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Brain Gangliosides: Increased Incorporation of (1-^{3}H)Glucosamine during Training[1]

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DUNN, A. J. AND E. L. HOGAN. *Brain gangliosides: increased incorporation of (1-^{3}H)glucosamine during training.* PHARMAC. BIOCHEM. BEHAV. 3(4) 605–612, 1975. – The incorporation of (1-^{3}H)glucosamine into biochemical fractions of brain was studied in mice trained in a conditioned avoidance jump-up task, in mice yoked to the trained animals, and in undisturbed mice in their home cages. The (^{3}H)glucosamine was injected subcutaneously 15 min before 15 min of training, and the mice killed after a total pulse time of one hour. There was a consistent and significant increase of about 21 percent of the incorporation of the ^{3}H into the brain ganglioside fraction when trained mice were compared with quiet mice. This increase was not observed in any of the other chemical fractions studied, including the total chloroform-methanol-soluble compounds, the non-ganglioside lipids, and the chloroform-methanol-insoluble compounds (radioactivity principally in glycoprotein). Yoked mice showed an intermediate level of incorporation, exhibiting only a 12 percent increase in the ganglioside fraction. When the individual ganglioside species were analyzed by thin-layer chromatography the changed incorporation was not specific for any ganglioside species.

Gangliosides (^{3}H)Glucosamine Conditioned avoidance training Glycoproteins

GANGLIOSIDES are glycolipids containing sialic acid and are significantly concentrated in neurons rather than glia [2,12]. After subcellular fractionation they are found in fractions rich in membranes, the highest concentration being in the synaptosomal and microsomal fractions [11, 20, 21, 22]. There is general agreement that neuronal gangliosides are concentrated at synapses [6]. Gangliosides are apparently important in nerve excitation, restoring excitability in depleted [1] and neuraminidase-treated preparations [23,24] so that they may be involved in synaptic conduction. For this reason ganglioside metabolism has recently been studied in relation to stimulation by light [3], gross environmental stimulation [15], and the acquisition of a behavioral task in rats [9]. In view of these considerations we have studied the incorporation of radioactive glucosamine into brain gangliosides during conditioned avoidance training of mice. Preliminary data from these studies have previously been reported [4].

METHOD

Behavioral Procedures

C57Bl/6J male mice 8–10 weeks old were obtained from Jackson Laboratories, Bar Barbor, Maine. D(1-^{3}H) glucosamine was purchased from Amersham-Searle, Arlington Heights, Illinois.

Mice were housed in a community cage until injection. After injection they were individually caged. Twenty-five μCi (^{3}H)glucosamine in 25 μl was injected subcutaneously at the back of the neck. In experiments where animals were trained, training commenced 15 min after injection. After 15 min of training the mice were returned to their individual cages until sacrifice, 60 min after injection.

Conditioned avoidance training was performed in the jumpbox apparatus previously described [25]. In some experiments an automated version of this box was used in which there was a safe shelf on only one side of the box. This shelf was cleared by a piston so that mice were not handled during training [5]. Yoked animals received the same light, buzzer and shocks as the trained animals but in an adjacent compartment of the apparatus. Quiet animals were undisturbed between injection and sacrifice.

Biochemical Procedures

Mice were sacrificed by decapitation and the brain (less olfactory lobes) excised and immediately homogenized. Gangliosides were isolated by the method of Suzuki [17].

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The brain was homogenized in 8 ml of $CHCl_3$:CH_3OH (2:1, v/v) in an all-glass homogenizer with the pestle rotated at 300 rev/min. The homogenate was filtered through Whatman No. 1 filter paper into a 35 ml graduated conical tube with a ground glass stopper. The residue was rehomogenized in 4 ml of $CHCl_3$:CH_3OH (1:2, v/v) containing 5% water, and filtered through the same filter paper into the same tube. The paper was then washed with 4 ml of chloroform to give a combined filtrate containing $CHCl_3$:CH_3OH approximately 2:1 (v/v). The volume of this Folch extract was measured and two 0.2 ml aliquots taken for determination of radioactivity. Next, 0.2 volumes of 0.88% KCl were added and the mixture shaken and centrifuged at 1000 X g for 10 min at room temperature. The upper phase was removed and the lower phase extracted in sequence with 2 ml of theoretical upper phase containing KCl, and with 2 ml of theoretical upper phase without KCl. The extracts were combined with the first upper phase, the total volume measured, two 0.2 ml samples removed for determination of radioactivity, and the combined upper phase was dialyzed against 0.9% NaCl for 24 hr (3 changes) and distilled water for 2 days.

In some experiments the gangliosides were treated with enzymes to degrade contaminating nucleotide sugars [10]. To the dialysand were added one-ninth volume of 1M tris-citrate (pH 8.5), one thirty-second volume of 1M $MgCl_2$, and 5 μg each of alkaline phosphatase (Sigma, *E. coli* Type III) and phosphodiesterase (Sigma, *Crotalus adamanteus* Type II) per ml of dialysand. The mixture was incubated for 90 min at 37° and then dialyzed overnight against two changes of distilled water. Provided that the gangliosides were dialyzed initially against saline, this procedure did not cause a substantial reduction in the radioactivity recovered, nor did it alter the results obtained.

The dialysand was evaporated to dryness in a rotary evaporator in the presence of a small quantity of ammonium bicarbonate to prevent hydrolysis of sialic acid from the gangliosides. The gangliosides were redissolved in 1 ml of $CHCl_3$:CH_3OH (2:1, v/v) and two aliquots each taken for determination of radioactivity and sialic acid content. The remainder was resolved into individual ganglioside species by thin-layer chromatography using silica gel G plates, (medium hard, thickness 250 microns: Analtech Inc.) eluted with $CHCl_3$:CH_3OH:2.5 M NH_4OH (60:38:8, v/v/v). A two-stage procedure was used, drying for 30 min at 110° between runs. Gangliosides were detected under ultraviolet light after spraying with ammoniacal Rhodamine (0.12% Rhodamine 6G:CH_3OH:concentrated NH_4OH:H_2O, 1:50:35:14, v/v/v/v). No bands were detected with this spray that did not also stain with resorcinol reagent [18]. Bands were identified by comparison with known standards, scraped off, and the gangliosides eluted. Half of this eluate was used for sialic acid determination and half for determination of radioactivity.

Ganglioside sialic acid was determined by the thiobarbituric acid assay of Warren [19] after hydrolysis with 0.1M H_2SO_4 at 80° for 2 hr. Aliquots of total Folch extract, upper phase, and lower phase (minus interphase) were taken to dryness and counted with 0.5 ml of Soluene 100 (Packard Instrument Co.), and 10 ml of PPO in toluene (4 g/l). Gangliosides were taken to dryness, redissolved in 1 ml water and counted after the addition of 10 ml of Triton scintillator (Triton X-100:0.4% PPO in toluene, 1:2 v/v).

Radioactivity was determined in a Packard Model 3375 scintillation counter, correcting for quench using the automatic external standard, comparing samples with Packard sealed standards. Computation was carried out on an IBM 1130 computer. There was excellent agreement between the duplicate samples counted.

The residue from the ganglioside extraction was scraped off the filter paper and dissolved in 5 ml of 0.3M NaOH. Samples of this dissolved residue were taken for determination of radioactivity and for protein assay by the Lowry method [13]. For some experiments the radioactivity in this solution precipitated by 5% trichloroacetic acid was determined by filtration on glass fiber filters (Whatman GF/C). The residue on the filter was solubilized with 0.5 ml of Soluene 100 for 8 hours at 60°C and counted with PPO scintillator. The trichloroacetic acid precipitation was also tested following incubation of the dissolved chloroform-methanol residue with 1M HCl at 80° for 2 hr.

RESULTS

The observed time course of the incorporation of subcutaneously injected (1-^{3}H)glucosamine into various chemical fractions of mouse brain is shown in Fig. 1. Note that the greatest incorporation of ^{3}H was into the chloroform-methanol-insoluble fraction at all times studied. A part of this radioactivity was precipitated by 5% trichloroacetic acid, the proportion increasing with incorporation time. The strong treatment (0.3M NaOH) needed to solubilize the residue prior to precipitation may have depressed these percentages. When the residue was subjected to mild treatment with hydrochloric acid prior to precipitation with trichloroacetic acid, very little radioactivity was precipitated. This suggests that much of the radioactivity was in carbohydrate attached to protein as glycoprotein.

The ganglioside fraction was the only one to show significantly increased radioactivity after one hour, although there was a slight increase in the total radioactivity of the chloroform-methanol extract which includes the gangliosides. Incorporation of ^{3}H into the non-ganglioside lipid fraction did not change appreciably after 1 hr.

When the gangliosides were separated into subspecies by thin-layer chromatography the ^{3}H from the (^{3}H)glucosamine became incorporated into the different species at approximately the same rate (Fig. 2). Thus the percentage of radioactivity found in each ganglioside species was approximately the same at all times (Table 1). These data do not indicate specific precursor-product relationships among the various ganglioside species, and suggest that an earlier step in ganglioside synthesis was rate limiting. Nevertheless, there was no apparent lag in the appearance of ^{3}H in gangliosides. Clearly the direct precursors for ganglioside synthesis derived from glucosamine must be different from those used for glycoprotein biosynthesis. The precursors must also separate into the aqueous phase of the Folch extract or the chloroform-methanol-residue fraction since there is insufficient radioactivity in the lipid fraction to account for the subsequent increase in the gangliosides.

A series of nine separate experiments was performed to determine whether or not conditioned avoidance training affected the incorporation of (1-^{3}H)glucosamine into gangliosides and other chemical classes of mouse brain. In four of these experiments manual training was used as described by Zemp *et al.* [25] and in the remaining five experiments

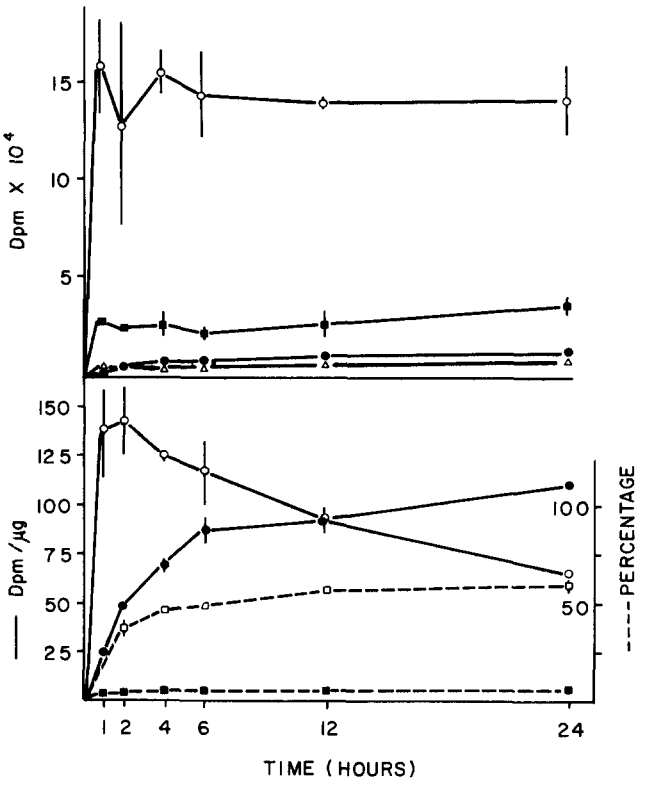

FIG. 1. Time course of the incorporation of subcutaneously injected (1-^{3}H) glucosamine into chemical fractions of the brains of quiet mice. Upper figure: •, ganglioside; ○, chloroform-methanol residue; ▪, chloroform-methanol (Folch) extract; △, lipid (lower phase of extract). Lower figure: •, ganglioside specific radioactivity dpm/μg sialic acid; ○, chloroform-methanol residue specific radioactivity dpm/50 μg protein; □, percentage of residue precipitated by 5% trichloroacetic acid; ▪, percentage of residue precipitated by 5% trichloroacetic acid following hydrolysis with 1M HCl for 2 hr at 80°C. Bars indicated the range of values obtained for different mice. (The absence of bars indicates that the range is encompassed by the symbol.) Data were obtained from duplicate observations on two mice for each time point.

an automated version of the apparatus was used [5]. All the trained mice learned the task in the 15 minute training session (approximately 30–35 trials). For the manual training the mean number of shocks received by the trained (and yoked) mice was seven for a mean total of 21 sec of shock. In the automated training a mean of 9 shocks was received per animal for a mean total of 45 sec of shock. The automated task was more difficult by virtue of the smaller floor space in the apparatus and the presence of an escape shelf on only one side of the box (as opposed to four in the manual version). The biochemical results using either method of training were indistinguishable and have been combined in the analysis of the data.

The particular time parameters chosen for this experiment were designed to permit sufficient time before training (15 min) for uptake of precursor into the brain to occur equally in all groups of animals. It was presumed that any changes in ganglioside biosynthesis would be detected shortly after training had finished, and 30 min were allowed for such to appear. No other time parameters have yet been studied.

Table 2 summarizes the data from one sample experiment while Table 3 shows a summary of the data from the nine experiments. The incorporation of ^{3}H into the various fractions is expressed as a ratio comparing trained mice with quiet mice and trained mice with yoked mice. The incorporation of radioactivity into the ganglioside fraction of trained mice was consistently higher than that of quiet mice. This was true in all experiments in which trained and quiet mice were run. It was statistically significant in three out of the nine experiments and was highly significant when the results of all the experiments were combined. The increased radioactivity in the ganglioside fraction was also observed when the specific radioactivity of the gangliosides was computed, thus correcting for the slightly variable recovery of this fraction. Significant changes were not seen in any other chemical fraction. The comparison of trained mice with yoked mice showed somewhat similar results. The only fraction to show a significant increase in the incorporation of radioactivity was the ganglioside fraction, but this increase was much smaller and less significant than that observed in the trained/quiet comparison. The ganglioside specific radioactivity change was not significant for trained versus yoked mice but data for this comparison were only available for three experiments.

Analyses were performed to determine whether there was any relationship between the number of shocks received, the total seconds of shock or the acquisition rate and the radioactivity determined in gangliosides for each trained mouse. No relationships were observed. Thus the increased incorporation is apparently related to the training experience itself or to aspects not also experienced by yoked animals.

The gangliosides extracted in these experiments were separated by thin-layer chromatography to determine whether the increased radioactivity with training was associated with any particular ganglioside species. A summary of the analysis for the experiment of Table 2 is given in Table 4. The most powerful comparison for the detection of such differences is that of the percentage distributions of radioactivity and sialic acid. While significant changes were detected in the radioactivity for the G_{DIA} fraction on this particular experiment, this was not true of other experiments. An overall analysis of the results of all the experiments did not reveal consistent differences in any particular ganglioside species and the occasional significant results obtained were probably due to chance.

DISCUSSION

The time course of labelling of brain gangliosides with (1-^{3}H)glucosamine is comparable to that previously observed with (U-^{14}C)glucosamine [14] or (1-^{14}C)glucosamine [8]. In these latter cases also, no differential labelling of the ganglioside species was observed. One must thus conclude that the processes of the interconversion of the ganglioside species are fast compared with earlier steps in the synthetic route from glucose or glucosamine. Radioactive glucosamine is incorporated into gangliosides both as the hexosamine of the oligosaccharide chain and as sialic acid. Maccionni *et al.* [14] have recently concluded from labelling studies with (6-^{3}H)glucosamine, that no independent turnover of the sialic acid groups of rat brain gangli-

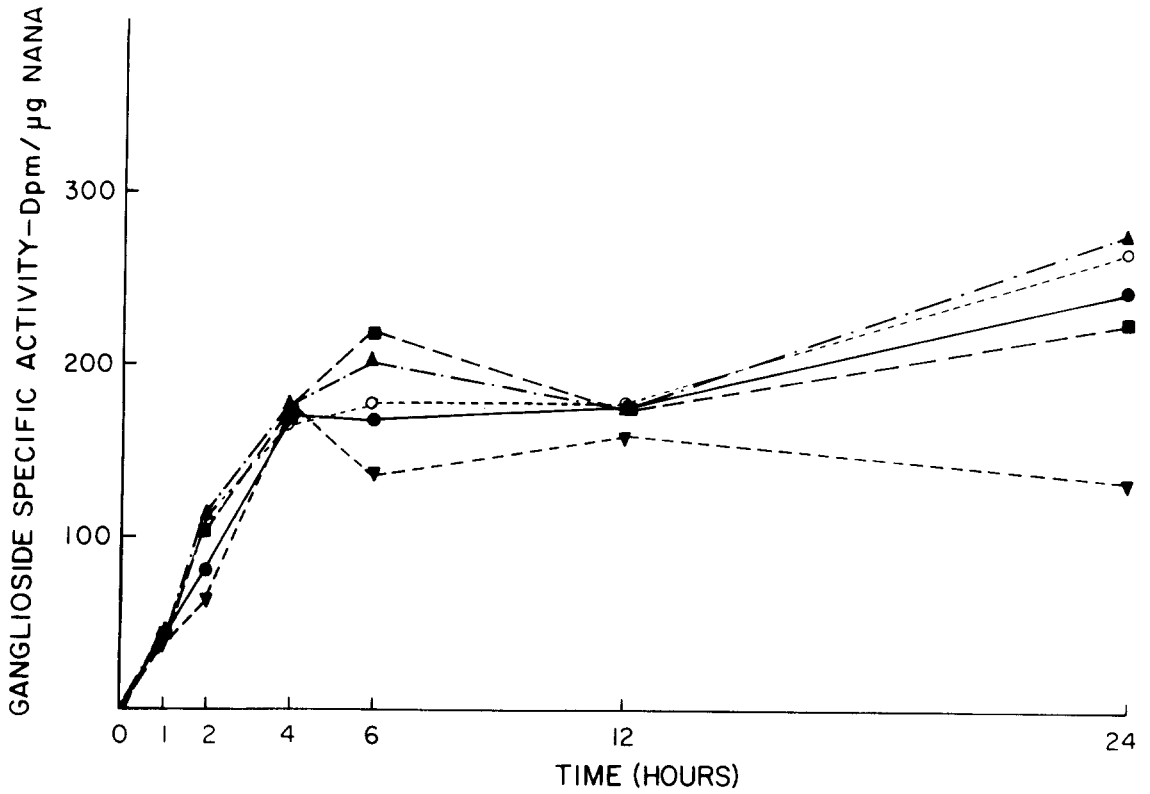

FIG. 2. Time course of the incorporation of (1-^{3}H)glucosamine into mouse brain gangliosides. Duplicate chromatographic separations were performed on two mice for each time point. ▼, G_{M1}; ●, G_{D1A}; ▲, G_{D2}; ○, G_{D1B}; ■, G_{T1}. The same experiment as in Fig. 1 and Table 1.

TABLE 1

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY FROM (1-^{3}H)GLUCOSAMINE IN GANGLIOSIDES

Ganglioside	Time (hr) 1	2	4	6	12	24	Mean for All Times
G_{M1}	6.2	3.9	6.6	5.7	6.3	4.6	5.5
G_{D1A}	32.9	29.8	37.6	31.9	36.0	36.1	34.0
G_{D2}	14.4	12.6	13.4	10.7	13.9	14.8	13.3
G_{D1B}	12.7	12.0	10.7	9.4	12.1	11.0	11.3
G_{T1}	34.0	41.7	31.7	42.3	31.7	33.7	35.9

The radioactivity of the gangliosides of the experiment of Figs. 1 and 2 expressed as a percentage of the total recovered from the thin-layer chromatography plate. Duplicate chromatographic separations were performed on each of two mice for each time point.

TABLE 2

INCORPORATION OF (1-^{3}H)GLUCOSAMINE INTO CHEMICAL FRACTIONS OF MOUSE BRAIN

	Fraction				
Treatment	C-M Soluble	C-M Residue	Lipid	Gangliosides	Gangliosides s.r.
Trained	52.5 ± 2.1	117 ± 11	21.6 ± 1.1	1.95 ± 0.18	77.8 ± 3.0
Yoked	57.1 ± 1.4	122 ± 6	22.5 ± 1.3	1.83 ± 0.18	67.3 ± 4.8
Quiet	57.4 ± 3.3	109 ± 3	20.4 ± 1.9	1.67 ± 0.17	65.3 ± 5.8
T/Q	0.91	1.07	1.06	1.16	1.19*
T/Y	0.92	0.96	0.96	1.06	1.17
Y/Q	1.00	1.12	1.10	1.10	1.03

The ^{3}H dpm (in 1000s ± standard error of the mean) recovered in the various chemical fractions after a training experiment using the automatic jumpbox. The gangliosides on this experiment were purified using phosphodiesterase and phosphatase treatment [9]. The dpm are corrected to the total amount of brain tissue. C-M Soluble: chloroform-methanol soluble (i.e., Folch extract); C-M Residue: Chloroform-methanol residue; Lipid: lower phase of Folch extract; Ganglioside: dialyzed upper phase of Folch extract. Ganglioside specific radioactivity (s.r.) is expressed as dpm/μg sialic acid as determined by hydrolysis and the thiobarbituric acid assay [14].

Number of animals: trained (5), yoked (6), quiet (6)

*$p<0.05$, Student's t-test

TABLE 3

INCORPORATION OF (1-^{3}H)GLUCOSAMINE INTO CHEMICAL FRACTIONS OF MOUSE BRAIN: COMPARISON OF BEHAVIORS

Comparison	C-M Soluble	C-M Residue	Lipid	Ganglioside	Ganglioside s.r.
Trained/Quiet	1.03	1.08	1.05	1.21*	1.17*
	(0.91–1.18)	(1.03–1.20)	(0.94–1.30)	(1.14–1.28)	(1.12–1.21)
Trained/Yoked	1.05	1.03	1.00	1.12†	1.08
	(0.93–1.10)	(0.96–1.13)	(0.95–1.13)	(1.06–1.21)	(1.00–1.20)

The mean of the radioactivity in each chemical fraction for each group of animals on each experiment was calculated. The numbers presented are the geometric means for all experiments, of the ratio of the means for each group in each separate experiment. The numbers in parentheses are the range of the ratio of the means obtained in the separate experiments. Eight separate experiments involving 41 trained and 41 quiet animals are included in the trained/quiet comparison. Five separate experiments involving 25 trained and 27 yoked animals are included in the trained/yoked comparison. The ganglioside specific radioactivity data was treated similarly but only 7 experiments were involved in the trained/quiet comparison and 3 in the trained/yoked comparison. Probabilities determined by a chi-squared analysis of the probability values obtained by t-comparisons on individual experiments.

*$p<0.001$

†$p<0.05$

TABLE 4

RADIOACTIVITY AND SIALIC ACID CONTENT OF GANGLIOSIDE SPECIES

Ganglioside	Trained	Yoked	Quiet	All
Sialic Acid – Percentage Distribution				
G_{M1}	6.2 ± 0.5	5.5 ± 0.3	6.0 ± 0.3	5.9 ± 0.2
G_{D1A}	35.8 ± 2.0	33.5 ± 1.9	30.4 ± 3.0	33.6 ± 1.3
G_{D2}	9.5 ± 0.8	8.9 ± 0.4	8.6 ± 0.6	9.0 ± 0.4
G_{D1B}	13.5 ± 1.0	16.4 ± 1.5	16.6 ± 1.7	15.4 ± 0.8
G_{T1}	35.0 ± 2.5	35.7 ± 3.0	38.5 ± 5.0	36.2 ± 1.8
Radioactivity – Percentage Distribution				
G_{M1}	9.5 ± 1.6	7.9 ± 1.1	6.7 ± 1.4	8.2 ± 0.8
G_{D1A}	33.7 ± 1.4*	34.6 ± 2.3*	27.0 ± 3.4	32.4 ± 1.5
G_{D2}	10.2 ± 1.0	9.0 ± 0.3	9.8 ± 1.6	9.6 ± 0.5
G_{D1B}	14.2 ± 1.0	18.3 ± 1.5	16.3 ± 1.9	16.2 ± 0.9
G_{T1}	32.4 ± 2.6	30.1 ± 2.9	40.3 ± 5.8	33.5 ± 2.2
Specific Radioactivity (dpm/μg sialic acid)				
G_{M1}	102 ± 11	92 ± 7	93 ± 18	95 ± 7
G_{D1A}	75 ± 5	69 ± 5	70 ± 9	71 ± 7
G_{D2}	88 ± 18	71 ± 9	84 ± 22	80 ± 8
G_{D1B}	79 ± 3	79 ± 12	71 ± 12	77 ± 5
G_{T1}	76 ± 7†	57 ± 4	61 ± 10	64 ± 4

The Gangliosides of the Experiment of Table 2 Separated by Thin-layer Chromatography. Radioactivity and Sialic Acid Expressed as a Percentage (± Standard Error of the Mean) of the Total Recovered from the Plate.

*different from quiet $p<0.05$ †different from yoked $p<0.05$

osides occurs. These authors also explained the lack of precursor-product relationships among the ganglioside species by suggesting that each species is synthesized in its entirety by a multienzyme complex, and that intermediates do not become free and equilibrate with each other. To explain the data one must also presuppose that the activity of each system is in proportion to the brain content of each ganglioside species. None of these data indicates the molecular form of the radioactive precursors over the extended period of synthesis. From the data presented here and that of Holian *et al.* [8] the precursors must be distinct from those used in glycoprotein biosynthesis since the specific radioactivity of glycoproteins reaches a maximum much earlier, after a period considerably shorter than their half-life.

The problem of the precursors is a critical one for the study of changes in rates of synthesis. Other than measuring the total radioactivity of the soluble fraction, no attempt was made to determine the specific radioactivity of precursors in the present series of experiments for a number of reasons: (1) Technically it is very difficult to extract gangliosides and precursors in the same experiment. (2) Since

glucosamine is incorporated into more than one part of the ganglioside molecule, and since the pathways for each are several steps long, it is not clear which precursor should be measured. (3) The precursors could only be measured at the end of the experiment, which does not adequately take account of intermediate fluctuations. (4) The analysis may be negated because of the different cellular locations of the precursors, especially since gangliosides are at least principally synthesized in neurons.

That precursor pool changes were not responsible for the results obtained is suggested since no change of radioactivity was observed in the total soluble fraction nor in the chloroform-methanol-residue (probably principally glycoprotein) or lipid fractions. These observations are, however, not conclusive. An aid to the resolution of this problem would be the use of a more specific precursor. Quarles and Brady [16] have shown that (^{3}H)N-acetylmannosamine is a relatively specific precursor for the sialic acid of glycoprotein and gangliosides. However, our observations indicate that this precursor does not cross the blood-brain barrier, so that intracranial injections would have to be used. Unfortunately, such injections are generally detrimental to the mice, impairing their acquisition, giving relatively poor reproductibility of injections and uneven intracerebral distribution of radioactivity.

The changes observed in these experiments do suggest that there are changes associated with conditioned avoidance training in the synthesis of gangliosides or their related metabolism. That the yoked mice also show a small effect is not unexpected, since these mice, too, are undergoing a novel (and in a sense a training) experience. These results are also consistent with those of Maccioni *et al.* [15] who observed increases in the incorporation of (6-^{3}H)glucosamine into rat brain gangliosides in response to environmental (light and sound) stimulation. Irwin and Samson [9] studied the incorporation of (1-^{14}C)glucosamine into brain gangliosides of rats trained to swim in a water maze. They observed both specific and non-specific decreases in the incorporation. The difference between their results and those presented here may be accounted for by the differences in precursor, route of injection (intracranial), task and species.

Our finding of the lack of a disproportionate change in the labelling of a single ganglioside species resembles the finding of a light-induced increase in concentration of total gangliosides without change in the relative proportion of gangliosides in rod outer segments of calf retina [3]. It is possible that changes in the sialyl residues of the gangliosides mediating changes in membrane permeability do occur, but at such rapid rates as to elude demonstration by available analytical methods.

That no correlation was observed in the trained animals between the ganglioside specific radioactivity and parameters of the training such as number of shocks or acquisition rate is not entirely surprising. Learning intensity need not be a continuous function; ceiling phenomena may be present since the animals are overtrained in the task, and further we need not expect to observe correlations between the extent of learning and the magnitude of the biochemical effects of every link in the chain of memory consolidation. We do not suggest that the gangliosides are part of this chain, however the results reported here are consistent with earlier reports and with the hypothesis that brain ganglioside synthesis may be influenced by environmental stimulation. The increased functional activity of the gangliosides indicated by the increased incorporation does suggest a relationship to brain function.

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