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Hydrocortisone stimulates the development of oligodendrocytes in primary glial cultures and affects glucose metabolism and lipid synthesis in these cultures

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Cultures of glial cells were prepared from the brains of one-week-old rat pups. After one day in culture, serum was omitted from the medium and replaced by a combination of growth-stimulating hormones and other factors that enhanced the percentage of oligodendrocytes in the cultures. We investigated the effects of hydrocortisone on the development of oligodendrocytes, on the activities of oligodendrocyte-specific enzymes and on glucose- and lipid-metabolism of the glial cells. (1) Hydrocortisone greatly enhanced the survival of glial cells in culture. (2) The development of galactocerebroside-positive cells and the specific activity of 2′,3′-cyclic-nucleotide 3′-phosphodiesterase were stimulated by 50 nM hydrocortisone, whereas these effects were partly reversed at higher concentrations of the hormone. (3) The specific activity of glycerol-3-phosphate dehydrogenase was markedly stimulated by hydrocortisone; 1 μM or higher concentrations of hydrocortisone were required for an optimal effect. (4) The consumption of glucose and the production of lactate were lowered by hydrocortisone whereas the oxidation of [6-^{14}C]glucose to $^{14}CO_2$ was not affected. (5) Incorporation of [^{35}S]sulfate into sulfolipids was greatly enhanced by hydrocortisone and [^{14}C]incorporation from [1-^{14}C]acetate into cholesterol and fatty acids was also stimulated but to a smaller extent. These results show that hydrocortisone (i) exerts a general trophic function on glial cells in our culture system; (ii) enhances the ratio of oligodendrocytes over astrocytes, possibly by directing bipotential progenitor cells to develop into oligodendrocytes; (iii) specifically induces glycerol-3-phosphate dehydrogenase in oligodendrocytes.

INTRODUCTION

Steroid hormones are important for the normal development and functioning of the brain[23,24]. The level of corticosterone in the blood of rat pups is low shortly after birth but rises in the second postnatal week to reach a peak at the end of the third week[12]. This increase of plasma corticosterone is preceded by a dramatic rise of plasma thyroid hormones[40] between day 7 and 12. Oligodendrocytes start to synthesize myelin in rat brain after the first postnatal week and the rate of myelin deposition increases until the end of the third week[28]. Hence, it is plausible that glucocorticosteroids, hereafter referred to as corticosteroids, may play a role in the process of myelination in rat brain.

On the other hand, we should note that experiments carried out in vivo led to a different conclusion, viz. that corticosteroids lower the rate of myelination[26,27], because the administration of high doses of various corticosteroids retarded myelination in rat pups[27].

This effect may be mediated by a reduction of the somatotropin (growth hormone) secretion by the pituitary gland as a result of high corticosteroid levels. Simultaneous administration of somatotropin counteracts the inhibitory effect of corticosteroids on myelination[27]. It is known that somatotropin promotes

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the production of somatomedins[11]. Since somatomedin C (insulin-like growth factor I) has recently been shown to be a strong activator of oligodendrocytic development[25], this sequence of events could well explain the inhibition of myelination by pharmacological doses of corticosteroids in vivo. Adrenalectomy of rat pups led to a general retardation of growth whereas myelination was relatively unimpaired; interestingly, myelin of the adrenalectomized animals contained about 10% less galactolipids and phosphatidylcholine than that of controls[26] indicating a specific lesion in myelin lipid synthesis as a result of adrenalectomy. These studies clearly demonstrate the difficulties of sorting out cause and effect of hormonal influences on glial development in intact animals.

Therefore, primary cultures of glial cells maintained in a chemically-defined medium are an important tool to investigate the action of hormones and growth factors on these cells. This study reports on the effects of oligodendrocyte-enriched glial cultures derived from one-week-old rat brain and maintained in a serum-free medium[16].

MATERIALS AND METHODS

Animals, isolation of brain cells and culture

Neurone-free oligodendrocyte-enriched glial cultures were prepared[16] with further modifications[19]. Briefly, cells were isolated from the cerebra of 6–8 days-old rat pups, by trypsinization, trituration and sieving. Subsequently they were suspended in Dulbecco's Modified Eagle Medium containing 10% (v/v) newborn-calf serum and plated on poly-L-lysine-coated culture dishes. These oligodendrocyte-enriched glial cultures were maintained after one day in a serum-free medium[33] with further modifications[19]. Cytosine-1-β-D-arabinofuranoside (10 μM) was added to the medium after 48 h in culture to prevent astroglial overgrowth. The cultures consisted of about 65% oligodendrocytes, 30% astrocytes and 5% other cells at 7 days in vitro[16].

Enzyme assays

The cultures were washed twice with ice-cold Hank's buffered salt solution and the cells were scraped from the culture dishes and suspended in a small volume of phosphate-buffered saline (200 μl per culture dish of 20 cm^2). The cells were homogenized by sonication (2 × 15 s; maximal output), an aliquot was removed for the assay of protein[21] and subsequently dithioerythritol and bovine serum albumin were added (final concentrations, 5 mM and 1% (w/v), respectively). The activity of glycerol-3-phosphate dehydrogenase (G3PDH) was measured on the same day, whereas samples for 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) assays could be stored for at least 2 weeks at −20 °C without loss of enzymatic activity.

G3PDH activity was measured spectrophotometrically (Aminco dual wavelength, DW2a, spectrophotometer; 340–400 nm). The reaction medium (final volume, 1 ml) contained 50 mM Tris (pH 7.5), 1 mM EDTA, 0.15 mM NADH, 0.5 mM lithium dihydroxyacetone phosphate (Sigma, St. Louis, MO, U.S.A.). The reaction was started by adding a sample of the homogenate (10–100 μg protein) and the rate at which the absorbance declined was recorded during 2 min. This rate was corrected for the decline that occurred in control incubations carried out in the absence of dihydroxyacetone phosphate.

The activity of 2′,3′-cyclic nucleotide 3′-phosphodiesterase was measured spectrophotometrically with 2′,3′-cyclic $NADP^+$ as substrate[36]. The enzyme was activated by preincubating (10 min, 0 °C) 50 μl of the cell homogenate (10–50 μg of cellular protein with 75 μl of Triton X-100/Tris (0.7% (v/v)/70 mM, pH 7.5). Thereafter, 375 μl icecold H_2O was added and 50 μl was used to start the enzymatic reaction; final volume, 1 ml.

Enzyme activities were measured at 30 °C and the assays were carried out in duplicate.

Metabolic experiments

Glucose and lactate concentrations were assayed spectrophotometrically using enzymatic methods as described previously[38]. Incubations with [6-^{14}C]glucose were carried out with cells grown in flasks (25 cm^2, Nunc, Denmark) and $^{14}CO_2$ was collected and counted as detailed elsewhere[39].

The incorporation of ^{14}C from [1-^{14}C]acetate into lipid fractions was determined[38]. After addition of carrier lipid to an aliquot of the sonicated cell suspension, total lipids were extracted and saponified[15]. Cholesterol and fatty acid fractions were obtained by subsequent extractions with petroleum ether and the fraction that contained sterols was purified by precip-

itation of cholesterol with digitonin[20].

Experiments with [^{35}S]sulfate were carried out with culture medium without streptomycin sulfate; the final sulfate concentration in this medium was about 5 μM. The ^{35}S incorporation into sulfolipids was measured as described by Flynn et al.[10]. All experiments were repeated at least 3 times; typical results are shown.

RESULTS

Glucocorticosteroids stimulate the development of oligodendrocytes in culture

Primary cultures of glial cells derived from one-week-old rat pups can be maintained in a serum-free medium that favours the development of oligodendrocytes[16]. This medium contains 0.35 μM insulin, 3 nM triiodothyronine (T_3) and 55 nM hydrocortisone as hormonal supplements and other ingredients such as transferrin, bovine serum albumin and selenite. Whereas insulin has a general trophic function for cells in culture[4], T_3 (ref. 19) and hydrocortisone stimulate the development of oligodendrocytes in our culture system specifically.

Fig. 1 shows that omission of hydrocortisone from the medium drastically lowers the number of oligodendrocytes (galactocerebroside-positive cells[31]). At day 4, the earliest time point at which identification is possible, the number of oligodendrocytes in cultures grown in a hydrocortisone-free medium is about 70% of the control and gradually drops to 23% at day 10. The relative number of oligodendrocytes, expressed as percentage of the total number of cells, was about 60% in the control cultures (Fig. 1) but varied (50–80%) among cell isolations. This percentage was consistently lower in cultures grown in a hydrocortisone-free medium. Apparently, hydrocortisone affects this culture system in two ways: (i) It improves the survival of glial cells in culture; (ii). It enhances the percentage of oligodendrocytes.

The specific activities of oligodendrocyte-asso-

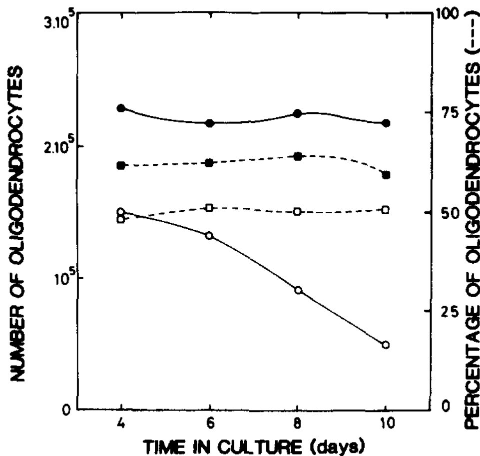

Fig. 1. Effect of omission of hydrocortisone on the development of glial cells in culture. Cells were grown in complete medium (●, ■) or in medium without 55 nM hydrocortisone (○, □). The oligodendrocytes (number of galactocerebroside-positive cells per 20 cm^2) were counted (●, ○) and expressed as the percentage of the total number of cells (■, □).

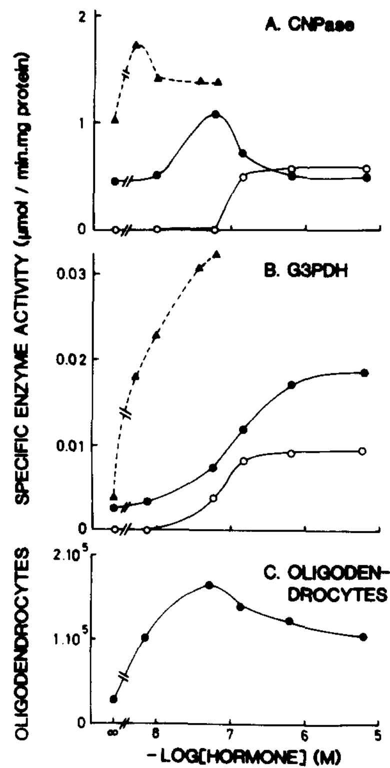

Fig. 2. Dose-dependent stimulation by glucocorticosteroids of oligodendroglial development in culture. The serum-free culture medium contained various concentrations of hydrocortisone (●, ○) or dexamethasone (▲) as indicated. Cells were harvested after 4 days (○) or 6 days (●, ▲) in culture and the protein content and activities of CNPase (panel A) and G3PDH (panel B) were measured. The number of oligodendrocytes per culture was assessed after 7 days in culture. The experiments with dexamethasone were carried out with a different cell preparation which contained a higher percentage of oligodendrocytes as reflected by the higher specific activities as compared to the results with hydrocortisone. Values are means of triplicates (S.D. less than 15%).

ciated enzymes, CNPase and G3PDH, were increased upon addition of hydrocortisone to the medium. Various concentrations of hydrocortisone and dexamethasone were tested as shown in Fig. 2. Concentrations of hydrocortisone in excess of 10^{-7} M appeared to be suboptimal both with regard to the number of oligodendrocytes and to the specific activity of CNPase. The higher activity of CNPase in cells cultured in the presence of corticosteroids probably reflects a higher percentage of oligodendrocytes in the cultures (Fig. 1) rather than a specific induction of the enzyme. The specific activity of G3PDH, on the other hand, was stimulated at least 7-fold by dexamethasone and hydrocortisone, which is in line with the induction of this enzyme in oligodendrocytes by corticosteroids[35,42].

The specific activities of CNPase and G3PDH increased during the development of the cells in culture (Figs. 2 and 3). Maintenance of the cells in hydrocortisone-free medium resulted in low levels of G3PDH (Fig. 3B). These basal G3PDH levels varied with the

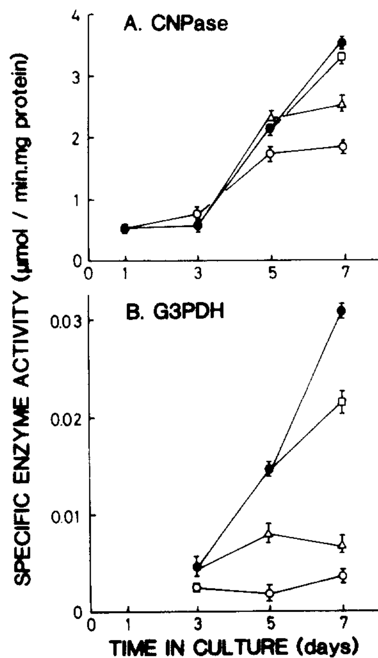

Fig. 3. The effect of omission of hydrocortisone from the medium at various timepoints in culture on the activities of oligodendrocyte-specific enzymes. Cells were grown in complete medium (●) or in medium from which hydrocortisone was omitted after day 1 (○), day 3 (△) or day 5 (□) in culture. Cells were harvested as indicated and the specific activities of CNPase (panel A) and G3PDH (panel B) were determined.

TABLE I

Stimulation of the activity of glycerol-3-phosphate dehydrogenase (G3PDH) by hydrocortisone at various time-points in culture

Oligodendrocyte-enriched glial cultures were grown in serum-free medium. The effects of omission and readdition of 0.25 µM hydrocortisone on the activity of G3PDH were evaluated. Cells were harvested after 4 and 6 days in culture.

Culture condition	*Serum-free medium*	*Specific activity of G3PDH (nmol/min mg protein)*	
		4 days in culture	*6 days in culture*
A	With hydrocortisone	10.1 ± 0.4	26.7 ± 0.3
B	Without hydrocortisone	n.d.*	4.6
C	As A but hydrocortisone omitted at days 5 and 6	10.1 ± 0.4	9.5 ± 0.2
D	As B but hydrocortisone added at days 5 and 6	n.d.*	13.6 ± 1.2

* Not detectable.

relative number of astrocytes in the culture (data not shown). Omission of hydrocortisone from the medium after 3 or 5 days resulted in lower specific activities of G3PDH, indicating that hydrocortisone had to be present at least until day 5 to achieve maximal expression of this enzyme (Fig. 3B). The effects of hydrocortisone on CNPase (Fig. 3A) were qualitatively similar but less marked than those on G3PDH.

In a complementary experiment (Table I) cells were grown during 4 days in a hydrocortisone-free medium and were subsequently switched to a medium with hydrocortisone. After 6 days in vitro the level of G3PDH in these cells was about 3-fold higher than in cells maintained in a medium without hydrocortisone; the specific activity being about half of that of cells grown in hydrocortisone-containing medium during the whole period.

Influence of hydrocortisone on the metabolism of oligodendrocyte-enriched glial cultures

We reported earlier that glycolysis and lactate production are high, whereas aerobic oxidation of glucose is low in this culture system[38]. Fig. 4 illustrates how various doses of hydrocortisone affect these parameters. At the highest concentration of hydrocortisone in the medium, glucose consumption and lactate synthesis were reduced by one third. The production of $^{14}CO_2$ from [6-^{14}C]glucose was not affected, indi-

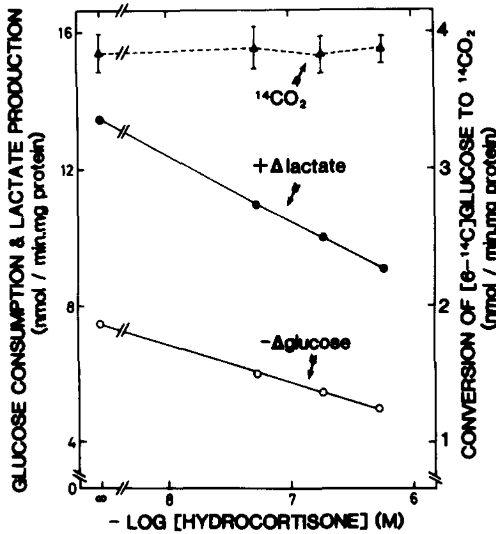

Fig. 4. Glucose metabolism of glial cells during 5 days in serum-free medium supplemented with various concentrations of hydrocortisone. After 1, 3 and 5 days in culture, the cells were given fresh medium (containing 6 mM D-glucose). At 20 h after the last medium change, samples of the media were taken for the estimation of glucose and lactate. Incubation with 6 mM [6-^{14}C]glucose (spec. act., 0.4 Ci/mol) were carried out after 5 days in culture during 5 h. $^{14}CO_2$ production was measured (Materials and Methods); values are means ± S.D. (n = 3).

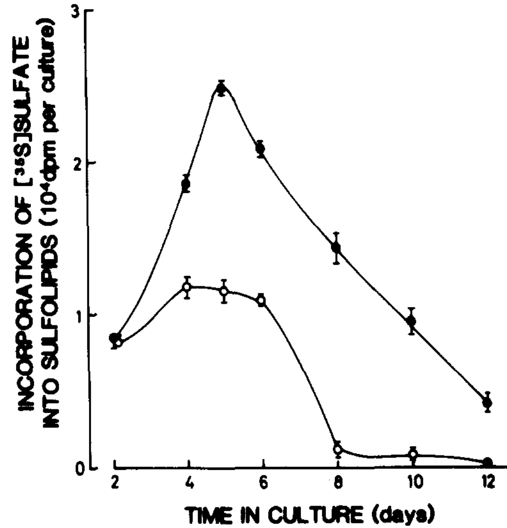

Fig. 5. Effect of hydrocortisone on the incorporation of [^{35}S]sulfate into sulfolipids. Cultures were grown in complete medium (●) or in medium without hydrocortisone (○). Cells were incubated with 5 μM [^{35}S]sulfate (0.5 Ci/mmol) during 24 h and cells were harvested at the indicated time points. Means ± S.E.M. of triplicate incubations are presented.

cating that Krebs-cycle activity was not inhibited.

Oligodendrocytes and astrocytes in serum-free culture media actively synthesize fatty acids and cholesterol from various precursors[17,19]. Table II shows that lipogenesis and cholesterogenesis from [1-^{14}C]-acetate were markedly stimulated by hydrocortisone.

Finally, we investigated the effect of hydrocortisone on sulfolipid synthesis. Sulfolipid synthesis is an oligodendrocyte-specific process[18] with a rather sharp optimum between days 4 and 6 in vitro[19]. A much less pronounced optimum was observed when the cells were maintained in a medium without hydrocortisone where the rate of ^{35}S incorporation dropped to a very low level after one week in culture (Fig. 5).

TABLE II

Enhanced synthesis of lipids by glial cultures grown in the presence of hydrocortisone

Oligodendrocyte-enriched glial cultures were grown in serum-free medium in the presence or absence of hydrocortisone. After 5 days the cultures were incubated during 4 h with 0.1 mM [1-^{14}C]acetate (spec. act., 12 Ci/mol) and the ^{14}C incorporation into lipids was analysed. Means ± S.D. are presented of triplicate incubations in nmol/mg protein·h.

Serum-free culture medium	*^{14}C incorporation of [1-^{14}C]acetate*		
	Total lipids	*Fatty acids*	*Cholesterol*
Without hydrocortisone	2.8±0.1	1.8±0.1	0.33±0.03
+ 0.25 μM hydrocortisone	3.8±0.2	2.6±0.1	0.48±0.05

DISCUSSION

The aim of this investigation was to characterize the effects of corticosteroids on the development of glial cells isolated from the brains of one-week-old rat pups; cultures being maintained in a serum-free medium[16]. Adrenal steroids can stimulate genomic activity in the brain as in non-neural target tissues[23]. Specific receptors for various steroid hormones have been found in discrete brain areas, various types of neural cells[24] and in C_6 glioma cells[3]. The induction by corticosteroids of glycerol-3-phosphate dehydrogenase in oligodendrocytes[22,35] and of glutamine synthetase in astrocytes[14] is therefore probably a direct effect of the steroid binding to a cytosolic receptor.

Corticosteroids stimulate the development of oligodendrocytes in culture

Survival and maturation of oligodendrocytes in serum-free culture medium requires supplementation

of the medium with hormones. A high concentration of insulin has been shown to be important[6,8,18,35] and inclusion of thyroid hormones is recommended by most[1,8,19,37] but not all[6,35] authors. The present results show that physiological concentrations[12] of hydrocortisone (10–50 μM) and T_3 (3 nM) promoted the development of oligodendrocytes (Figs. 1 and 2A) defined as galactocerebroside-positive cells[31]. This conclusion is further supported by the observation that hydrocortisone stimulated the specific activity of CNPase (Figs. 2 and 3) and that this hormone enhanced ^{35}S incorporation into sulfolipids (Fig. 5). High concentrations of hydrocortisone (0.1–10 μM) resulted in lower numbers of oligodendrocytes and impaired CNPase activity (Fig. 2). This inhibitory effect of high levels of corticosteroids on the development and function of oligodendrocytes is in agreement with previous observations[7,33,37].

The hydrocortisone-induced synthesis of glycerol-3-phosphate dehydrogenase in the brain, that occurs specifically in oligodendrocytes at the time of myelin synthesis, has been studied extensively by De Vellis et al.[42]. We could confirm this in our culture system (Fig. 2B) and showed that the induction is reversible (Table I) and requires the continuous presence of hydrocortisone for its full expression (Fig. 3).

In short, our present working hypothesis is that corticosteroids affect the development of cells in this culture system in two ways: (i) more progenitor cells[9] are committed to become oligodendrocytes; (ii) the synthesis of specific proteins, such as glycerol-3-phosphate dehydrogenase, is induced.

Whether corticosteroids affect oligodendrocytes directly or indirectly remains to be elucidated. Our culture system is enriched in oligodendrocytes (50–80%) but also contains astrocytes. Therefore, it is possible that astrocytes rather than oligodendrocytes are the primary target of corticosteroids. Astrocytes might respond by secreting lipocortin[29,41] or other factors[2] and thus stimulate oligodendrocytes indirectly. On the other hand, the induction of G3PDH appears to be a direct effect of corticosteroids on oligodendrocytes in primary cultures prepared from neonatal rat brain[22].

Corticosteroids reduce lactate formation but enhance lipid synthesis by glial cells

Very little is known about metabolic effects of corticosteroids on glial cells in culture. Previously we reported that aerobic oxidation of glucose contributes less than 1% to its utilization and that more than 30% is converted into lactate[39]. The present data show that hydrocortisone lowered the rate of anaerobic glycolysis but did not affect the production of $^{14}CO_2$ from [6-^{14}C]glucose in the Krebs cycle (Fig. 4).

Glial cells growing in a serum-free medium actively synthesize fatty acids and cholesterol[17,38]. Both astrocytes and oligodendrocytes contribute to the observed de novo lipid synthesis, but the rates are higher in oligodendrocytes than in astrocytes[17]. The present results indicate that hydrocortisone must be present in the medium to achieve optimal rates for ^{14}C incorporation from [1-^{14}C]acetate into fatty acids and cholesterol (Table II).

These data on glucose metabolism and lipid synthesis suggest that hydrocortisone influences the metabolism of glial cells in culture. However, an alternative explanation could be that hydrocortisone enhances the percentage of oligodendrocytes in the cultures (Fig. 1) and that the observed changes in metabolism reflect the enrichment of the cultures with regard to this cell type.

Sulfolipids are characteristic components of myelin[30] and their synthesis occurs exclusively in oligodendrocytes in our culture system[17]. Our experiments indicate that both T_3 (ref. 19) and hydrocortisone (Fig. 5) are needed to induce maximal rates of sulfolipid synthesis in neurone-free, oligodendrocyte-enriched glial cultures. These results complement data reported by other groups working with different model systems[13,37].

Towards an optimal, serum-free culture medium for oligodendrocytes

The search for factors promoting the attachment and growth of neural cells in culture necessitates the replacement of serum by a chemically defined medium[5]. For our purpose we adapted the serum-free but rather complex medium developed by Romijn et al.[33] for the culture of neurons. This medium proved to be beneficial both for the growth and for the development of oligodendrocytes. The absence of serum[32] and the presence of insulin, triiodothyronine[19], hydrocortisone, transferrin and selenite were the most important features of this medium to achieve optimal numbers of oligodendrocytes after one week in cul-

ture[18].

Insulin, transferrin and selenite are common ingredients of most serum-free media developed for oligodendrocytes (e.g. refs. 4, 8, 33, 34). Triiodothyronine and hydrocortisone appear to be essential for the optimal development of oligodendrocytes in our culture system. Eccleston and Silberberg[8] reported that T_3 but not hydrocortisone is essential for optimal development of oligodendrocytes, using the number of galactocerebroside-positive cells as criterium. The formulations for serum-free media by Bottenstein[5] and by Saneto and de Vellis[34] notably lack T_3 and hydrocortisone. The model system of the latter authors[34] requires preculture of mixed glial cells in conventional serum-containing medium for at least one week, whereafter a purified preparation of oligodendroblasts is subcultured without serum with addition of insulin, transferrin and fibroblast growth factor during another week. T_3 did not promote cell proliferation; data on influences of T_3 and hydrocortisone on differentiated properties of the oligodendrocytes were not presented. Bottenstein[5] prepared dissociated neonatal rat brain cultures in serum-containing medium. After two days, cells were transferred to a serum-free medium containing bovine insulin, human transferrin, sodium selenite and biotin. These cultures contained up to 40% oligodendrocytes (galactocerebroside-positive cells). Effects of other hormones were not reported.

We conclude that there is as yet no consensus about an optimal, serum-free medium for the primary culture of oligodendrocytes. This is not surprising in view of the variety of experimental animals used as source of brain cells; the species and especially the age of the animal are important parameters for the development of the cultures. Furthermore, the possibility that corticosteroids work in an antagonistic or a synergistic manner with respect to other hormonal supplements complicates the analysis of their effects in the various serum-free media. Experiments to analyse these hormonal interactions are in progress.

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