Brain Research, 102 (1976) 267–281

EFFECTS OF CERAMIDE ANALOGS ON MYELINATING ORGAN CULTURES

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(Accepted July 11th, 1975)

SUMMARY

Analogs of ceramide which inhibit galactocerebrosidase also demyelinate or inhibit myelination in organ cultures of rat cerebellum. The potency of the analogs in culture correlated with their effectiveness as inhibitors of cerebrosidase, but not with their effectiveness as inhibitors of galactosyl transferase. The most effective compound was the decanoyl amide of 3-phenyl-2-amino-1,3-propanediol with *erythro*-conformation. Stimulators of cerebrosidase also demyelinated cultures. With both groups of compounds, myelin sheaths became distorted, then broke into lipid droplets. Axons were preserved, but neurons showed some nuclear changes and granularity. Metabolic studies with the most effective inhibitor showed that glucose incorporation into cerebroside and other alkali-stable lipids was initially depressed compared to proteins and total lipids.

INTRODUCTION

Arora, Lin and Radin have synthesized compounds, some of which inhibit[1,3] and some of which stimulate[4] the degradative enzyme galactosyl ceramide β-galactosidase (cerebrosidase). These compounds are of general interest because they are among the first analogs available to interfere with specific steps of galactolipid metabolism. The compounds are of special interest because the enzyme they affect is markedly decreased in Krabbe's disease[21], a dysmyelinating disorder. Thus, the inhibitors may provide a unique opportunity to examine mechanisms by which

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decreased levels of a degradative enzyme lead to pathologic changes in the nervous system[16].

We examined these compounds in myelinating organ cultures of rat cerebellum where direct application of the analogs and observation of the cultures was possible. We asked the following questions.

(1) Is the ability of an analog to demyelinate or inhibit myelination related to its *in vitro* effect on cerebrosidase?

(2) When demyelination occurs, is there preservation of axons and neurons?

(3) Is metabolism of cerebroside in fact affected in the organ cultures?

(4) Do other metabolic changes occur and if so, what is their temporal relationship to the changes in cerebroside metabolism?

MATERIALS AND METHODS

Analogs

Three series of compounds synthesized by Arora and Radin[3,4] were screened in the organ cultures. The first two series were (1) hydroxy- and (2) non-hydroxy-analogs of ceramide which inhibited cerebrosidase[3]. The third series included N-acyl derivatives of 2-amino-2-methyl-1-propanol, several of which stimulated cerebrosidase[4]. The structures of the compounds tested are shown in Tables I and II. The synthetic analogs were stored desiccated at −20 °C until use. Analogs were dissolved in chloroform–methanol and the desired aliquots were transferred to sterile glass screwtop tubes and dried with nitrogen at room temperature. Nutrient medium was added, and the mixture was sonicated for 5–10 min in a water-bath sonicator until clear. With analogs containing longer chain fatty acids, a fine cloudy suspension resulted.

Preparation and maintenance of cultures

The methods for preparing and maintaining organ cultures from rat cerebellum were essentially those of Bornstein and Murray[7] as modified by Silberberg[18,19]. Newborn Sprague–Dawley rats, less than 24 h old, were anesthetized by exposure to cold (−20 °C for 7 min). The cerebellum was removed, stripped of meninges, and cut into 9–10 fragments. Each fragment was placed on a collagen-coated coverslip in a 35 mm × 10 mm plastic tissue culture dish. About 0.4 ml of nutrient medium was added to each dish. The routine maintenance nutrient medium was 20% Earle's minimal essential medium, 40% (v/v) Earle's balanced salt solution (BSS), and 40% (v/v) fetal calf serum (Microbiological Associates, Bethesda, Md.) supplemented with glucose to 6 mg/ml, 0.4 units of zinc-free insulin/ml (a gift of Squibb Institute for Medical Research, New Brunswick, N.J.), 0.002% phenol red, and 1.7 μg/ml tetracycline. In some experiments, 10 m*M* HEPES buffer was included in all media. The cultures were maintained at 34.5 °C in a CO_2 incubator and fed twice weekly.

Four days before addition of analogs, cultures were transferred from plastic dishes to the Maximow slide assemblies described by Bornstein and Murray[7]. These assemblies require smaller amounts of medium and allow more detailed observation

of the cultures. Cultures were selected for histological normalcy, then divided randomly into control and experimental groups, so that the control group contained cultures from several animals.

In each experiment, 4–10 control cultures were observed with the experimental cultures from the same explant. Cultures were observed and photographed at various times after addition of analogs. For histology, selected cultures were fixed in buffered formalin and stained as whole mounts with a modified Holmes silver impregnation[22].

To determine whether a compound *demyelinated cultures*, we transferred well-myelinated cultures to Maximow assemblies at 15 days *in vitro* (DIV), then fed the cultures normal or experimental medium at 18–19 DIV. The cultures were observed at 4–8 h intervals. In the control groups, spontaneous demyelination was rarely observed; 249 of the 256 control cultures remained well-myelinated throughout the experimental period. A well-myelinated culture was one which had 20 or more visible myelinated axons (Fig. 1a, b). Cultures were considered demyelinated when (a) no myelin was visible, (b) the number of myelinated axons decreased to less than three-fourths, or (c) all of the myelin was grossly lumpy and irregular.

To determine whether a compound *inhibited myelin formation*, we transferred cultures to Maximow assemblies at 4 DIV, then substituted the normal medium with experimental medium at 8–9 DIV. Control cultures were fed the normal medium. Cultures were observed for several days to determine whether myelination occurred. In the controls, 52 of 55 cultures myelinated between 10 and 12 DIV.

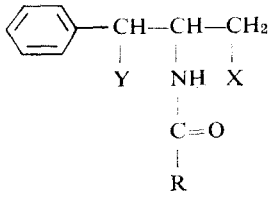
Glucose incorporation

For measurement of glucose incorporation in myelinated cultures, 19-day-old cultures were exposed to D-[U-^{14}C]glucose for 4–48 h. Control and experimental cultures were each fed 20 μl of medium containing 1 μCi of radioactive glucose, spec. act. 1.8 mCi/mmole (New England Nuclear, Boston, Mass.). To stop incorporation, we washed the cultures twice for 15 min in 10 ml of cold BSS, then scraped the cultures from the cover slips into tubes containing 1 ml of 0.32 *M* sucrose. Generally, 5–10 cultures were pooled for each time point and frozen at −20 °C for 1–10 days before extraction.

The pooled cultures were homogenized in a power-driven glass-to-glass homogenizer, then mixed with 0.2 ml of a 20% (w/v) homogenate of cerebellum from a 19 day rat to minimize losses during extraction. Aliquots of 50 μl were removed and mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.) for measurement of total radioactivity. For lipid extraction, each homogenate was mixed with 12 Vol. (24 ml) of chloroform–methanol (1:1), 150 μg of cerebroside, 150 μg of sulfatide, and 6000 counts/min of [6-^{3}H]cerebroside prepared by the borohydride procedure[15]. After 60 min at room temperature, the samples were centrifuged at 1500 rev./min for 10 min, the supernatant was removed, and the remaining pellet was washed with 3 ml of chloroform–methanol. The pellet was transferred to a counting vial with several washes of chloroform–methanol. The solvent was evaporated and the residue was dissolved in 1 ml of NCS (Nuclear Chicago, Des Plaines, Ill.), with heating to 50 °C when necessary. Toluene scintillation solvent and a drop

TABLE I

STRUCTURES OF CERAMIDE ANALOGS TESTED IN MYELINATING ORGAN CULTURES: INHIBITORS OF GALACTO-CEREBROSIDASE*



Amide	*Structure*				*% Inhibition* (0.3 mM)*
	—X (1)	*—Y (3)*	*R*	*Configuration*	
1	H	OH	C_9H_{19}	DL-erythro	3
2	H	OH	$C_{15}H_{31}$	DL-erythro	0
3	OH	H	C_9H_{19}	L-form	18
4**	OH	OH	C_9H_{19}	DL-erythro	26
5	OH	OH	C_9H_{19}	DL-erythro	48
6	OH	H	$CHOHC_{14}H_{29}$	L-form	36
7	OH	OH	$CHOHC_{14}H_{29}$	DL-erythro	54

* From previous studies by Arora and Radin[1,3].
** P-NO_2 group on phenyl ring.

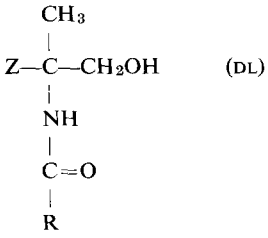
of $SnCl_2$ in 0.1 *N* HCl were added, and radioactivity was measured. The pellet contains primarily proteins and nucleic acids.

The combined chloroform–methanol supernatants were partitioned by adding chloroform and 0.74% KCl containing 5% glucose, mixing well, and centrifuging for 10 min at 1500 rev./min. One milliliter of the upper phase was mixed with 10 ml of Aquasol for measurement of soluble radioactivity. The lower phase was washed 3 times with 0.2 Vol. of theoretical upper phase. A portion of the washed lower phase was removed for measurement of total lipid radioactivity. In preliminary experiments, we evaporated the lower phase to dryness, extracted the residue 3 times with chloroform–methanol to redissolve the lipids, and measured the radioactivity in the residue (presumably some of the proteolipid protein which has been denatured). The residue contained less than 1% of the total lower phase counts, so the evaporation step was routinely omitted.

The remaining washed lower phase was subjected to alkaline cleavage to degrade phospholipids[10]. Portions of the resulting washed lower phase were used for the measurement of radioactivity or for separation by thin-layer chromatography in chloroform-methanol–H_2O (65:25:4). The carrier cerebroside and sulfatide were visualized by brief exposure to iodine. Each lane was divided into 8–12 areas which were scraped directly into vials for counting by addition of toluene scintillation solvent. Recovery of [^{14}C]cerebroside was determined from the recovery of [^{3}H]-cerebroside.

TABLE II

STRUCTURES OF CERAMIDE ANALOGS TESTED IN MYELINATING ORGAN CULTURES: STIMULATORS OF GALACTOCEREBROSIDASE*



Amide	*−Z*	*R*	*%Stimulation (0.3 mM)**
8	H	C_9H_{19}	0
9	CH_3	$C_{17}H_{35}$	10
10	CH_3	$C_{15}H_{31}$	15
11	CH_3	$C_{13}H_{27}$	28
12	CH_3	$C_{11}H_{23}$	29
13	CH_3	C_9H_{19}	55

* From previous studies by Arora and Radin[4].

RESULTS

Effects of inhibitors of cerebrosidase

From the series of aromatic amides synthesized from 3-phenyl-2-amino-1,3-propanediol and tested by Arora and Radin[3], the best inhibitors of cerebrosidase were the analogs with a DL-erythro-1,3-propanediol grouping[3], which resembles sphingosine stereochemically (Table I). Compounds with a hydroxyl group at position 1 alone retained some inhibitory activity, while compounds with a hydroxyl group at position 3 alone were inactive[3].

The most effective inhibitors of cerebrosidase *in vitro*, the 1,3-propanediol analogs, were also the most potent compounds in demyelinating cultures or inhibiting myelination (Table III). The 1-hydroxy compound we tested (amide 3) demyelinated only 1 out of 10 cultures during the 48 h they were observed, but effectively inhibited myelination in younger cultures. The two analogs with a 3-hydroxyl group (amides 1 and 2) did not demyelinate or inhibit myelination.

The ability of the most potent compound, a 1,3-propanediol analog, amide 5, to demyelinate organ cultures was examined in more detail. The lower the concentration of the analog, the longer before demyelination occurred: more than 96 h at 0.3 m*M*, compared to 24 h at 3.0 m*M*.

Two compounds similar to the analogs described above except that they contained α-hydroxy hexadecanoic acid were also tested in the cultures (Table I). Again,

TABLE III

EFFECTS OF INHIBITORS OF GALACTOCEREBROSIDASE ON CULTURES

Cultures were observed at 4–8 h intervals after addition of new medium (controls) or medium containing inhibitor. Ratios show number of cultures exhibiting demyelination or lack of myelination (0/8 means all 8 cultures myelinated) compared to the total number of cultures exposed to the inhibitor. Data in parentheses show the average time when demyelination occurred. In the inhibition experiments, cultures containing inhibitors were last observed on 14–15 DIV, 48–72 h after all of the control cultures had myelinated. Data for demyelination are sums of 2–4 experiments, for inhibition of myelination, 2.

Amide	*Demyelination ratio*		*Myelination inhibition ratio*
	(1 mM amide)	*(3 mM amide)*	*(0.3 mM amide)*
1 (3-OH)		0/5 (48 h)	
2 (3-OH)	0/3 (48 h)	3/27 (48 h)	0/8
3 (1-OH)	0/3 (48 h)	1/10 (48 h)	6/16
4 (diol)		20/32 (12 h)*	15/15
5 (diol)	15/22 (48 h)	17/20 (24 h)	8/8**
6 (1-OH)		1/4 (48 h)	2/8
7 (diol)		24/32 (48 h)	8/8

* Myelin in remaining cultures distorted and lumpy.
** Lethal to cultures by 24 h after addition of analog.

the 1,3-propanediol derivative was more damaging to the cultures than the analog with the hydroxyl group in the 1-position alone.

Cultures exposed to analogs which demyelinated showed the following course of degeneration by bright-field microscopy.

(a) At an early stage of demyelination, myelin appeared lumpy and distorted, but still largely intact (Fig. 1c). Many normal-appearing neurons were evident (Fig. 1d). Some swollen cells with clear watery cytoplasm, diffuse refractile granules and clumped acentric nuclei began to appear. These cells were tentatively identified as glial cells since they contained small nuclei and no Nissl substance.

(b) Myelin then became more distorted, and broke into myelin balls and lipid droplets (Fig. 1e). The number of intact myelin sheaths was markedly reduced. More watery swollen cells appeared. Large neurons began to appear granular (Fig. 1f), but swelling was rarely noted. The neuronal nuclei appeared normal, but were sometimes difficult to see because of the granular cytoplasm.

(c) At late stages of demyelination, few if any intact myelin sheaths could be found. Many lipid droplets accumulated, Neurons showed some evidence of swelling, with granular acentric nuclei and perinuclear Nissl substance. Axon preservation was observed at this stage (Fig. 2).

Cultures exposed to analogs which inhibited myelination appeared similar to the controls except for absence of myelin and some granularity. The neurons appeared healthy. The one exception was amide 5 (Table IV) which appeared toxic to the whole culture; in older cultures, this toxic effect was not observed.

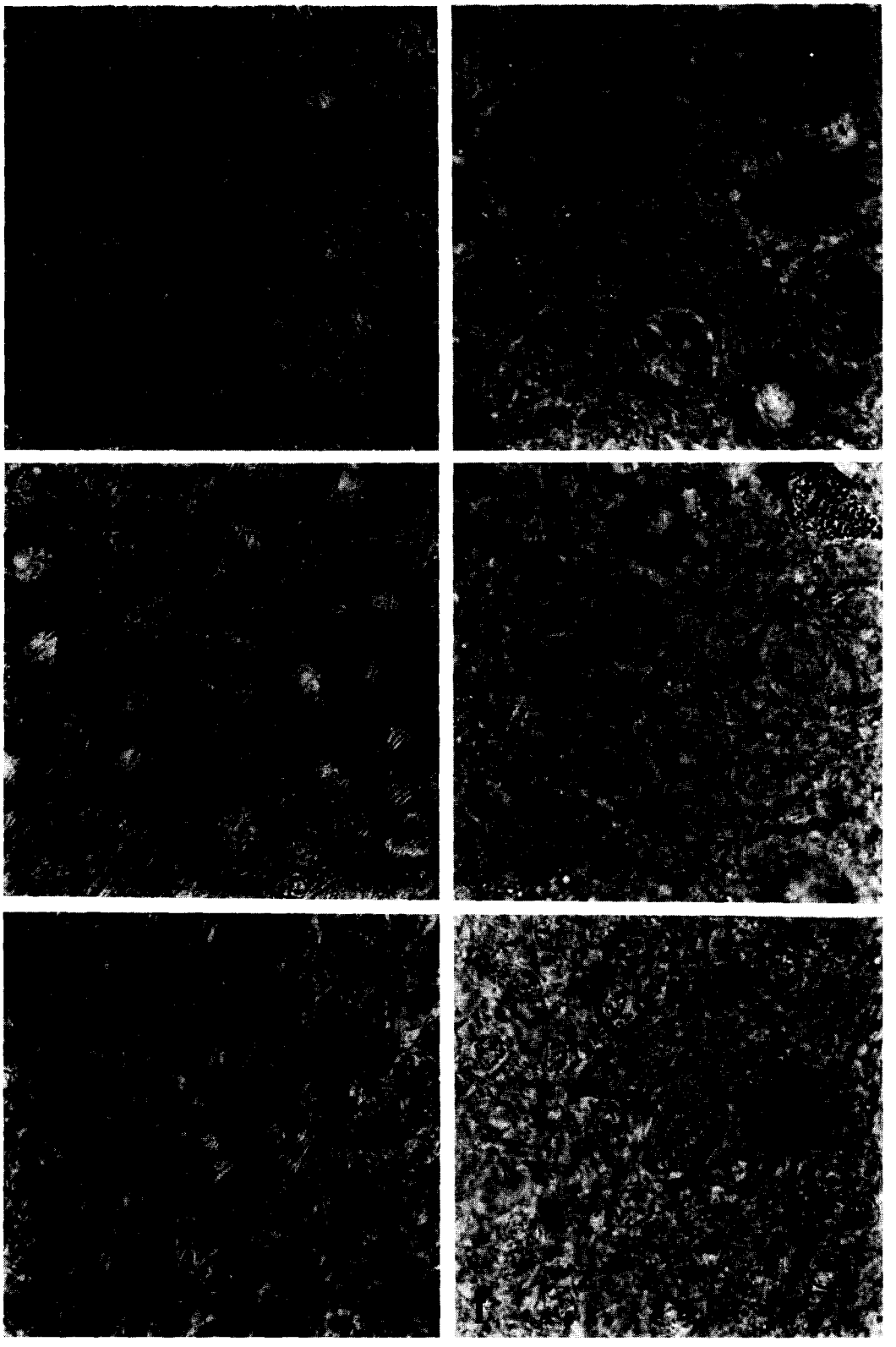


Fig. 1. Bright-field micrographs of living cultures during demyelination by amide 5 (1 mM) × 800, original magnification. a: well-myelinated control culture, 18 DIV. b: neurons in another control culture. c: another culture 6 h after addition of cerebrosidase inhibitor amide 5. d: normal appearing neurons in the same culture. e: intermediate stage of demyelination, 48 h after addition of amide 5, with formation of lipid droplets. f: granular neurons in another culture, 48 h after addition of amide 5.

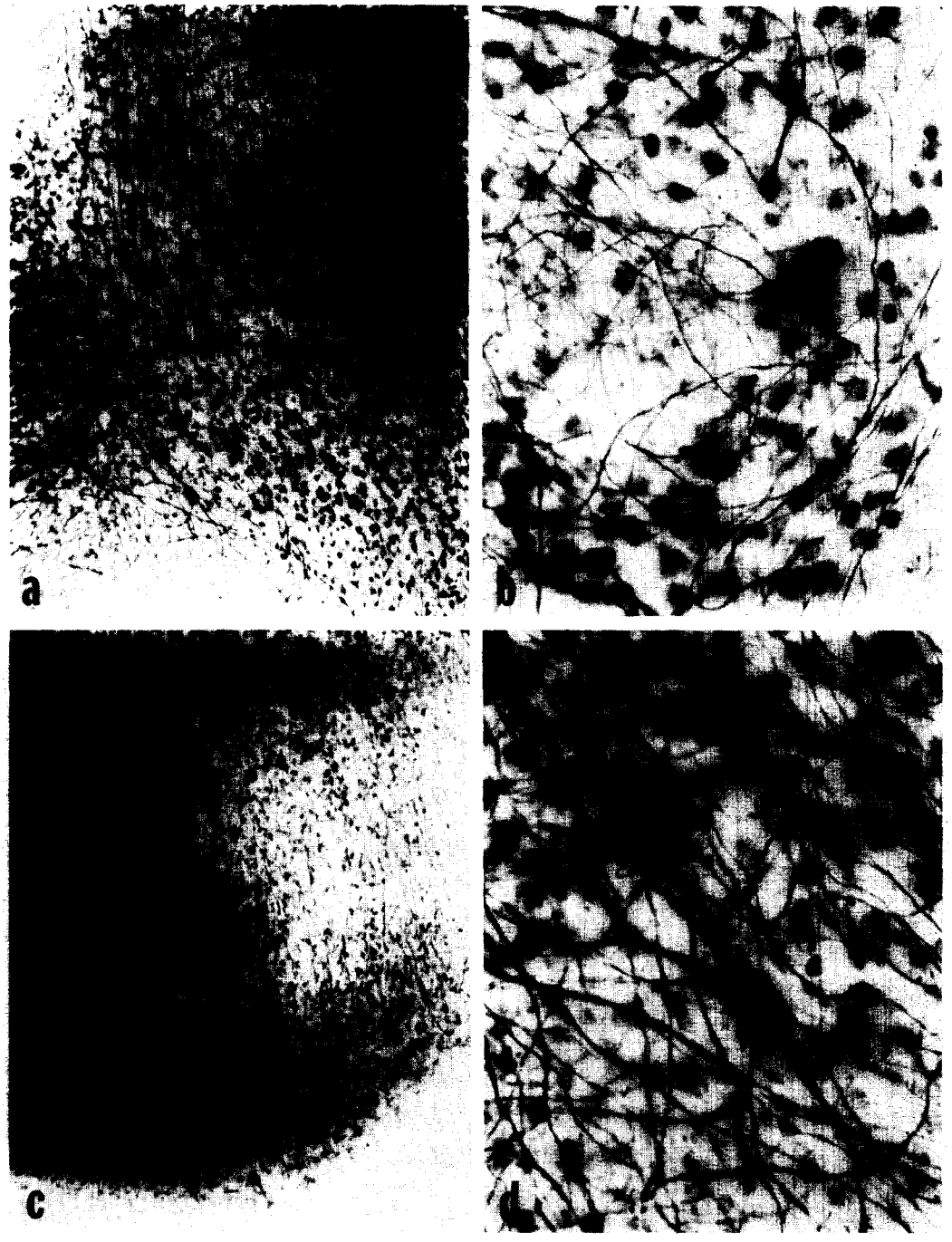


Fig. 2. Axons in control and demyelinated cultures, 19 DIV (modified Holmes silver stain). a: control culture. × 100, original magnification. b: same culture. × 400, original magnification. c: another culture demyelinated by amide 5 (1 m*M*), with normal appearing axons. × 100, original magnification. d: same culture, × 400, original magnification.

TABLE IV

EFFECT OF STIMULATORS OF GALACTOCEREBROSIDASE ON CULTURES

The cultures were exposed to stimulators and observed as described in Table III. Ratios show number of cultures exhibiting demyelination or lack of myelination compared to the total number of cultures exposed to the stimulator. Data in parentheses show the average time when demyelination occurred. The data for demyelination with amides 9, 10, 11 and 13 are from a single typical experiment; the data for amide 12 is the sum of results from the above experiment plus two other experiments in which it was compared with amide 8. The inhibition experiments were done with a single batch of cultures, and were last observed on 14–15 DIV, 72 h after all of the control cultures had myelinated.

Amide	*Demyelination ratio (3 mM amide)*	*Myelination inhibition ratio (0.3 mM amide)*
8	11/11 (10 h)	
9	0/8 (60 h)	0/8
10	1/8 (36 h)	
	7/8 (60 h)	1/15
11	3/8 (12 h)*	2/12
12	14/20 (10 h)*	2/4
13	7/7 (9 h)	3/6

* Myelin in remaining cultures distorted and lumpy.

Effects of stimulators of cerebrosidase with fatty acids of differing chain lengths

Aliphatic derivatives of 2-amino-2-methyl propanol stimulated the activity of cerebrosidase when tested *in vitro*[4]; the N-decanoyl derivative was the most effective (Table II).

We noted good correlation between the reported potency of these homologs to stimulate cerebrosidase and their ability to demyelinate or inhibit myelination in organ cultures (Table IV). The decanoyl derivative demyelinated more rapidly than those with longer chain fatty acids. When the methyl group on the 2-position was omitted (amide 8), the analog had no stimulatory effect on cerebrosidase activity[4]. However, amide 8 at 3 m*M* consistently demyelinated cultures within 10 h while control cultures remained normal for several days. At present we have no explanation for this discrepancy; perhaps this analog has a toxic impurity or exerts some unknown metabolic effect.

Metabolic studies

In control cultures, glucose incorporation into non-lipid material (chloroform–methanol insoluble pellet) and into the total lipid fraction was rapid between 0 and 4 h, then somewhat slower between 8 and 36 h (Fig. 3). Glucose incorporation in cultures containing one of the most potent inhibitors of cerebrosidase, amide 5, showed an initial stimulation, then depression of non-lipid labeling (Fig. 3). Incorporation of glucose into total lipids was depressed by 4 h, and remained depressed. The 1 m*M* concentration of analog depressed glucose incorporation to a greater extent than did the 0.3 m*M* concentration. Incorporation of glucose into cerebroside followed the same pattern as that observed for total lipid in each case.

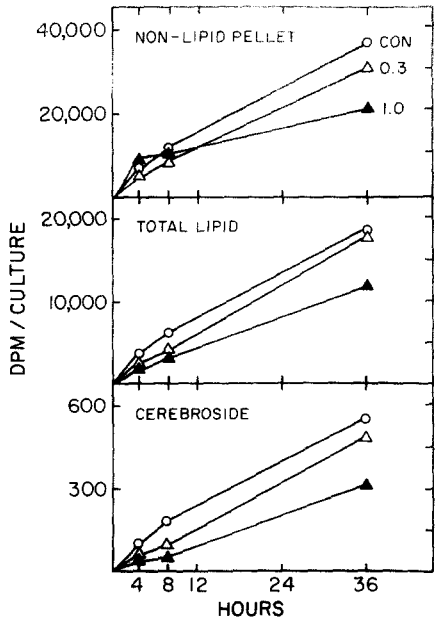


Fig. 3. Time-course of glucose incorporation into non-lipid, lipid and cerebroside fractions of myelinated organ cultures. Data represent results from a single typical experiment with 5–8 cultures/group at each time point. Cultures were exposed to [^{14}C]glucose alone (controls, ○———○), or [^{14}C]glucose plus amide 5 (0.3 m*M*, △———△; 1.0 m*M*, ▲———▲), on the 19th day after explant.

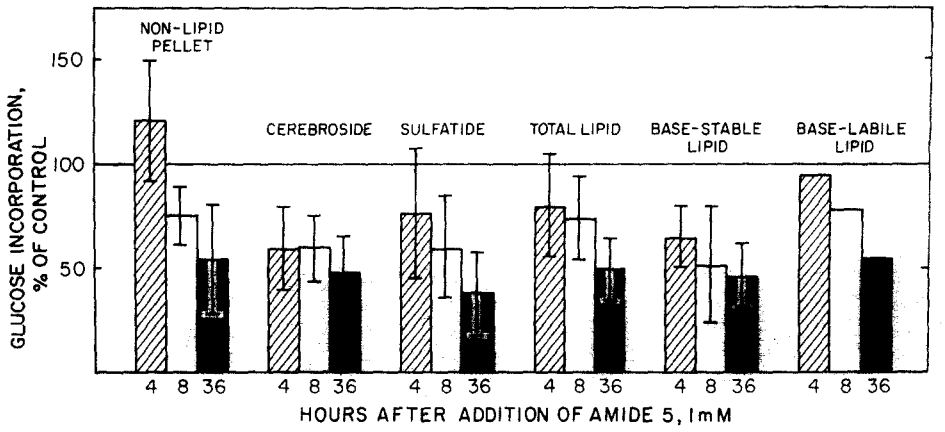


Fig. 4. Glucose incorporation into various fractions of myelinated organ cultures exposed to 1 m*M* amide 5, expressed as percentage of control values. Data represent the averages of results from 4 to 5 separate experiments at each time point, with standard deviations.

Results of several experiments with the analog were compared by expressing glucose incorporation into the various fractions as a per cent of that observed in the control cultures in each experiment (Fig. 4). Again, some stimulation of non-lipid labeling, and the early depression of labeling of alkali-stable lipids were apparent.

TABLE V

COMPARISON OF GLUCOSE INCORPORATION IN CULTURES WITH STRONG AND WEAK INHIBITORS OF CEREBROSIDASE

Cultures in the first experiment were harvested 36 h after the addition of the amides and the [^{14}C]glucose. In this experiment, the cultures with amides 1 and 2 appeared similar to the control at 36 h, with all cultures still well-myelinated as at the beginning of the experiment. Of the 5 cultures exposed to amide 3, one demyelinated between 18 and 36 h, and the remaining cultures all had swollen glial cells and lumpy myelin. In the second experiment, cultures were harvested at 4 h, when 7 of the 8 cultures exposed to amide 4 showed early signs of demyelination (primarily lumpy myelin).

Amide	*Non-lipid pellet (counts/min/culture)*	*Total lipids*	*Cerebroside*	*Cerebroside / Total lipid × 100*
None	57,980	37,380	818	2.2
1 (3 m*M*)	40,740	28,300	512	1.8
2 (3 m*M*)	38,350	35,490	532	1.5
3 (3 m*M*)	33,730	28,380	258	0.9
None	10,072	8730	115	1.3
4 (3 m*M*)	3836	4370	38	0.9

TABLE VI

GLUCOSE INCORPORATION IN CULTURES WITH AMIDE 5 (STRONG INHIBITOR OF CEREBROSIDASE) AND INHIBITORS OF PROTEIN SYNTHESIS

At 4 h, all cultures appeared similar to controls. By 8 h, the cultures exposed to amide 5 had much distorted myelin although little demyelination had yet occurred; the cultures exposed to cycloheximide had some distorted myelin, and many sheaths appeared thinner, eventually 'melting away', while the cultures exposed to chloramphenicol showed little change.

Addition	*Non-lipid pellet (counts/min/culture)*	*Total lipids*	*Cerebroside*	*Cerebroside / Pellet × 100*
4 h				
None	4970	5340	97	2.0
Chloramphenicol (1 m*M*)	4360	4920	86	1.9
Cycloheximide (0.3 m*M*)	1730	6300	90	5.2
Amide 5 (1 m*M*)	8400	5890	90	1.1
8 h				
None	6920	4810	177	2.6
Chloramphenicol (1 m*M*)	7020	5460	180	2.6
Cycloheximide (0.3 m*M*)	3780	9350	413	10.9
Amide 5 (1 m*M*)	11,000	4150	131	1.2

Incorporation of glucose into cerebroside was more depressed than into total lipids or alkali-labile lipids at 4 h. This analog has little effect on ceramide galactosyl transferase *in vitro*[17].

In another series of experiments, the 3-hydroxy derivatives, amides 1 and 2, had little effect on cerebroside labeling compared to total lipid, while the 1-hydroxy derivatives, amides 3 and 4, caused a marked depression (Table V). This depression is again coincidental with early signs of demyelination, as with amide 5. Amides 1 and 2 were previously found to inhibit cerebroside synthesis[2,17] but not cerebrosidase *in vitro*, while amides 3 and 4 inhibited both activities.

The effects of the diol analog were compared to those of chloramphenicol and cycloheximide, known inhibitors of protein synthesis in mammalian mitochondria and endoplasmic reticulum, respectively. Chloramphenicol and the 1,3-propanediol analog (amide 5) were tested in the cultures at 1 m*M*, and cycloheximide at 0.3 m*M*. Cultures containing chloramphenicol showed no signs of demyelination up to 72 h, while those with cycloheximide and the analog were demyelinated by 24 and 48 h, respectively. Glucose incorporation was measured at the end of 4 h and 8 h (Table VI). With 1 m*M* chloramphenicol, we saw little effect on labeling of non-lipid, lipid or cerebroside fractions compared to controls. Cycloheximide strongly decreased non-lipid labeling, and by 8 h stimulated lipid labeling. As previously observed, the analog depressed labeling of lipids, including cerebroside, and stimulated non-lipid labeling at early time points.

DISCUSSION

Our observations in myelinating cultures indicate that inhibition or stimulation of cerebrosidase activity can inhibit myelination and cause demyelination. The strong correlations between degree of interference with myelin status and degree of interference with cerebrosidase activity support the interpretation that this enzyme is the primary enzyme affected in the amide-treated cultures. Studies on the amides carried out subsequent to this study have shown that some of them have the additional ability to interfere with galactocerebroside synthesis[17]. For example, amide 7 acts as a competitive substrate for the rat brain galactosyltransferase, blocking ceramide utilization, so there is the possibility that this amide yielded sufficient abnormal myelin to cause interference with myelin metabolism. However amide 5 and the aliphatic amides, which also interfered effectively in myelination, have a minor effect on cerebroside synthesis, while amide 1, which inhibits cerebroside synthesis slightly, had no effect on myelin in the cultures for the periods studied.

There are the additional possibilities that the amide analogs of ceramide acted on the cultures by virtue of ability to interfere with other sphingolipid enzymes or through non-specific toxic effects. However, it seems unlikely that the hypothetical interference could correlate so well with the observed order of effectiveness of the analogs.

Our observations on the incorporation of labeled glucose into cerebroside, which showed a correlation between the reported degree of inhibition of cerebrosidase

and degree of inhibition of cerebroside synthesis in the cultures, suggest that there is a tight coupling between cerebroside synthesis and degradation, and that excessive slowing seems to upset a fine balance needed to maintain myelin deposition. The same need for fine balance is seen in our finding that stimulation of cerebrosidase also induced demyelination.

Only a few clues are available on the role of cerebrosidase in myelin deposition and maintenance[14]. That the enzyme acts *in vivo* is known from turnover studies, particularly the demonstration in young rats that there is a rapidly decaying pool of cerebroside following injection of labeled acetate[11]. That the enzyme is needed for normal brain development, particularly myelin *deposition*, is evident from the relationship of the enzyme to the pathology of Krabbe's disease[21]. Possibly the enzyme functions during myelination to 'remodel' the winding membrane or to eliminate 'internodal crowding'[5].

Evidence for a significant role for cerebrosidase in the *maintenance* of myelin comes from the observation[8] that the specific activity of the enzyme in rat brain rises 4-fold between 7 and 24 days and drops only slightly in adulthood. In contrast, the enzyme which synthesizes cerebroside[9] and the enzymes which synthesize[13] and degrade[10] cerebroside sulfate also increase markedly during active myelin deposition, yet decrease considerably when the period of rapid myelination ends.

When we examined the overall metabolic effects of amide 5, one of the strong inhibitors of cerebrosidase, we observed depressed incorporation of [^{14}C]glucose into alkali-stable lipids, including cerebroside, before labeling of other lipids was markedly affected. The alkali-stable lipid fraction includes galactolipids, sphingomyelin and lysoplasmalogens, all enriched in myelin, and depressed incorporation of glucose into these lipids may indicate early damage to oligodendroglial and myelin metabolism. The early stimulation of incorporation of [^{14}C]glucose into protein in our experiments may simply reflect decreased utilization of carbohydrate for lipid synthesis, or some more specific effect. The decreased labeling seen in all fractions by 36 h in demyelinating cultures may be a combination of fewer viable cells to incorporate [^{14}C]glucose, and degradation of some components labeled earlier.

We also altered the metabolism of the cultures by inhibiting protein synthesis with cycloheximide. Demyelination occurred, followed by degeneration of all cell types. The metabolic effects were different from those observed with inhibition of cerebrosidase, with decreased incorporation of glucose into proteins, and increased incorporation into all lipids, including cerebroside and other alkali-stable lipids. Chloramphenicol was tested because it bears a structural resemblance to the propanediol analogs; Arora and Radin[3] previously reported it had no effect on cerebrosidase activity, and we found no effects on cerebroside metabolism in the cultures. Smith and Hasinoff[20] reported that chloramphenicol inhibits both lipid and protein synthesis in brain slices at a concentration of 5 m*M*. We saw no effect on total lipid or protein synthesis in the cultures at a 5-fold lower concentration.

The demyelination we observed with the analogs was similar to that described in cultures exposed to sera from rabbits with experimental allergic encephalomyelitis (induced by injection of whole spinal cord)[6] or to sera from patients with multiple

sclerosis[12]. That is, distorted myelin and glial swelling were the first signs of abnormality, with neurons, axons and ependymal cells appearing normal until the late stages of demyelination. Unlike the experiments with sera, we did see abnormalities in large neurons with higher concentrations (1–3 m*M*) of the more potent analogs. Axon preservation was seen after demyelination, as noted by Silberberg in cultures exposed to some metabolites of phenylketonuria[18]. Except in the case of amide 5 (strongest inhibitor of cerebrosidase) in younger cultures, we did not see rapid vacuolization or death of the cultures. Nor did we observe the spherical 'beaded' myelin figures apparently caused by low glucose in the medium[12].

The ceramide analogs which inhibit or stimulate cerebrosidase are potentially tools for determining the role of cerebroside degradation in myelination and in the metabolic economy of the cell. More specifically, some of the inhibitory analogs may provide a model of Krabbe's disease, in culture and possibly *in vivo*. Finally, glucose incorporation into various fractions appears to be a useful technique for determining the specific metabolic effects of various demyelinating agents added to organ cultures.

ACKNOWLEDGEMENTS

This study was supported by funds from the John A. Hartford Foundation and U.S.P.H.S. Grants NS-08719 and NS-93192.

We thank Dr. Guy McKhann for his helpful advice and support during the course of this work.

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