Changes in sterol biosynthesis accompanying cessation of glial cell growth in serum-free medium

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C-6 glioma cells, grown in medium supplemented with 5% delipidated foetal calf serum, were induced to enter a quiescent state by removing serum from the medium. Within 24 h there was a 75–80% decline in the rate of incorporation of [^{14}C]acetate or $^3\text{H}_2\text{O}$ into digitonin-precipitable sterols. Experiments with [^3H]mevalonolactone as a labelled sterol precursor suggested that the decline in sterol synthesis was regulated primarily at a point in the pathway before the formation of mevalonate. The specific activities of 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase decreased sharply in conjunction with the decline in sterol synthesis in the serum-free cultures; however, the activity of acetoacetyl-CoA thiolase was altered only slightly. The magnitude of the initial decline in reductase activity was not affected when 50-mM-NaF was included in the preincubation and assay buffers to prevent activation of physiologically inactive enzyme. However, after 6 h of serum deprivation the decline in 3-hydroxy-3-methylglutaryl-CoA reductase activity was due to a decrease in the amount of latent activity. The sterol concentration in C-6 cells was unchanged after 24 h in serum-free medium, although a 20% decrease in the sterol/fatty acid molar ratio occurred as a result of a small increase in the fatty-acid concentration. Incorporation of $^3\text{H}_2\text{O}$ into fatty acids was inhibited in the serum-deprived glial cells; however, this inhibition developed more slowly and was not as pronounced as the diminution in sterol synthesis. The results suggest that in C-6 glia, which resemble the glial stem cells of the developing brain, the decreased demand for membrane sterols in the quiescent state results in a decline in sterol synthesis, mediated primarily through co-ordinate changes in the activities of 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase.

The marked increase in the cholesterol content of the brain during the perinatal period of development (Wells & Dittmer, 1967; Cuzner & Davison, 1968) is consistent with the important role of this sterol as a structural component of cellular membranes and the myelin sheath. Since cholesterol carried by plasma lipoproteins does not cross the blood/brain barrier in appreciable quantities (McIlwain & Bachelard, 1971; Pardridge & Mietus, 1980), neural cells apparently must rely on cholesterol synthesized *in situ* for the formation of new membranes. This hypothesis is supported by the data of Kandutsch & Saucier (1969), which demonstrate a high rate of sterol synthesis *de novo* from [^{14}C]acetate in the mouse brain during the early postnatal period.

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

Several studies have shown that the activity of HMG-CoA reductase, which is generally regarded as the rate-limiting enzyme in the cholesterol biosynthetic pathway in mammalian tissues and cultured cells (for review see Volpe, 1978), is elevated in developing brain (Kandutsch & Saucier, 1969; Sudjic & Booth, 1976; Aragon *et al.*, 1978; Maltese & Volpe, 1979a; Ness *et al.*, 1979). However, a report of high cytosolic HMG-CoA synthase activity in developing rat brain has raised the possibility that enzymes proximal to the reductase may be involved in the regulation of sterol synthesis in neural tissues (Shah, 1978).

The tremendous cellular heterogeneity of the brain, and the consequent overlap of a number of different sterol-requiring processes (e.g. cell proliferation, myelination, neuronal arborization) during development, make it extremely difficult to study the

regulation of sterol synthesis as it relates to specific cellular events *in vivo*. C-6 glioma cells, originally derived from a rat tumour, have proven to be useful as a model system for the study of glial biochemistry and physiology in a homogeneous cell population (for review see Benda, 1978). These cells appear to be comparable with the glial stem cells present in developing brain, since they exhibit properties of both astrocytes and oligodendroglia (Bissell *et al.*, 1974; Volpe *et al.*, 1975; Parker *et al.*, 1980). After arrest of growth, induced by serum-deprivation, C-6 glioma cells exhibit increased activity of the oligodendroglial marker enzyme, 2':3'-cyclic nucleotide 3'-phosphohydrolase, and undergo a morphological transformation reminiscent of glial differentiation (Maltese & Volpe, 1979b).

C-6 glia can proliferate in medium supplemented with delipidated serum. Under these conditions the cells, like the glial stem cells in the developing central nervous system, must rely on endogenous sources for their supply of membrane sterol. The aim of the present investigation was to study the response of the sterol biosynthetic pathway during the transition of C-6 glia from a proliferating to a quiescent state, after removal of delipidated serum from the medium. By elucidating the enzymic basis for changes that occur during this transition, we hoped to obtain evidence that would suggest which enzymes normally are involved in adjusting the rate of sterol synthesis to comply with the demand for membrane cholesterol in the glioblasts of the developing brain.

Experimental

Materials

The following isotopes were purchased from New England Nuclear, Boston, MA, U.S.A.: [*acetyl*- ^{14}C]acetyl-CoA (sp. radioactivity 58.0 Ci/mol), DL-3-hydroxy-3-[*methyl*- ^3H]methylglutaryl-CoA (sp. radioactivity 10.9 Ci/mmol), [1,2- ^3H]cholesterol (sp. radioactivity 52.9 Ci/mmol), [4- ^{14}C]cholesterol (sp. radioactivity 54 Ci/mol), RS-[5(n)- ^3H]mevalonolactone (sp. radioactivity 2.0 Ci/mmol), [*methyl*- ^3H]thymidine (6.7 Ci/mmol), $^3\text{H}_2\text{O}$ (1 Ci/g), and [U- ^{14}C]palmitic acid (sp. radioactivity 1.0 Ci/mmol). [1- ^{14}C]Acetic acid (sodium salt; sp. radioactivity 60.2 Ci/mol) was obtained from Amersham, Arlington Heights, IL, U.S.A. Foetal calf serum (Kansas City Biological, Lenexa, KS, U.S.A.), cholesterol test kits (Boehringer Mannheim, Indianapolis, IN, U.S.A.), 3a70B scintillation fluid (Research Products International, Elk Grove, IL, U.S.A.) and plastic tissue culture flasks (Corning; Corning, NY, U.S.A.) were obtained from the designated sources. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cell culture

C-6 glioma cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were maintained in monolayer culture in Higuchi (1970) medium, modified as described previously (Maltese & Volpe, 1979b). Stock cultures were supplemented with 10% (v/v) foetal calf serum and were subcultured 3 days after reaching confluency. Cultures used for experiments were between the tenth and thirtieth passages in our laboratory. The methods for harvesting and subculturing C-6 glioma cells have been described previously (Volpe & Marasa, 1975). Periodic tests for the presence of *Mycoplasma* contamination (performed by M.A. Bioproducts, Walkersville, MD, U.S.A.) were negative. Cell viability was assessed routinely by Trypan Blue dye exclusion (Patterson, 1979). During the course of all experiments, the culture medium was changed at 24 h intervals.

Preparation of delipidated serum

Foetal calf serum was delipidated by extraction with butan-1-ol/di-isopropyl ether (2:3, v/v) as described by Cham & Knowles (1976). The delipidated serum was dialysed for 48 h at 4°C against 50 vol. of phosphate-buffered saline (Dulbecco & Vogt, 1954) to remove any residual organic solvent. Assays (see below) indicated that this treatment removes >95% of the cholesterol and fatty acid present in foetal calf serum.

Protein determination

Total cellular protein was determined as described previously (Maltese & Volpe, 1979b), by using a microbiuret method (Munkres & Richards, 1965). When protein was measured in cell extracts used for enzyme assays, the method of Lowry *et al.* (1951) was used. Bovine serum albumin served as the standard for all determinations.

DNA synthesis

Cells were incubated with medium containing [^3H]thymidine (0.4 $\mu\text{Ci/ml}$) for 1 h, and the rate of DNA synthesis was estimated by measuring the incorporation of radioactivity into trichloroacetic acid-precipitable material (Maltese & Volpe, 1979b).

Sterol synthesis

Cells growing in 25 cm² flasks were incubated in 3 ml of medium containing [^{14}C]acetate (0.5 $\mu\text{Ci/ml}$, 1 h incubation), $^3\text{H}_2\text{O}$ (5 mCi/ml, 3 h incubation) or [^3H]mevalonolactone (10 $\mu\text{Ci/ml}$, 3 h incubation). After removing the medium and washing the monolayers three times with cold Tris-buffered saline [0.14 M-NaCl/3.5 mM-Tris/HCl (pH 7.4)], the cells were dissolved in 2 ml of 0.5 M-KOH. The solutions were heated at 70°C for 5 min and

portions were removed for protein determination by the microbiuret method. The remaining KOH solution was used for isolation of 3- β -hydroxysterols by precipitation with digitonin as described previously (Volpe & Hennessy, 1977). Radioactivity was determined by mixing the digitonin-precipitated material (dissolved in 1 ml of methanol) with 10 ml of 3a70B scintillation fluid and counting for radioactivity in a Packard Tricarb liquid-scintillation spectrometer. All results were corrected for recovery of internal standards ($[^3\text{H}]$ cholesterol in cells pulse-labelled with $[^{14}\text{C}]$ acetate, or $[^{14}\text{C}]$ cholesterol in cells pulse-labelled with $^3\text{H}_2\text{O}$ or $[^3\text{H}]$ mevalonolactone), which were added before beginning the isolation of sterols. Recoveries of the internal standard routinely ranged between 70 and 80%.

Fatty-acid synthesis

In experiments where incorporation of radioactivity from $^3\text{H}_2\text{O}$ into fatty acids was measured, cells were incubated with the labelled precursor and harvested with 0.5 M-KOH as described above under 'Sterol synthesis'. Fatty acids were saponified and isolated essentially as described by Volpe & Marasa (1975) and radioactivity incorporated into the product was corrected for recovery of internal standard ($[^{14}\text{C}]$ palmitate) added before saponification.

Enzyme assays

Cells to be used for determination of enzyme activity were frozen in liquid N_2 and held at -70°C . Assays were performed within 1 week of the time of harvest, although all of the enzymes assayed in the present study were stable at -70°C for 1 month. Cell pellets to be assayed for acetoacetyl-CoA thiolase or HMG-CoA synthase were suspended in 20 mM-potassium phosphate (pH 7.0)/1 mM-EDTA, and were disrupted by three rapid freeze-thaw cycles with liquid N_2 . Microscopic observation indicated that this procedure disrupts >95% of the cells. Cytosolic fractions were obtained after centrifuging the broken-cell preparations at 100 000 g for 60 min. Supernatant fractions to be assayed for HMG-CoA synthase were dialysed for 18 h against 100 vol. of 20 mM-potassium phosphate (pH 7.2)/0.1 mM-EDTA/0.5 mM-dithiothreitol, to inactivate any HMG-CoA lyase activity (Ramachandran *et al.*, 1978).

The activity of acetoacetyl-CoA thiolase (EC 2.3.1.9) in cytosolic fractions was determined by the spectrophotometric method, which measures the decrease in A_{300} due to the CoA-dependent cleavage of acetoacetyl-CoA (Clinkenbeard *et al.*, 1973; Balasubramaniam *et al.*, 1977). The reaction mixture consisted of 0.1 M-Tris/HCl (pH 8.2)/0.1 mM-EDTA/0.12 mM-acetoacetyl-CoA/90 μM -CoA and

30 μg of protein, in a total volume of 1 ml. The reaction was started by adding the CoA after a 2 min preincubation. Under these conditions the amount of acetoacetyl-CoA cleaved was linear with time for 6 min at 25°C and was proportional to protein concentration within the range 20–60 μg . One unit of enzyme activity cleaves 1 μmol of acetoacetyl-CoA/min at 25°C .

Cytosolic HMG-CoA synthase (EC 4.1.3.5) activity was assayed by a modification of the radiochemical method of Clinkenbeard *et al.* (1975a), as described by Balasubramaniam *et al.* (1977). The method measures the synthesis of 3-hydroxy-3-methyl- $[^{14}\text{C}]$ glutaryl-CoA from $[^{14}\text{C}]$ -acetyl-CoA in a reaction mixture consisting of 0.1 M-Tris/HCl (pH 8.0)/10 μM -acetoacetyl-CoA/0.1 mM-EDTA/20 mM-MgCl₂/0.6 mM- $[^{14}\text{C}]$ acetyl-CoA (0.24 μCi) and 40 μg of cytosolic protein in a total volume of 0.2 ml. Blank reactions contained heat-inactivated protein. The reaction was linear with time for 15 min at 30°C and was proportional to protein concentration within the range 20–60 μg . After termination of the reaction by acidification of the assay mixture, unreacted acetyl-CoA was removed by drying the samples at 95°C and radioactivity incorporated into the non-volatile HMG-CoA was determined (Balasubramaniam *et al.*, 1977). One unit of enzyme activity formed 1 nmol of HMG-CoA/min at 30°C .

Cells to be assayed for HMG-CoA reductase (EC 1.1.1.34) were suspended in 50 mM-potassium phosphate (pH 7.4)/5 mM-dithiothreitol/1 mM-EDTA (and 50 mM-NaF where indicated). The cells were disrupted by rapid freeze-thawing as described above, and the broken-cell preparations were preincubated at 37°C for 30 min immediately before the assay. The reaction mixture consisted of 0.1 M-potassium phosphate (pH 7.4)/2.5 mM-dithiothreitol/1 mM-EDTA/2.5 mM-NADPH/75 μM -3-hydroxy-3- $[^3\text{H}]$ methylglutaryl-CoA (sp. radioactivity 10 Ci/mol) and 100 μg of protein, in a total volume of 0.2 ml. Where indicated 50 mM-NaF was also included in the assay mixture. Blanks contained heat-inactivated protein. The reaction was started by addition of the 3-hydroxy-3- $[^3\text{H}]$ methylglutaryl-CoA and was terminated after 1 h at 37°C , by addition of 20 μl of 5 M-HCl. $[^{14}\text{C}]$ Mevalonate (2000 d.p.m.) was added to each sample and conversion of mevalonate into the lactone form was carried out by incubating the acidified reaction mixture for 30 min at 37°C . The mevalonolactone was isolated from unreacted 3-hydroxy-3- $[^3\text{H}]$ -methylglutaryl-CoA by an ion-exchange method (Maltese & Volpe, 1979c), and the results were corrected for recovery of the internal standard (routinely 60–65%). Under the conditions described, the rate of reaction was linear with time up to 1.5 h and was proportional to protein concentration within

the range 50–200 μg . One unit of enzyme activity formed 1 pmol of mevalonate/min at 37°C.

Determination of sterol, fatty acid and total lipid phosphorus

Cell pellets were suspended in 2 ml of 0.9% (w/v) NaCl and total lipids were extracted with methanol and chloroform (saline / methanol / chloroform, 3:4:8, by vol.) as described by Johnson (1979). A 2 ml portion of the organic phase was dried under N_2 and resuspended in 1 ml of 20% (w/v) methanolic KOH containing [^3H]cholesterol (200 000 d.p.m.). The sample was incubated at 70°C for 30 min to hydrolyse sterol esters, and sterols were extracted into light petroleum (b.p. 37.9–56.8°C). After evaporating the light petroleum under N_2 , the residue was dissolved in 0.21 ml propan-2-ol and a 10 μl portion was removed for determination of the recovery of the [^3H]cholesterol standard. The remaining 0.2 ml of the solution was assayed for 3- β -hydroxysterols, by using a commercially available colorimetric assay kit (see under 'Materials').

Fatty acids were quantified by a modification of the method of Lauwerys (1969). A 1 ml portion of the organic phase (see above) was evaporated under N_2 and 1 ml of 20% (v/v) methanolic KOH containing [^3H]palmitate (200 000 d.p.m.) was mixed with the residue. After saponification at 70°C for 1 h, the solution was acidified with HCl and fatty acids were extracted into pentane. A portion was removed for determination of the recovery of [^3H]palmitate, and the remainder of the pentane was evaporated under N_2 . The residue was dissolved in 3 ml of pentane, and 3 ml of chloroform and 3 ml of Lauwerys' copper reagent was added sequentially. The tubes were shaken for 2 min and the phases were separated by centrifugation. A 3 ml portion of the upper phase was combined with 0.5 ml of 0.1% (w/v) sodium diethyldithiocarbamate in butan-1-ol, and the A_{440} was determined. Fatty acid was quantified by reference to a palmitate standard curve (0.01–2.0 μmol).

Total lipid phosphorus was determined in chloroform/methanol cell extracts (200–400 μl of organic phase, dried under N_2) by the method of Ames (1966).

Results

Effects of removal of serum on cell growth

C-6 glioma cells, cultured in medium supplemented with 5% delipidated foetal calf serum, attained a growth rate comparable with that previously described for cells cultured in medium containing 10% foetal calf serum (Maltese & Volpe, 1979b). However, cells supplemented with delipidated serum entered stationary phase at a lower cell density than did cells cultured with whole serum.

Removal of delipidated serum from the culture medium had a profound effect on cell growth, as evidenced by the nearly complete cessation of protein accumulation in the serum-deprived cultures (Fig. 1a). The marked decrease in incorporation of [^3H]thymidine into trichloroacetic acid-precipitable material in the serum-free glial cultures (Fig. 1b) indicated that few, if any, of the cells were able to enter the S-phase of the cell cycle after 24 h. The

Fig. 1. Effects of serum deprivation on growth of C-6 glial cells

Cells were plated in 25 cm^2 flasks at a density of 6×10^4 cells/ cm^2 . After 2 days of growth in medium supplemented with 5% (v/v) delipidated foetal calf serum, cultures either were washed and changed to serum-free medium (●) or were allowed to continue growing in serum-supplemented medium (O). At the indicated times, we determined the total protein per flask (a), or the rate of incorporation of [^3H]thymidine into trichloroacetic acid-precipitable material (b), as described in the Experimental section. Each point represents the mean ($\pm$ s.d.) for separate determinations performed on three cultures.

cessation of cell proliferation was not accompanied by a decline in cell viability, measured by Trypan Blue dye exclusion. Moreover, as described below (Fig. 2a), the arrest of cell growth was rapidly reversed by readdition of delipidated serum to the serum-deprived cultures.

Effects of removal of serum on sterol synthesis

Within 24 h after delipidated serum was removed from the culture medium, C-6 cells exhibited an 80% decline in their ability to incorporate radioactivity from [^{14}C]acetate into digitonin-precipitable sterols (Table 1a). Little or no additional decline in the rate of sterol synthesis was observed after the first day of serum deprivation. Similar data were obtained when $^3\text{H}_2\text{O}$ was used as a labelled precursor to measure sterol synthesis (Table 1a). Thus, the decreased incorporation of radioactivity from [^{14}C]acetate was not due to a decline in acetate uptake or activation to acetyl-CoA, or to an increase in the size of the intracellular acetate pool in the serum-deprived cells.

After 48 h, addition of delipidated serum to the serum-deprived quiescent cultures promoted a resumption of cell proliferation (Fig. 2a). This was accompanied by a sharp increase in the rate of sterol synthesis (Fig. 2b). The small decrease in the rate of sterol synthesis, observed on the second day after readdition of serum, was associated with a decline in the growth rate of the cultures as they reached confluency.

We attempted to localize the regulatory site responsible for the suppression of sterol synthesis during cessation of glial cell growth, by comparing

the rates of sterol synthesis from [^{14}C]acetate and [^3H]mevalonolactone at 6 and 24 h after removal of delipidated serum from the medium (Table 1b). Although there was a small decline in the rate of sterol synthesis from [^3H]mevalonolactone, the data suggest that the decrease in sterol synthesis in serum-deprived C-6 cells is controlled primarily at one or more points in the biosynthetic pathway between acetyl-CoA and mevalonate.

Effects of removal of serum on enzymes of the sterol biosynthetic pathway

We next determined the effects of serum deprivation on the activities of the three enzymes that catalyse the synthesis of mevalonate from acetyl-CoA (acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase). The specific activity of acetoacetyl-CoA thiolase underwent only a 20% decline after 24 h of serum deprivation (Fig. 3a); however, the activity of cytosolic HMG-CoA synthase decreased by approx. 85% as the cells entered a quiescent state (Fig. 3a). The rapid 60% decline in synthase activity that occurred during the first 6 h after removal of serum paralleled the 58% decline in [^{14}C]acetate incorporation into sterols (Table 1b), and the 53% decline in [^3H]thymidine incorporation (Fig. 1) that occurred during the same time interval.

Several studies have demonstrated that HMG-CoA reductase may exist in a latent form *in vivo*; i.e. the reductase activity in homogenates or microsomal fractions of liver, brain and cultured cells (L cells and hepatocytes) can be increased markedly by preincubating samples at 37°C before carrying out

Table 1. *Effects of serum deprivation on incorporation of [^{14}C]acetate, $^3\text{H}_2\text{O}$ and [^3H]mevalonolactone into sterols* C-6 cells were plated as described in the legend to Fig. 1. After 2 days of growth in medium supplemented with 5% (v/v) delipidated serum, the cultures were washed and changed to serum-free medium. At the indicated times, cells were incubated with [^{14}C]acetate for 1 h, with $^3\text{H}_2\text{O}$ for 3 h, or with [^3H]mevalonolactone for 3 h, and the incorporation of radioactivity into digitonin-precipitable sterols was determined as described in the Experimental section. Control values were obtained immediately before changing the cells to serum-free medium. Each value is a mean ($\pm$ S.D.) derived from separate determinations performed on three cultures.

	Sterol synthesis			
	[^{14}C]Acetate		$^3\text{H}_2\text{O}$	
	(c.p.m./mg protein)	(% of control)	(c.p.m./mg protein)	(% of control)
(a) Control	18 859 $\pm$ 620	100	959 $\pm$ 68	100
–Serum (24 h)	4043 $\pm$ 273	21.4	254 $\pm$ 40	26.4
–Serum (48 h)	3437 $\pm$ 507	18.2	214 $\pm$ 34	22.3
	[^{14}C]Acetate		[^3H]Mevalonolactone	
	(c.p.m./mg of protein)	(% of control)	(c.p.m./mg of protein)	(% of control)
(b) Control	20 221 $\pm$ 510	100	3438 $\pm$ 232	100
–Serum (6 h)	8724 $\pm$ 448	43.1	2444 $\pm$ 43	71.1
–Serum (24 h)	5806 $\pm$ 86	28.7	2406 $\pm$ 140	70.0

Fig. 2. Reversibility of the inhibition of sterol synthesis in serum-deprived glia

C-6 cells were plated as described in the legend to Fig. 1. After 2 days of growth in medium supplemented with 5% (v/v) delipidated serum, all of the cultures were washed and changed to serum-free medium. The cells were maintained in serum-free medium for 2 days, after which the remaining cultures were fed with fresh medium containing 5% delipidated serum. Cell growth (a) and sterol synthesis (b) were monitored throughout the experiment. At the designated times, the cultures were incubated with [¹⁴C]acetate and the total protein and incorporation of radioactivity into digitonin-precipitable sterols were measured as described in the Experimental section. Each point represents the mean ($\pm$ s.d.) for separate determinations performed on three cultures.

the assay (Berndt *et al.*, 1976; Nordstrom *et al.*, 1977; Maltese & Volpe, 1979a; Saucier & Kandutsch, 1979). The conversion of latent enzyme into an active form presumably proceeds through the action of phosphatase, since fluoride has been shown to block the increase in enzyme activity in all of these studies. We therefore preincubated glial cell homogenates and assayed HMG-CoA reductase both in the presence and absence of 50 mM-NaF (Fig. 3b). In the absence of fluoride, the activity of the reductase underwent a 52% decline after 6 h of serum deprivation, and finally reached a value that

Fig. 3. Effects of serum deprivation on the activities of acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase

Cells were plated in 75 cm² flasks at a density of 6×10^4 cells/cm². After 2 days of growth in medium supplemented with 5% (v/v) delipidated foetal calf serum, all of the cultures were washed and changed to serum-free medium. At the indicated times, parallel cultures were harvested and assayed for (a) acetoacetyl-CoA thiolase (●), HMG-CoA synthase (▲) and (b) HMG-CoA reductase (see the Experimental section). For the reductase assays, the cells from each culture were divided into two equal portions, to be preincubated and assayed either with (○) or without (●) 50 mM-NaF in the buffers. Each point represents the mean ($\pm$ s.d.) for separate assays performed on three cultures.

was only 11% of the activity measured in the cultures before removing delipidated serum. Addition of fluoride to the buffers did not affect the magnitude of the decline in reductase activity during the first 6 h of serum deprivation; however, in the presence of fluoride, the enzyme activities determined at 0 and 6 h after removal of serum were 55–60% lower than those measured in the absence of fluoride. Thus, although a substantial quantity of the reductase in C-6 glia appears to exist in a latent form, the decline in enzyme activity measured during the first 6 h after withdrawal of serum cannot be attributed to an increase in the ratio of latent/active enzyme. Changes in the amount of latent HMG-CoA reductase did occur after 6 h of serum deprivation. In the presence of fluoride, the activity of the enzyme remained relatively constant between

6 and 24 h, whereas the activity in samples without fluoride continued to decline until 24 h (Fig. 3b).

Effects of removal of serum on the sterol content of C-6 glia

To determine whether the sharp decline in the rate of sterol synthesis resulted in an alteration of the sterol content of serum-deprived C-6 glia, we compared the total sterol, fatty acid and lipid phosphorus concentrations of the cells before and 24 h after removing delipidated serum from the medium. The data (Table 2) clearly demonstrate that there was no change in the sterol concentration, expressed on a per mg of protein basis. The sterol/fatty acid and sterol/lipid phosphorus ratios underwent a small decline in the serum-free cultures; however, this apparently was due to a slight increase in the fatty acid and phospholipid concentrations in the serum-deprived cells, rather than to a decrease in the sterol concentration.

Effects of removal of serum on fatty-acid synthesis de novo

If sterol synthesis and cell growth were to cease in serum-free cultures, while fatty acid synthesis continued at a high rate characteristic of rapidly dividing cells, one might expect to detect a substantial decline in the sterol/fatty acid molar ratio. Thus, the relatively small change in this parameter (Table 2) suggested that the rate of fatty-acid synthesis in the serum-deprived cultures might be regulated in co-ordination with the rate of sterol

synthesis. Experiments in which the incorporation of radioactivity from $^3\text{H}_2\text{O}$ into fatty acids was measured indicated that fatty-acid synthesis was indeed reduced in serum-deprived glia, but that the magnitude of the decline after 24 h (31.2%) was approximately half as great as the decline in sterol synthesis. Moreover, fatty-acid synthesis continued to decrease after 24 h, whereas the decline in sterol synthesis was essentially complete after the first day of serum deprivation. Thus, the change in fatty-acid synthesis is not as abrupt as the change in sterol synthesis, and does not appear to be closely correlated with the cessation of DNA synthesis.

Discussion

A number of studies have shown that inhibition of sterol synthesis in cultured cells leads to a cessation of cell proliferation, unless exogenous sterol is supplied in the culture medium (Brown & Goldstein, 1974; Kandutsch & Chen, 1977; Cornell *et al.*, 1977; Doi & Endo, 1978; Goldstein *et al.*, 1979; Chang *et al.*, 1979). Moreover, recent studies have demonstrated that an increase in synthesis of mevalonate must precede or accompany the entrance of synchronized mammalian cells into the S phase of the cell cycle (Quesney-Huneeus *et al.*, 1979; Habenicht *et al.*, 1980). These observations support the hypothesis that sterol synthesis may be coupled to the regulation of cell growth under certain circumstances (Chen *et al.*, 1978; Kandutsch *et al.*, 1978).

The present data demonstrate that sterol synthesis undergoes a marked decline as C-6 glioma cells pass from a rapidly proliferating state to a quiescent state after delipidated serum is removed from the culture medium. Brown *et al.* (1974) previously observed that the activity of HMG-CoA reductase in confluent fibroblasts in serum-free medium is lower than it is in cells maintained in medium containing lipoprotein-deficient serum. Fogelman *et al.* (1977) suggested that this suppression of HMG-CoA reductase activity might be due to a decline in efflux of cellular sterol, caused by removal of an extracellular sterol acceptor present in serum. Thus the low rate of cholesterologenesis in serum-deprived C-6 glioma cells may be similarly related to a change in sterol efflux. However, the correlation between the cessation of DNA synthesis and the decline in sterol synthesis after removal of delipidated serum raises the alternative possibility that the decreased rate of cholesterologenesis reflects an accumulation of non-dividing glial cells, which require less sterol for membrane biogenesis. Although additional studies are needed to distinguish between these alternatives, the observation that HMG-CoA reductase activity also declines as C-6 glioma cells enter a quiescent state at high cell density (W. A. Maltese & J. J.

Table 2. *Effects of serum deprivation on the sterol, fatty-acid and phospholipid contents of C-6 glia*

Cells were plated in 75 cm² flasks at a density of 6×10^4 cells/cm². Control cells were harvested after 2 days of growth in medium supplemented with 5% delipidated serum. Cells designated as —serum were maintained for an additional 24 h in serum-free medium. Protein, sterol, fatty-acid and lipid-phosphorus determinations were performed on whole cells as described in the Experimental section. Each value is the mean ($\pm$ S.D.) for three separate determinations. Two flasks of cells were pooled for each determination.

	Control	—Serum
Sterol (nmol/mg of protein)	47.3 $\pm$ 2.4	46.5 $\pm$ 4.1
Fatty acid (nmol/mg of protein)	175.2 $\pm$ 4.7	208.6 $\pm$ 14.9
Lipid phosphorus (nmol/mg of protein)	158.7 $\pm$ 2.8	187.8 $\pm$ 5.9
Sterol/fatty acid (molar ratio)	0.270 $\pm$ 0.015	0.224 $\pm$ 0.030
Sterol/lipid phosphorus (molar ratio)	0.298 $\pm$ 0.020	0.247 $\pm$ 0.015

Volpe, unpublished work) suggests that changes in the rate of cell proliferation may be of primary importance in the regulation of sterol synthesis in this cell line.

The co-ordinate decline in the activities of HMG-CoA synthase and HMG-CoA reductase, which occurs in conjunction with the decrease in the rate of sterol synthesis in quiescent glial cells, is consistent with the results of previous studies showing that factors that inhibit or stimulate hepatic sterol synthesis and HMG-CoA reductase activity (e.g. cholesterol feeding, cholestyramine) also produce corresponding changes in the activity of cytosolic HMG-CoA synthase (Clinkenbeard *et al.*, 1975b). Co-ordinate regulation of the synthase and the reductase also has been demonstrated in the adrenal gland (Balasubramaniam *et al.*, 1977). The finding that a decline in HMG-CoA synthase activity accompanies the decline in sterol synthesis in C-6 glioma cells is of particular interest in view of the data of Ramachandran *et al.* (1978) demonstrating that HMG-CoA synthase, rather than HMG-CoA reductase, regulates the rate of cholesterol synthesis in HeLa cells in response to glucocorticoids. Thus, pending further study, we suggest that the concept of HMG-CoA reductase as the sole regulatory enzyme controlling sterol synthesis in the glia of the developing nervous system may represent an oversimplification.

The mechanisms underlying the suppression of HMG-CoA synthase and HMG-CoA reductase activities in response to serum deprivation are presently unknown. Inactivation of the reductase via a phosphorylation mechanism, as described in several reports (Nordstrom *et al.*, 1977; Beg *et al.*, 1978; Ingebritsen *et al.*, 1978; Mitropoulos *et al.*, 1980), would be compatible with the rapidity of the decline in enzyme activity; however, the rapid turnover of the reductase, reflected in a half-life of approximately 3 h (Dugan *et al.*, 1972), also would permit abrupt changes in enzyme synthesis or degradation to produce a rapid decrease in activity. The results of the assays carried out in the presence and absence of fluoride clearly indicate that the initial decline in HMG-CoA reductase activity, immediately after withdrawal of serum, is not due to conversion of active enzyme into a latent form that can be activated during preincubation *in vitro*. However, our data do suggest that when the enzyme is assayed in the absence of fluoride, degradation of latent (i.e. physiologically inactive) enzyme may account for most of the decline in reductase activity measured after 6 h of serum deprivation.

If cell growth (i.e. the approximate doubling of cell mass in preparation for mitosis) were to continue in the absence of an adequate supply of sterols, one might expect to observe a marked decrease in the cellular sterol concentration. Therefore, the fact that

the sterol/protein ratio of the serum-deprived glial cells remained constant constitutes strong evidence for a close temporal relationship between the cessation of glial cell cycling and the suppression of sterol synthesis. The decline in the rate of fatty-acid synthesis may be secondary to the decline in sterol synthesis and the cessation of cell growth, since the fatty-acid/protein ratio increased by approx. 20% in the serum-starved glial cells, whereas the sterol/fatty-acid ratio underwent a corresponding decrease. Nevertheless, the change in the rate of fatty-acid synthesis apparently was sufficient to prevent the development of severe imbalances in the cellular sterol/fatty-acid and sterol/phospholipid ratios, which might have led to disruption of vital cell functions that are influenced by membrane lipid composition. Future studies with isolated glial membrane fractions will be necessary to ascertain the extent to which the whole-cell lipid ratios determined in the present experiments reflect the lipid composition of the cellular membrane network.

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