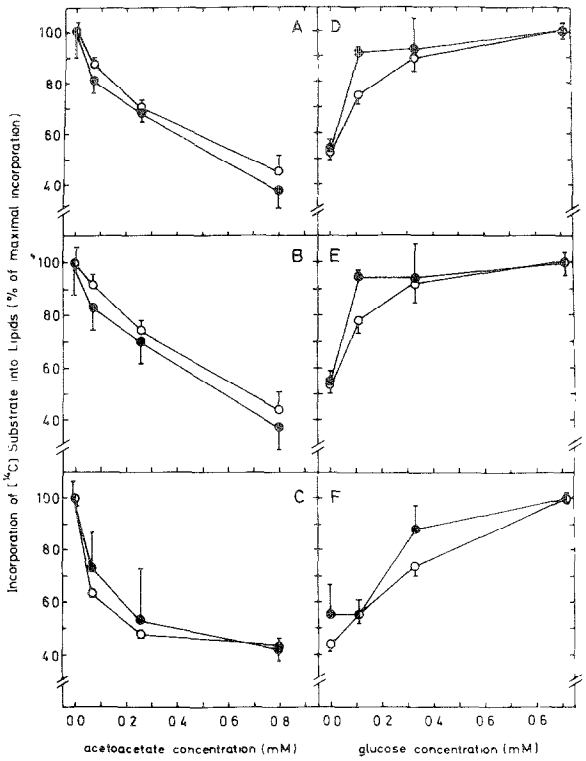

Fig. 2. Relationship between acetoacetate and glucose as precursors for lipid synthesis by astrocytes and by oligodendrocytes. ^{14}C incorporation into fatty acids (○) and into cholesterol (●) from 0.41 mM [U-^{14}C]glucose (18 Ci/mol) with varying concentrations of acetoacetate (panels A–C) and from 0.47 mM [3-^{14}C]acetoacetate (5 Ci/mol) with varying concentrations of glucose (panels D–F). A and D, untreated cultures; C and F, cultures from which oligodendrocytes had been removed by treatment with anti-galactocerebroside serum and guinea-pig serum, representing the contribution of astrocytes; B and E, A minus C and D minus F, respectively, representing the contribution of oligodendrocytes. Triplicate cultures were incubated for 4 h. Data are means ± S.E. expressed as percentage of the maximal incorporation. Absolute values for maximal incorporation into fatty acids were (pmol/h per culture) (A) 168.0 ± 5.0; (B) 141.0 ± 5.3; (C) 27.0 ± 0.3; (D) 37.4 ± 1.4; (E) 28.8 ± 1.7; (F) 9.9 ± 1.3; and into cholesterol (A) 126.0 ± 4.0; (B) 116.0 ± 4.1; (C) 9.0 ± 0.1; (D) 32.2 ± 3.2; (E) 27.7 ± 3.5; (F) 4.5 ± 0.3. Changes in the substrate concentrations during the incubation period were less than 10%.

cholesterogenesis in oligodendrocytes. The ratios for [3-^{14}C]acetoacetate and for [U-^{14}C]glucose were very similar. Hence, preferential use of acetoacetate for cholesterogenesis was not observed.

Absolute rates of lipogenesis and cholesterogenesis

In order to estimate the absolute contributions of acetoacetate and glucose to the synthesis of fatty acids and cholesterol we incubated cultures with ^3H_2O (see Ref. 24) in combination with either [U-^{14}C]glucose or [3-^{14}C]acetoacetate. Assuming that ^3H_2O is incorporated into fatty acids and cholesterol at a rate of approx. 1 mol per mol of C_2 units [24], we found that [3-^{14}C]acetoacetate contributed 38 ± 7 and 32 ± 2% of the acetyl groups to lipogenesis and cholesterogenesis, respectively. [U-^{14}C]Glucose contributed 4 ± 1% to lipogenesis and 7 ± 2% to cholesterogenesis Omission of pyruvate from the medium only slightly enhanced the contribution of [U-^{14}C]glucose to the synthesis of these lipids.

TABLE II

DISTRIBUTION OF LABEL FROM [U-^{14}C]GLUCOSE AND FROM [3-^{14}C]ACETOACETATE BETWEEN THE SYNTHESIS OF CHOLESTEROL AND OF FATTY ACIDS

Data are derived from the experiments shown in Fig. 2. Ratios were calculated for the various substrate concentrations. Significant differences were not found; means ± S.E. are presented in the table.

	dpm in cholesterol/dpm in fatty acids after incorporation of label from	
	[U-^{14}C]glucose	[3-^{14}C]acetoacetate
Total cultures	0.80 ± 0.05	0.85 ± 0.07
Oligodendrocytes	0.89 ± 0.05	0.87 ± 0.07
Astrocytes	0.48 ± 0.03	0.37 ± 0.04

Discussion

We developed an isolation procedure and culture system [18] for oligodendrocytes from whole cerebra of 1-week-old rats, to study ketone body and lipid metabolism in this cell type. These cultures are highly enriched in oligodendrocytes (up to 80%), but also contain other cells, mainly astrocytes [18]. It is possible to remove the population of galactocerebroside-positive cells (oligodendrocytes) quantitatively by complement-mediated cytolysis [25] and hence to measure the metabolic 'background'. This method allows us to gather information on the metabolic contribution of astrocytes and of oligodendrocytes in mixed glial cell cultures.

The data in Table I support the conclusion that treatment with anti-galactocerebroside serum and complement completely removes the population of oligodendrocytes from the cultures. The incorporation of [6-^{3}H]galactose into galactocerebrosides was greatly reduced by this treatment and the synthesis of sulfatides was no longer detectable. On the other hand, the incorporation of [6-^{3}H]galactose into neutral lipids and phospholipids was less affected, indicating that the astrocytes in the culture remained metabolically active. The observation that guinea-pig serum alone damages oligodendrocytes is interesting in view of the reported effect of complement on myelin (Ref. 23 and references therein). Parenthetically, it may be noted that some galactocerebroside-positive cells developed again after complement treatment and prolonged culture in serum-free medium (cf. Refs. 26 and 27).

Our results show that both oligodendrocytes and astrocytes actively utilize acetoacetate for lipid synthesis: maximal rates of incorporation of [3-^{14}C]acetoacetate into fatty acids and into cholesterol are approx. 5-times higher than the rates found with [U-^{14}C]glucose as substrate (Figs. 1 and 2). The incorporation of label from [3-^{14}C]hydroxybutyrate into lipids was similar but slightly lower than the incorporation of label from [3-^{14}C]acetoacetate (J.E.C. Sykes, unpublished data).

Oligodendrocytes synthesize relatively more cholesterol than astrocytes do, both from acetoacetate and from glucose (Table II). This conclusion is also supported by the observation that incorporation of [6-^{3}H]galactose into neutral lipids (mainly cholesterol) is inhibited more by treatment with complement than the incorporation of [6-^{3}H]galactose into phospholipids (Table I). The cultures, used to collect the data shown in Fig. 2 and Table II, contained $70 \pm 10\%$ oligodendrocytes. Calculations of the contribution per oligodendrocyte and per astrocyte indicate that oligodendrocytes synthesize more fatty acids than astrocytes: 2- and 1.25-times as much from acetoacetate and glucose, respectively. Oligodendrocytes synthesize more cholesterol as well: 5.5-times more from acetoacetate and 2.7-times more from glucose. This corroborates the concept that oligodendrocytes have a high capacity for lipogenesis and, especially, for cholesterogenesis [1,2].

We expected to find that acetoacetate was more cholesterogenic than glucose, especially in oligodendrocytes, because of a cytosolic acetoacetyl-CoA synthetase [12–14] and its enrichment in oligodendrocytes [17]. However, to our surprise, we did not find preferential incorporation of [3-^{14}C]acetoacetate into cholesterol, when compared to [U-^{14}C]glucose as substrate. Possible explanations for the discrepancy between these results and conclusions derived from in vivo experiments [7,9,10] are: (i) the presence of a blood-brain barrier in vivo and the possible modification of circulating substrates by cells that constitute this barrier before these substrates are used as lipid precursors by oligodendrocytes; (ii) the activity of acetoacetyl-CoA synthetase may be reduced in or even be absent from our culture system; (iii) the actual acetoacetate concentration in the brain may be outside the range of acetoacetate concentrations that we have tested.

The present results show that [3-^{14}C]acetoacetate and [U-^{14}C]glucose were incorporated into cholesterol and fatty acids in the same proportion, regardless of the concentration of these substrates. Therefore, we conclude that acetoacetyl-CoA synthetase does not play an important role in rat-brain oligodendrocytes and astrocytes in vitro, and that the discrepancy with observations in vivo [7,9,10] warrants further investigation.

We used the incorporation of ^3H_2O into fatty acids and cholesterol to estimate total rates of lipid synthesis [24] and measured the contribution of acetoacetate and glucose in parallel incubations. We calculated that acetoacetate contributes 30–40% to lipogenesis and cholesterogenesis, whereas glucose contributes only 5% at saturating concentrations of both substrates. This shows that acetoacetate is indeed an important precursor for lipid synthesis in glial cells. It also indicates that other components in our culture medium such as glutamine, other amino acids and pyruvate contribute substantially to the synthesis of fatty acids and cholesterol and that endogenous substrates may also serve as lipid precursors.

The incorporation of [U-^{14}C]glucose is reduced by 60% by the addition of 0.8 mM acetoacetate (Fig. 2; panels A–C), an observation that is compatible with the finding that acetoacetate contributes many more acetyl groups to lipid synthesis

than glucose. On the other hand, addition of glucose to the medium stimulates the incorporation of [3-^{14}C]acetoacetate into lipids (Fig. 2; panels D–F). Apparently, glucose is necessary for the production of NADPH in the pentose phosphate pathway. Panels D, E and F of Fig. 2 illustrate that any dilution of [^{14}C]acetyl-CoA derived from [3-^{14}C]acetoacetate by acetyl groups from added glucose is masked by this stimulatory effect of glucose. Consistent with this explanation is the observation that 6-aminonicotinamide, an inhibitor of the pentose phosphate pathway, blocks the stimulatory effect of glucose on lipid synthesis from acetoacetate by mixed glial cultures (J.E.C. Sykes, personal communication).

In short, we conclude from experiments with oligodendrocyte-enriched cultures that: (i) both astrocytes and oligodendrocytes actively use acetoacetate for lipogenesis and cholesterogenesis; (ii) oligodendrocytes synthesize more cholesterol than astrocytes; (iii) acetoacetate contributes one third of the acetyl groups to lipid synthesis, whereas glucose contributes only about 5%, at optimal concentrations of both substrates; (iv) the distribution between fatty acid synthesis and cholesterogenesis is the same for acetyl groups derived from acetoacetate as for those from glucose.

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