Vitamin A deficiency and glycolipid sulfation

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ABSTRACT   The relationship between vitamin A deficiency and the formation of the sulfatides in brain was investigated in vivo in rats of various ages. By varying litter size and by pair feeding, the control animals had body weights similar to those of the deficient rats. No significant differences in the concentrations or in the total amounts of sulfatides were detected in the brains of vitamin A deficient or control rats, weanling or adult, or in most of the preweanling rats studied at the period of rapid myelination. Except for relatively small, malnourished, vitamin A deficient preweanling rats, variation in body weight within animals of the same age group, did not by itself influence the content of brain sulfatides. Vitamin A deficient animals also showed equal or greater incorporation of ^{35}S into sulfatides compared to controls.

Deficient rabbits were able to incorporate ^{35}S into the sulfatides of brain and ocular tissues to the same or greater extent as did control animals.

Thus, vitamin A deficiency does not interfere in the sulfation of glycolipids or perhaps generally in the processes of biological sulfation as has been proposed previously.

SUPPLEMENTARY   KEY   WORDS   sulfatides · rat brain · rabbit ocular tissues · malnutrition · pair feeding · Florisil chromatography · azure A assay · myelination · cerebroside sulfate

Controversy exists concerning the influence of vitamin A on sulfate metabolism. Such widely disparate results have been reported concerning the influence of vitamin A deficiency on the processes involved in biological sulfation as increases and decreases in the sulfation of mucopolysaccharides, different effects on different tissues, and variation of effect among different mucopolysaccharides (1–7).

Contradictory findings have also appeared concerning the influence of vitamin A deficiency on some of the enzymatic processes involved in sulfate activation. Reports showing decreases in the sulfurylase and sulfokinase activities in tissues from vitamin A deficient animals have appeared (8–14), while in other studies no effect which can be attributed solely to the deficiency was discernible in these enzymatic activities (15–21).

As vitamin A deficiency is established, the test animal is subject to a variety of nutritional stresses and also has a lowered resistance to infection. The basis for some of the discrepancies between the various studies on sulfation may have been revealed by reports which showed the marked influence on key reactions involved in sulfation by the nutritional state of the animal (14, 19, 21). Alteration in protein intake, starvation, levels of food consumption, type of diet, and body weight were shown to have marked effects on the activity of enzymes involved in sulfate activation. Procedures such as pair feeding, which attempt to balance the body weight of control animals with that of the test animal, have been widely used as compensatory measures in order to minimize some of the extraneous effects due to inanition that might obscure those effects caused by the deficient state under investigation. In studies dealing with the suckling rat, variation in litter size can also be used to influence the body weight of the progeny.

The sulfatides, in addition to the mucopolysaccharides, are a class of naturally occurring sulfated compounds whose biosynthesis also includes the sequence of metabolic events involved in sulfate activation (22–24). When compared with the array of mucopolysaccharides, the sulfatides are relatively uniform in type and structurally simple. Sulfatide metabolism presents itself, therefore, as a relevant system and possibly a less complex one for the study of the influence of vitamin A deficiency on these processes. Several studies have shown that the most rapid phase of sulfate uptake into rat brain sulfolipids occurs in the very young rat, i.e., at about 3 weeks of age which

coincides with the period of rapid myelination (23, 25, 26). Special emphasis was given to this period in the present study of the possible involvement of vitamin A deficiency in these processes. The present report deals with the effects in vivo of vitamin A deficiency on the sulfatides of rat brain during the period of rapid myelination, in the weanling rat, in the adult rat, and in brain and ocular tissues of young rabbits.

After the present investigations had been completed, Clausen (27) reported that vitamin A deficiency in the rat during the period of myelination resulted in a decrease in the formation of sulfatide in brain. The present findings do not agree with those of Clausen.

From these studies on the content of sulfatides and(or) the incorporation of $Na_2{}^{35}SO_4$ into the sulfatides of brain and ocular tissues, it is concluded that the sulfation of glycolipids is not interfered with by vitamin A deficiency per se.

METHODS

Vitamin A Deficiency in the Preweanling Rat

In order to produce a state of vitamin A deficiency in the suckling rat, females were partially depleted of vitamin A before mating. Except in cases of extreme deficiency, rats of the age investigated in most of these studies i.e. up to 23-days-old and termed preweanling, do not show the anomalies usually associated with vitamin A deficiency that had been initiated in the weanling (28). For the purposes of the present work, therefore, an operational definition of vitamin A deficiency has been employed: a liver vitamin A content that was below the level of detection by the Carr-Price reaction (29), i.e. less than 0.01 μmole vitamin A per g wet wt liver. Experiments in which the Vitamin A content of all of the members of a litter did not conform to this definition were discarded. In most of these investigations the effect of vitamin A deficiency on the sulfation of glycolipids was studied in the animals of the various age groups before signs of severe malnutrition associated with vitamin A deficiency were evident. Extensive vitamin A deprivation of the mother before mating, however, resulted in progeny that displayed many of the signs usually associated with the deficiency. The body weights of preweanling rats were manipulated by dietary restriction of the mother and(or) by adjusting the number of animals in the litter.

Animals and Diets

Procedure A. Female Sprague-Dawley rats weighing about 250 g were fed either a diet deficient in vitamin A or the same diet supplemented with vitamin A (19.8 USP units vitamin A per g diet). The rats were fed their respective diets ad lib. for periods of 1–2 weeks, after

which they were mated. Feeding was continued ad lib. for the animals on the deficient diets until their progeny were killed. Ad lib. feeding was allowed for the animals on the vitamin A supplemented diets until they gave birth. A modified paired-feeding procedure was then carried out on the mother and progeny that were receiving the vitamin A supplemented diet in which the food intake of the animals receiving vitamin A supplementation was restricted to amounts equal to or slightly greater (about 12%) than the weight of the food consumed by the animals on the deficient diet. In addition, the litter sizes of the deficient groups were varied as indicated.

Procedure B. Female Sprague-Dawley rats, about 160 g each, were pair fed normal or vitamin A deficient diets for 5 weeks prior to mating, after which feeding was ad lib. Body weight of the progeny was controlled by varying the size of the litters.

Progeny were also obtained after maintaining a female rat on the vitamin A deficient diet for a prolonged period (19 weeks) before giving birth.

Procedure C. Adult female rats of approximately the same weight were obtained, after being used for mating purposes in some of these studies, and thus had varied nutritional backgrounds. However, all of the "deficient" females from this group were fed the vitamin A deficient diet ad lib. for a total period of 6 months before they were killed. Pair feeding of adult female rats on the vitamin A adequate diet was initiated 4 months prior to termination of the experiment. Each rat, deficient and control, had given birth to one litter prior to this regime.

Adult male rats were pair fed vitamin A deficient or normal diets for a period of 5 months before being killed.

Procedure D. Male, weanling Sprague-Dawley rats (45–51 g) were divided into six groups of three animals each; the body weight within each group differed by not more than 1 g. Each animal was fed a diet deficient in vitamin A. Animals I and III of each group were fed ad lib., while animal II was pair fed to animal I. Animals II and III were given 2000 units of vitamin A (water-dispersible form of vitamin A palmitate) (30) per week by stomach tube, while animal I was given the same volume of water by stomach tube. The experiment was terminated either at the weight plateau stage or at the time when the animals had started losing weight (Fig. 1).

Procedure E. Sprague-Dawley rats were obtained 2 weeks pregnant from the supplier and were fed ad lib. the vitamin A adaquate diet. After birth, the litter sizes were reduced to three, five, six, and nine animals, and the ad lib. feeding was continued.

Procedure F. From two litters of weaned albino rabbits of similar body weight (800–900 g) two groups of six animals each were selected at random. The animals were fed ad lib. diets that were either deficient or adequate in vitamin A.
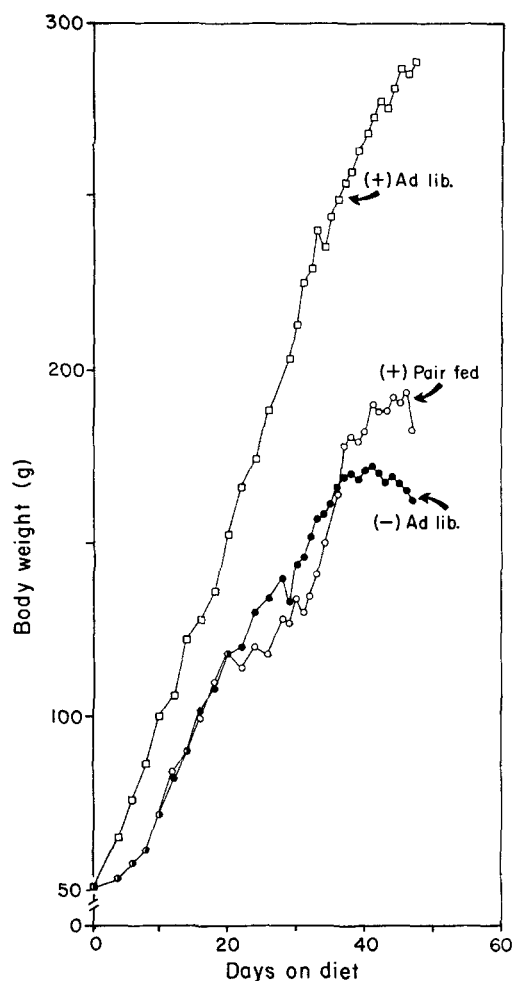
FIG. 1. The body weight responses of weanling rats to vitamin A deficient and vitamin A adequate diets. These growth curves are from a representative group of three animals fed according to procedure D (see Methods). Food consumption and body weight were measured daily. All of the animals received a powdered diet deficient in vitamin A. A water-dispersible form of vitamin A palmitate was fed by stomach tube (2000 U/wk) to the animals whose growth curves are designated by (+), while the same volume of water was administered to the rat whose growth curve is labeled (−). The rat (+) Pair Fed was pair fed to the rat that did not receive the supplemental source of vitamin A. The other two animals were fed their respective diets ad lib.

Isotope Studies

35S Administration, Treatment of Tissues, and Counting Procedures. The specific activity of $Na_2{}^{35}SO_4$ was adjusted to 390 $\mu Ci/\mu mole$. The solutions were then diluted with 0.15 M NaCl for injection as follows: 20–30 $\mu Ci/ml$ for preweanling rats; 250 $\mu Ci/ml$ for adult rats; 2000 $\mu Ci/ml$ for rabbits.

18-day-old rats, under light ether anesthesia, were injected intravenously (tail vein) with $Na_2{}^{35}SO_4$ (0.4 $\mu Ci/g$ body weight) and then returned to their mothers. On the 23rd day of life, the animals were killed by decapitation, and the whole brains and livers were removed and stored at −20°C until analysis. Adult rats under light

ether anesthesia were injected intravenously (femoral vein) with $Na_2{}^{35}SO_4$ as above, and after 2 days they were killed by decapitation, and the tissues were frozen until analysis.

After the appearance of corneal opacification and (or) low blood vitamin A concentrations in rabbits on the deficient diet, the rabbits from deficient and control groups were injected with $Na_2{}^{35}SO_4$ intravenously (ear vein, 0.4 $\mu Ci/g$ body weight). After 1 week the animals were killed by air embolism, and the brains, livers, and eyes were removed. The brains were dissected immediately into gray and white matter, and the eyes were dissected into the following areas: cornea, lens, choroid, retina, iris plus ciliary body, and optic nerve. The identity of the ocular areas was verified histologically. All dissections were performed in the cold.

Radioactivity was measured by liquid scintillation counting in a Packard 314 X Tricarb scintillation spectrometer. Lipid extracts (see below) were evaporated to dryness in counting vials and stored overnight in a dessicator over KOH and under vacuum. Counting was performed using 15 ml of a solution containing 5 g 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene, 50 ml of methanol, and 950 ml of toluene (23). All samples were corrected for quenching by internal standardization using a solution of 35S in toluene. Counts were corrected for decay. Counting efficiency was about 60%.

Radioactivity on thin-layer plates was determined after scraping the gel from the dry plate into counting vials and adding 15 ml of the scintillation fluid. No decline of counts with time was observed other than that caused by isotopic decay, and little or no quenching was detected.

Lipid Extraction

Individual whole brains from each of the animals were homogenized with C–M 2:1 according to the procedure of Folch, Lees, and Sloane Stanley (31). The extracts were filtered through solvent-washed filter paper, diluted to a known volume, and an aliquot was washed according to the procedures of Folch et al. (31), except that 0.1 M sodium sulfate was used as the aqueous phase (32). The lower phase was washed four times with "pure solvents upper phase" (also prepared with 0.1 M Na_2SO_4). The interfacial emulsion that formed during this process was retained in the lower phase. After washing, methanol was added to the chloroform extract until one phase formed, and the solution was diluted to a known volume. An aliquot was then evaporated to dryness under nitrogen at 40°C in tared weighing bottles. The evaporation was repeated in the presence of excess water to insure disruption of proteolipids (33). The residue was then stored at room temperature in a desiccator over KOH under

vacuum and then weighed (weight of lipid used in Tables 1-7).

The pooled ocular and brain tissues of rabbits were treated in a manner similar to that described above for rats, except that the entire extracts were carried through these procedures. The residue resulting from the homogenization and extraction of corneal tissue with C–M 2:1 was further subjected to Soxhlet extraction for 4 hr using C–M 1:1. The extract was then evaporated to dryness, dissolved in C–M 2:1 and washed (31).

Florisil Chromatography

The washed lipid extracts were further purified by Florisil chromatography. Florisil was washed sequentially with C–M 95:5, 2:1, 1:1, methanol, and water. Fines were removed by resuspension in water several times. After being dried overnight at 100°C, the washed Florisil was heated at 600°C for 1 hr (34) and then deactivated to a final water concentration of 7% (35). The dried lipid extracts were dissolved in C–M 95:5 and washed on to columns packed with Florisil (columns 1 cm in diameter, fitted with porous glass plates and teflon stopcocks). In the case of tissues from the preweanling rats, 1 g of Florisil was used per mg of dried lipid, while 1 g per 10 mg of lipid was employed for tissues from the adult and weanling rats. Elution was carried out batchwise at a flow rate of 5 ml/min using C–M 95:5, C–M 2:1, and C–M 1:1 in the proportions of 20, 35, and 20 ml/g Florisil, respectively. The fractions were evaporated to dryness on a rotary evaporator at 30°C, and the residues were dissolved in C–M 1:1 for analysis.[1]

Analytical Procedures

Sulfatide, Vitamin A, TLC. Sulfatide was measured by the azure A colorimetric procedure of Kean (32) on the fractions purified by Florisil chromatography. The vitamin A content of liver was measured by the procedure of Ames, Risely, and Harris (36) employing the SbCl₃ procedure of Carr et al. (29). The vitamin A content of plasma was determined by the procedure of Kimble (37).

Thin-layer plates were prepared from Silica Gel G (Brinkman Instruments Inc., Westbury, N. Y.) as previously described (32). The following solvent systems (all v/v) were employed: (*a*) chloroform–methanol–water 65:35:8; (*b*) *n*-propanol–concentrated NH₄OH–water 12:1:2; (*c*) chloroform–methanol–water 65:25:4. Materials on the chromatograms were detected by charring the plates according to the procedure of Privett and Blank (38). Glycolipids were detected by the α-naphthol spray of Feldman, Feldman, and Rouser (39). When

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[1] Blank column separations were routinely performed with each set of determinations, since small amounts of azure A reactive materials were detected in some batches of Florisil.

materials were to be recovered from thin-layer plates, they were located by exposure of the dry plates to iodine vapor for 1 min.

In addition to the use of spray reagents, the migration of sulfolipids was detected after TLC by scraping off 1 cm sections of gel from the starting line to the solvent front into screw cap culture tubes for performance of the azure A assay (32), and into counting vials for the determination of radioactivity.

MATERIALS

The diet used in feeding procedures A, B, C, and E (see Methods) was a vitamin A deficient test diet for rats prepared and pelleted by General Biochemicals, Chagrin Falls, Ohio. The control diet had, in addition, 19.8 IU of vitamin A per g. The vitamin A deficient diet used in procedure D was a test diet in powdered form obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Vitamin A deficient diet for rabbit used in procedure F was prepared and pelleted by General Biochemicals according to the formulation of Strain, Houser, and Hunscher (40). The control diet had in addition, 4.75 IU of vitamin A per g.

The following materials were obtained from the indicated sources: water-dispersible vitamin A palmitate (250,000 USP units/g), from General Biochemicals; bovine sulfatides, Supelco, Inc., Bellefonte, Pa.; azure A (total dye content 92%), from National Aniline Division of Allied Chemical Corp., New York; vitamin A acetate, crystalline, from Mann Research Labs Inc., New York; Na₂³⁵SO₄, from New England Nuclear Corp., Boston, Mass.; Florisil, from Floridin Co., Pittsburgh, Pa. All solvents and other chemicals used in this study were reagent grade.

RESULTS

Preweanling Rats

Sulfatide Content. The total amount of sulfatide (μmoles per brain)[2] and its concentration (μmoles per mg of lipid or per g [wet weight] of tissue) in the brains of preweanling vitamin A deficient rats was similar to that of control animals (Tables 1 and 2). In these studies the body weights of the control animals were maintained similar to those of the deficient animals. The small, but statistically significant, difference between the deficient and the normal that could be detected when the data were calculated in terms of dry weight of lipid (Table 1), was not reproduced when nutritional procedure B was followed (Table 2). Irrespective of the differences in

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[2] In the interest of brevity, not all of these relationships were presented in the Tables. They may be calculated from the data.

TABLE 1 EFFECT OF VITAMIN A DEFICIENCY ON THE CONTENT OF SULFATIDES AND THE INCORPORATION OF ^{35}S INTO THE SULFATIDES OF THE BRAINS OF PREWEANLING RATS*

	Deficient Group	Control Group	P Value
μmole Vitamin A/ g liver	<0.01	0.215 ± 0.0067	—
Litter size	11–9†	13	—
Body wt (g)	30 ± 0.35‡	30 ± 0.41	>0.6§
Brain wt (g)	1.30 ± 0.013	1.24 ± 0.014	<0.01
Sulfatide concentration			
μmoles/g of brain (wet wt)	1.31 ± 0.013	1.44 ± 0.051	>0.05
mμmoles/mg of lipid	21.8 ± 0.53	24.7 ± 0.69	<0.01
cpm			
per g of brain (wet wt)	46,500 ± 3280	51,100 ± 1590	>0.1
per mg of lipid	787 ± 50	879 ± 26	>0.05

* These animals were the members of single litters obtained after being reared according to procedure A (see Methods). Na$_2$^{35}SO$_4$ (0.4 μCi/g of body weight) was injected i.v. on the 18th day of life, and the animals were killed on the 23rd day. Sulfatide was isolated from the individual brains and analyzed as indicated in Methods. The dam of the deficient group had been on the vitamin A deficient diet for $4^{1}/_{2}$ weeks before giving birth.

† At the time of injection of the isotope, this litter was reduced from 11 animals to 9.

‡ Mean ± SEM.

§ The data were analyzed by Student's "t" test. On the basis of the 5% level of significance, a P value > 0.05 indicates that there was no significant difference between the groups.

body weights of the animals, there were no significant differences in the concentrations of sulfatide in the brains of the animals (Tables 1 and 2). In the animals of similar body weight there were no significant differences in the *total* amounts or concentrations of sulfatide in the brains of vitamin A deficient and control rats.

In Table 3 are data obtained from deficient animals of much smaller body weight. The larger litter size of deficient group A produced animals of relatively small body weight. There were significant differences in the sulfatide content of the brains of these animals as compared with the controls (Table 1). Presented separately are data from two runts of this litter (group A-2) for which the body weight was about 30% less than that of their litter mates. These animals had lower amounts of brain sulfatide than either their heavier litter mates or the controls in Table 1. Similar low levels of sulfatide were observed in the brains of the severely malnourished young rats that were obtained after prolonged vitamin A deprivation of the mother prior to mating (group B). When compared with the combined values of the control animals in Tables 1 and 2, the sulfatide content of the brains of these severely ill animals was significantly depressed ($P < 0.05$).

TABLE 2 EFFECT OF VITAMIN A DEFICIENCY ON THE CONTENT OF SULFATIDES AND THE INCORPORATION OF ^{35}S INTO THE SULFATIDES OF THE BRAINS OF PREWEANLING RATS

	Deficient Group	Control Group	P Value
μmole Vitamin A/ g liver	<0.01	0.110 ± 0.004	—
Litter size	6	10	—
Body wt (g)	51.2 ± 1.02	49.1 ± 0.63	>0.05
Brain wt (g)	1.49 ± 0.017	1.48 ± 0.022	>0.9
Sulfatide concentration			
μmoles/g of brain (wet wt)	1.40 ± 0.020	1.40 ± 0.041	>0.9
mμmoles/mg of lipid	21.8 ± 0.30	22.2 ± 0.62	>0.6
cpm			
per g of brain (wet wt)	115,000 ± 8770	62,800 ± 2110	<0.01
per mg of lipid	1804 ± 134	998 ± 35	<0.01

The animals were fed according to procedure B (see Methods). Experimental procedures and data presentation are the same as indicated in Table 1. The dam of the deficient group had been on the vitamin A deficient diet for 11 weeks before giving birth.

TABLE 3 CONTENT OF SULFATIDES AND THE INCORPORATION OF ^{35}S INTO THE SULFATIDES OF PREWEANLING, VITAMIN A DEFICIENT RATS OF RELATIVELY SMALL BODY WEIGHT

Group	A-1*	A-2*	B
Litter size	13	13	†
Animals per group	11	2	2
Body wt (g)	22 ± 0.54‡	15 ± 2	19 ± 0.3
Wet wt brain (g)	0.90 ± 0.04	0.99 ± 0.10	1.17 ± 0.07
Sulfatide concentration			
μmoles/g of brain (wet wt)	1.33 ± 0.058	0.75 ± 0.04	1.02 ± 0.04
mμmoles/mg of lipid	19.1 ± 0.48	14.5 ± 0.60	19.5 ± 2.2
cpm			
per g of brain (wet wt)	29,600 ± 2060	10,100 ± 25	14,570 ± 20
per mg of lipid	424 ± 28	196 ± 3	277 ± 20

* The animals in groups A-1 and A-2 were fed according to procedure A (see Methods), and were from the same litter. The animals in group A-2 refer to two runts from this litter. The dam of this litter had been on the deficient diet for 6 weeks before giving birth.

† The animals in group B represent the survivors from a litter obtained from a female that was maintained on a deficient diet for 19 weeks prior to giving birth. The size of the litter was reduced at birth from 13 to 8. This number was maintained until the 14th day of life after which many of these severely malnutritioned animals died. Five animals survived to the injection period (18th day) during which time the animals displayed many of the classical signs seen in severe vitamin A deficiency in the weanling. On the 22nd day two animals survived, and the experiment was terminated at this point.

‡ Group A-1, mean ± SEM; groups A-2 and B, average value with indicated range.

TABLE 4   EFFECT OF VITAMIN A DEFICIENCY (INITIATED IN THE WEANLING RAT) ON THE SULFATIDE CONTENT OF BRAIN*

	Deficient (I)	Pair-Fed Control (II)	Ad Lib. Control (III)	P Value (I–II)†	P-Value (I–III)†
μmoles Vitamin A/g liver	<0.01	0.98 ± 0.13	0.59 ± 0.03	—	—
Wet wt brain (g)	1.71 ± 0.027	1.78 ± 0.021	1.82 ± 0.051	>0.05	>0.05
Sulfatide concentration					
μmoles/g of brain (wet wt)	3.62 ± 0.17	3.42 ± 0.093	3.67 ± 0.12	>0.3	>0.8
mμmoles/mg of lipid	35.2 ± 1.5	34.2 ± 0.58	36.2 ± 0.75	>0.5	>0.5

* The rats were fed according to procedure D (see Methods).   Weanling rats (45–51 g) were maintained on their respective nutritional regimens until those on the deficient diet were in the weight plateau or early weight loss stage (Fig. 1). Weanling rats on the vitamin A deficient diet at the time of killing varied in weight from 100 to 192 g; their pair-fed controls, from 130 to 228 g; the ad lib. controls, from 202 to 289 g. In each of six groups, the deficient animal was lightest, the pair-fed control 12–30% heavier, and the ad lib. control from 40 to 180% heavier than the deficient animals.

† P values comparing groups II with I, and III with I.

Incorporation of ^{35}S. As mentioned previously, several studies have shown that the period from about 15 to 20 days of life is the optimal one for the incorporation of inorganic sulfate into the sulfatides of rat brain. In preliminary studies these observations were confirmed for both normal and vitamin A deficient animals. The period used in the present studies with preweanling rats spans this rapid stage of glycolipid sulfation, i.e., from the 18th day (sulfate injection) to the 23rd day of life (termination of the experiment).

Animals of similar body weight (procedure A) showed no significant differences in the extent of labeling or specific activity[2] of sulfatide between deficient and control groups (Table 1). The labeling of brain sulfatide of vitamin A deficient rats prepared by procedure B (Table 2) was greater than that of the controls.

Greatly diminished labeling of sulfatide was observed in brains of deficient rats of group A-1 (Table 3) whose body weight was much smaller than that of the control groups. This decrease was accentuated in the brains of the runts, who incorporated about half the radioactivity as compared with their larger litter mates. Similar effects were observed in the severely malnourished vitamin A deficient progeny (group B, Table 3).

Thus, in the period of rapid myelination, the extent of incorporation of ^{35}S into brain sulfatides by the vitamin A deficient rat was as great or greater than that of control animals of similar body weight.

Weanling Rats

The weanling rat has been used extensively for studies dealing with vitamin A deficiency and the processes of sulfation. Although these animals, at the time at which they showed signs of deficiency, were past the optimal period of myelination and sulfatide biosynthesis, it was of interest to measure the sulfatide content in brain of animals reared with the strict nutritional controls that were possible with this experimental model. As can be seen from the data in Table 4, the sulfatide content in the brains of rats maintained on vitamin A deficient diets

from the weanling stage to the weight plateau or early weight loss stage in their deficiency (Fig. 1), was essentially the same as that of their pair-fed controls. Nor were there any significant differences in the content of brain sulfatides between deficient animals and controls fed ad lib.

Adult Rats

Even after prolonged feeding of vitamin A deficient diets, it was difficult to deplete completely the vitamin A stores of mature rats that had previously had adequate nutrition. The vitamin A content in the livers of the adult animals used in this study were at the lower level of detectability, about 0.01 μmole/g wet wt liver, and thus should be considered only partially vitamin A deficient.

The adult rat has a limited capacity, compared with the young rat, to synthesize brain sulfatides. As can be seen from the data in Table 5, there were little differences between control animals (pair fed or ad lib., male or female) and the vitamin A depleted animals, in terms of sulfatide content or the ability to incorporate ^{35}S into the sulfatides of brain.

Body Weight

The results of some of the studies with the preweanling rat suggested that the nutritional status of the animal as reflected in body weight might influence the sulfation of glycolipids, which agrees with a previous report by Chase, Dorsey, and McKhann (41). These workers studied the formation of sulfatides in "poorly nourished" rats of small body weight, induced by maintaining artificially large litters (16–20 animals per litter). Did variation in body weight by itself, in preweanling animals that were *not* poorly nourished, influence the content of sulfatides and the incorporation of ^{35}S into brain sulfatides? The body weights of rats born from dams on vitamin A adequate diets were varied by adjusting the litter sizes from nine to three animals. The same experimental procedure was used with these animals as had been used previously when the effects of vitamin A

TABLE 5   The Effect of Vitamin A Depletion on the Content of Sulfatides and the Incorporation of ³⁵S into Sulfatides of Adult Rat Brain*

	Female			Male	
Vitamin A Status	Deficient†	Control (Pair-Fed)	Control (Ad Lib.)	Deficient†	Control (Pair-Fed)
Body wt (g)‡	281	304	318	525	525
Wet wt brain (g)	1.98	2.01	1.82	2.13	2.10
Sulfatide concentration					
μmoles/g of brain (wet wt)	5.91	5.32	5.94	6.20	5.38
mμmoles/mg of lipid	27.8	25.4	31.0	26.6	24.5
cpm					
per g of brain (wet wt)	3010	2850	2570	4030	3660
per mg of lipid	28	28	24	37	35

* The animals were fed according to procedure C (see Methods), with a single animal in each group.
† The vitamin A content of the livers of the depleted animals was at the lower limit of detectability, 0.01 μmole/g wet wt.
‡ The body weights did not vary more than 5% of this final weight during the last 2 months before the animals were killed.

TABLE 6   The Content of Sulfatide and the Incorporation of ³⁵S into Sulfatides in Brains of Normal Preweanling Rats with Different Body Weights

	I	II	III	IV	Mean
Adjusted litter size	3	5	6	9	
Body wt (g)	83 ± 1.8	64 ± 1.1	57 ± 1.1	51 ± 0.88	
Brain wt (g)	1.61 ± 0.021	1.56 ± 0.013	1.50 ± 0.020	1.50 ± 0.007	1.53 ± 0.012
Sulfatide concentration					
μmoles/g of brain (wet wt)	1.38 ± 0.009	1.58 ± 0.013	1.45 ± 0.018	1.39 ± 0.061	1.46 ± 0.027
mμmoles/mg of lipid	21.8 ± 0.59	24.7 ± 0.34	22.6 ± 0.41	22.5 ± 1.19	23.0 ± 0.43
cpm					
per g of brain (wet wt)	37,100 ± 2480	44,900 ± 2080	72,500 ± 871	66,300 ± 3050	
per mg of lipid	562 ± 57	708 ± 46	1148 ± 13	1060 ± 30	

Variation in body weight was accomplished by adjusting the size of the litters at birth. The rats were fed according to procedure E (see Methods). Experimental procedures and data presentation are the same as indicated in Table 1.

deficiency on the preweanling rat were studied. These vitamin A normal preweanling rats, differing in body weight from 51 to 83 g, contained a relatively constant level of brain sulfatides, irrespective of the differences in body weight (Table 6). Thus, variation in body weight by itself does not play a role in these processes and is of importance only when it is indicative of malnutrition.

The amount of ³⁵S incorporated into brain sulfatide differed in the rats of different body weight. Relatively heavier animals from litters of three to five animals had less radioactivity than lighter animals from larger litters. These observations are contrary to what would be expected if the lowered incorporation of ³⁵S in the deficient progeny in Table 3 were attributed solely to small body weight.

Rabbit Studies

Because of the small amount of tissue that can be obtained from the various regions of the eye, the tissues were pooled for analysis. Healthy weaned rabbits were 5–6 weeks old when placed on the vitamin A deficient diet.

After the animals had been on this diet for about 3 months, the plasma vitamin A values were about ¹/₁₀ that of animals on the control diet. At this time the body weights of the animals on the deficient diet were similar to those on the control diet (2327 ± 64 g and 2294 ± 170 g, respectively). At the time of killing four of the original six animals on the deficient diet survived, the others having succumbed to infection. Two of the survivors displayed visible corneal lesion. The vitamin A content of the livers of all of the animals on the deficient diet was about ¹/₁₀ of that of the controls.

Similar to the observations obtained with brains from young and adult rats, the vitamin A deficient rabbit was able to incorporate ³⁵S into the sulfolipids of ocular and brain tissues in amounts equal to or greater than that of animals on the control diets (Table 7).

Isolation and Identification of Sulfatide

As observed previously by Folch et al. (31) and Lees, Folch, Sloane Stanley, and Carr (42), washing of a chloroform–methanol 2:1 extract of tissues by aqueous

TABLE 7 THE EFFECT OF VITAMIN A DEFICIENCY ON THE INCORPORATION OF ^{35}S INTO THE SULFOLIPIDS OF BRAIN AND OCULAR TISSUES OF RABBITS*

Tissue	Vitamin A Status				Deficient/
	Deficient†	Control†	Deficient	Control	Control‡
	cpm/g wet wt		cpm/mg of lipid		
Retina	1370	909	57	44	1.5
Cornea	266	37	121	15	7.2
Optic nerve	1710	1340	7	7	1.3
Choroid	3170	2360	131	89	1.3
Iris and ciliary	1190	906	106	66	1.3
Lens	0	0	0	0	—
Brain					
White matter	13,200	12,700	99	96	1.0
Gray matter	3240	2740	62	57	1.2

* The rabbits were fed according to procedure F (see Methods). These analyses were made on the pooled tissues from four surviving deficient rabbits and five control rabbits.

† The concentration of vitamin A in the plasma at the time of killing was 1.5 mμmole vitamin A/ml for the controls, while essentially none was detected in plasma from the animals on the deficient diet. The vitamin A content of the livers of the deficient animals was at the lower level of detection by the method used, about 0.01 μmole/g liver.

‡ Ratio calculated from the wet weight data.

systems not containing salts results in substantial loss of sulfatide from the organic phase. Sodium sulfate was previously recommended (32) for the preparation of the solvents (31) when the azure A method was to be used for the assay of sulfatides. When 0.1 M Na$_2$SO$_4$ was used as the aqueous phase and in the preparation of pure solvents upper phase, no loss of sulfatide (0.025 μmole) was observed after nine successive washes as compared to an 85% loss from the lower phase at the end of four washes if salts were omitted during these extractions.

As previously shown (32) after Florisil chromatography, the colored complex formed between azure A and sulfatide is an accurate means of determining the sulfatide content of extracts of brain. The elution pattern from a column of Florisil of a sample of a "Folch washed" extract from normal young rat brain carried out as indicated in Methods is seen in Fig. 2. There was exact correspondence of radioactive ^{35}S and azure A reactive material in the C–M 2:1 fraction. The elution pattern of standard sulfatide from bovine brain was essentially the same as that of the radioactive, azure A reactive material from extracts of rat brain.

Since much less radioactivity was incorporated into brain sulfatides by adult as compared with young rats, larger amounts of lipid extracts were required for analysis. The elution pattern from Florisil of radioactive, azure A reactive material from extracts of adult rat brain, using a ratio of 10 mg lipid/g Florisil (10-fold more lipid than that used for the preweanling rats) was identical to that seen in Fig. 2.

Over 90% of the ^{35}S counts applied to the columns were recovered in the C–M 2:1 fraction. From 4 to 8% of the applied radioactivity was eluted in the C–M :11 fraction. The identity of this latter material has not as yet been established. About 70% of the azure A reactive material applied to the column was eluted in the C–M 2:1 fraction. This agrees with previous observations (32) describing the reactivity in the azure A assay of some compounds other than the sulfolipids, such as some of the phospholipids, materials which would not interfere with the determination of sulfatide after Florisil chromatography. Standard bovine sulfatide was recovered from Florisil in 93% yield in the C–M 2:1 fraction; 6.3% of the applied azure A reactive material was recovered in the C–M 1:1 fraction.

In addition to having elution patterns from Florisil similar to that of standard sulfatide, the radioactive, azure A reactive material which was eluted from Florisil with C–M 2:1, migrated on thin-layer plates (three different solvent systems) in one area as detected by radioactivity, azure A reactivity, and spray reagents, in a manner similar to that of standard sulfatide. With solvent system b, that could distinguish between cholesterol sulfate and sulfatide (43), no radioactive or azure A reactive material that migrated with the steroid sulfate was detected.

Similar observations were obtained with extracts of the brains of vitamin A deficient and control animals.

The concentration of sulfatides in the brains of the preweanling and weanling rats determined in this study are similar to those of animals of similar age reported by Wells and Dittmer (44), although higher values were obtained for the adult rats.
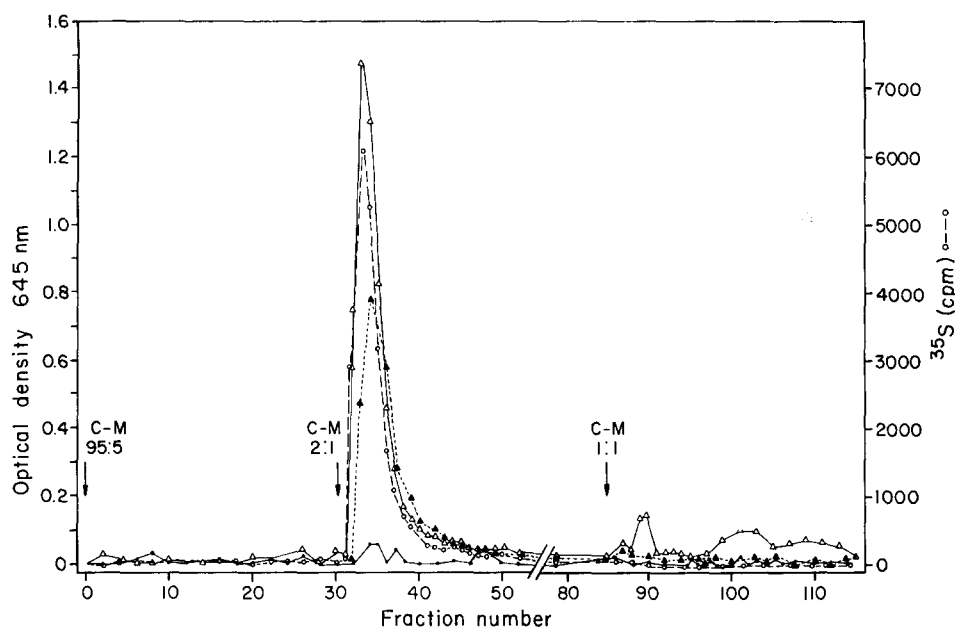
FIG. 2. Elution patterns of sulfatides from columns of Florisil. Samples in C–M 95:5 were applied to columns containing 8 g of Florisil. 5-ml fractions were collected at a flow rate of 5 ml/min. Radioactivity was measured by liquid scintillation spectrometry, and sulfatide was determined by the azure A assay (see Methods). Standard sulfatide from bovine brain (▲ - - -, azure A assay); blank column (● ———, azure A assay); aliquot of a "Folch washed" extract of preweanling rat brain containing 8 mg dry wt of lipid, (○ - - radioactivity, ³⁵S), (△—, azure A assay). The eluting solvents were changed from C–M 95:5 to C–M 2:1 to C–M 1:1 as indicated by the arrows.

DISCUSSION

Inherent in investigations of an effect caused by a nutritional variant are the complications that can arise from the existence of other phenomena, such as starvation and general malaise, which may be associated with the production of the nutritionally deficient animal. It is often difficult to dissociate effects due to these factors from those due to the primary lesion. Although there are no techniques that can completely resolve these difficulties, nutritional controls can be utilized that can minimize some of these effects. Employing such procedures, Rogers (21) showed that the specific activity of liver sulfurylase from vitamin A deficient rats was not significantly different from that of controls when animals of the same size were used, or when the amount of food administered to the two groups was the same.

The techniques of paired or restricted feeding of controls and manipulation of litter sizes, all of which influencing the body weight of progeny, were employed in the present study in order to reduce possible variations due to the effects of malnutrition associated with vitamin A deficiency which could produce animals of relatively small body weight. In the present studies these effects did not seem to play a role in sulfatide metabolism except in the preweanling rat studied at the optimal period of myelination. In addition, animals which were selected for most of these studies were at stages in their vitamin A deficiency where these secondary effects probably did

not exert a dominating influence, i.e., the animals were not rapidly losing weight nor were they beset with infection.

In most of the present studies, the chemical analyses indicated that vitamin A deficiency had no measurable effect on the sulfatide content of rat brain. This was true in the adult rat, the weanling and in most of the young rats examined during the period of rapid myelination.

Two groups of animals in this latter category, however, did show lower levels of brain sulfatides; the deficient animals of relatively small body weight (groups A-1 and A-2, Table 3) and the ill, surviving progeny of the extensively vitamin A depleted dam (group B, Table 3). These animals also incorporated much less ³⁵S into sulfatides than did the controls or the other groups of vitamin A deficient preweanling rats. Was the deficiency the cause of these lower values? Attention is called again to the much lower body weight of these animals, a deficit that ranged from one-third to one-half of that of the groups showing the higher content of and the greater ability to incorporate ³⁵S into sulfatide. In view of the similarity in sulfatide content and the equal or greater incorporation of isotope into brain sulfatides by the other deficient preweanling animals compared with controls (Tables 1 and 2), it is suggested that these lower values may reflect the effects of nonspecific stress from malnutrition and inanition rather than changes due primarily to the absence of vitamin A from the tissues of

these animals. Similar conclusions have been made by Geison, Rogers, and Johnson (19) and by Rogers (21) who studied the presumed effect of vitamin A deficiency on the activity of ATP-sulfurylase.

The present investigations showed also that differences in body weight of animals of the same age which were not nutritionally deprived (Table 4 and Table 6) did not result in significant differences in the content of brain sulfatides. In the studies with the vitamin A deficient preweanling rats, only in the cases of the relatively small animals did this factor seem to play an important role.

In studies unrelated to vitamin A deficiency, Chase et al. (41) demonstrated that malnourished young rats incorporated less than half the ^{35}S per g brain into sulfatides than did the control animals. These malnourished animals were obtained by the relatively innocuous "large litter" procedure whereby nutrition is reduced in quantity but still balanced. At 21 days of age these animals were about the same weight as the deficient rats used in the present study in group A-1, Table 3. The studies of Chase et al. (41) demonstrated the extreme vulnerability to nutritional trauma of the developing nervous system as reflected in sulfatide metabolism in the rat during the period of rapid myelination.

In the report by Clausen (27), a decrease in the rate of deposition and in the total amount of sulfatide in brain tissue of vitamin A deficient rats was described. Also, extracts from brains of vitamin A deficient rats showed a decreased ability to synthesize sulfatide and active sulfate as compared to extracts from normal animals. The vitamin A deficient animals used by Clausen were obtained after feeding a vitamin A deficient diet to pregnant rats 2 weeks prior to delivery and then maintaining the progeny on the deficient diet for 2–3 months. These animals were thus exposed, in varying degrees, to a vitamin A deficient regime for an extended period of time: in utero, from birth through the period of rapid myelination, and postweaning. As far as can be ascertained, pair feeding or other techniques to control the effects of malnutrition which very possibly existed in animals exposed to these conditions, were not used. As previously indicated, evidence has been presented (14, 19, 21), indicating that effects ascribed to vitamin A deficiency, e.g. decreased activity of enzymes involved in sulfate activation, may instead reflect the generally inadequate nutritional state of the animal. In addition, as demonstrated by Chase et al. in both in vivo and in vitro experiments (41), malnutrition by itself during the period of myelination can result in a decrease in the synthesis of the sulfatides of brain. The decrease in sulfatide formation under conditions of malnutrition is probably also related to the lower content of brain cerebrosides that has been observed in conditions of nutritional deprivation during the preweaning period (45–47). Some of these considerations may apply to some of the effects reported by Clausen.

Variation in the incorporation of ^{35}S into the sulfatides of brain was observed in the different experiments. Thus, vitamin A deficient rats prepared by procedure B, (see Methods and Table 2), incorporated more radioactivity into brain sulfatide as compared with controls, while there was little difference when the deficiency was induced by a different nutritional procedure (procedure A, Table 1). It is difficult to explain these results which perhaps reflect the complexities of studying by isotopic procedures alone in vivo effects on a lipid that is a constituent of both metabolically stable and labile brain structures (26) in single dose experiments during a period of rapid growth (48). These variations may reflect differences in the turnover of sulfatide or of pool size which may be influenced by these various nutritional states.

The important role played by vitamin A in vision prompted a study of the effect of vitamin A deficiency on the sulfation of glycolipids by ocular tissues. As was observed in the studies with rat brain, vitamin A deficient rabbits incorporated as much or more ^{35}S into sulfatides of ocular tissue as did the normal controls.

It is concluded from the studies reported here that the state of vitamin A deficiency, per se, does not result in an interference in the production of the sulfated cerebrosides, and perhaps of the processes involved in biological sulfation in general.

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