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RAPID COMMUNICATION

Incomplete Reversibility of an Experimentally Induced Hypocholinergic State: Biochemical and Physiological, but Not Behavioral, Recovery

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RUSSELL, R. W., R. A. BOOTH, D. J. JENDEN, A. S. CHANG, K. M. RICE, M. ROCH AND S. D. LAURETZ. *Incomplete reversibility of an experimentally induced hypocholinergic state: Biochemical and physiological, but not behavioral, recovery.* PHARMACOL BIOCHEM BEHAV **41**(2) 433-444, 1992.—In previous reports, we described the experimental development of a hypocholinergic state in rats following the total replacement of dietary choline by an artificial isostere, *N*-aminodeanol (NADe). NADe shares most of the physicochemical and biochemical characteristics of choline (Ch) but is utilized less efficiently in pathways leading to the formation of both acetylcholine and phospholipids. This experimental model mimics many of the features of human degenerative dementias. We now discuss the behavioral and physiological effects of restoring a normal diet after the hypocholinergic state has become well established. The procedure by which that state was induced has been described in detail in earlier publications. After replacing Ch in the diets of weanling rats for 270 days, NADe replaced 70-85% of the phospholipid-bound Ch in plasma, brain, and peripheral tissue. When dietary NADe was removed and Ch was restored in the diet, NADe disappeared and plasma and erythrocyte (RBC) choline levels returned to normal within 30-60 days. Quinuclidinyl benzilate (QNB) binding showed that muscarinic receptors continued to be depressed in animals remaining on the NADe diet, but returned to control levels in the reversal group. There were no differences in cholinesterase activity among the three treatments. Choline acetyltransferase activity returned to control levels, while continuing to be lower in the NADe animals. Liver lipids were elevated in the latter and not significantly different in the control and reversal groups. Among physiological functions, body weight increased more rapidly in the reversal group than in animals continuing on the NADe diet. Brain weights of the reversal animals were significantly greater than those of animals not reversed, but less than controls. Core body temperatures did not differ from controls at any time during the reversal period. Behaviorally, nociceptive thresholds indicative of sensory-reflexive and sensory-perceptual responses remained significantly below normal, that is, a hyperalgesic state. Reversal animals also remained hyperactive and displayed memory significantly poorer than those on the normal diet, that is, no improvement over animals continuing on NADe. In general, the results suggest that behavioral losses induced by NADe reflect persisting changes in the CNS, despite essentially complete recovery of biochemical parameters. The changes may be morphological or be associated with adaptive changes in other neurochemical events in the CNS.

False precursor (NADe)   False transmitter   Cholinergic function   Experimental hypocholinergic model
Progressive degenerative dementia

IN previous reports, we described the experimental development of a hypocholinergic state in rats that mimics many of the features of human progressive degenerative dementias (25,39). Our objective was to test a hypothesis about the etiology of such disorders. This model state is induced by chronic administration of an analog of choline, *N*-aminodeanol (NADe), that shares most of the physicochemical and biochemical characteristics of choline (Ch) but is handled less efficiently (30-34). It is taken up in competition with Ch by the high- and low-affinity Ch transport systems, acetylated by choline acetyltransferase (ChAT), and stored in vesicles as a classical false transmitter, *o*-acetyl-*N*-aminodeanol

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(AcNADe). AcNADe interacts with both muscarinic and nicotinic receptors in competition with acetylcholine (ACh), but is only 4 and 17%, respectively, as potent as ACh. This produces a profound hypocholinergic state, particularly at muscarinic sites. Like ACh, AcNADe is rapidly hydrolyzed by cholinesterase (ChE). NADe is also progressively incorporated in place of choline into phospholipid pathways.

As the hypocholinergic state develops in animals fed a Ch-free NADe diet, basic physiological ("vegetative") processes appear not to be affected. Apparently, the rate at which this state develops is sufficiently slow for adjustments to occur that allow the organism to adapt at a survival level to its environment. By contrast, more complex behavioral functions are affected progressively, cognitive processes (e.g., learning and memory) being most sensitive and showing the least adaptability. We now report the consequences of restoring the N animals to a normal Ch-containing diet after the hypocholinergic syndrome is fully developed.

METHOD

Animals

All animals in the present experiments were offspring of females purchased from Bantin and Kingman (Pleasanton, CA). They were housed in semibarrier cages to reduce the risk of infection from endemic disease pathogens frequently found in rat colonies (9). Filters were changed and cages washed weekly with 5% bleach solution and bactericidal soap. Litter in the base of each cage was changed twice weekly. All personnel were required to gown before entering the animal room. "Sentinel" animals, placed at various locations in the room, were used for periodic serological tests of possible infestations. Results were negative in all cases. These precautions were considered essential to prevent viral and mycoplasma infections, which may have profound effects on experimental results (1).

Dietary Treatments

Pups were weaned onto their respective diets 29 days postpartum, when they were allocated randomly to one of two diets, that is, experimental (containing NADe in place of choline) or control (C). At 210 days on the diets, the experimental group was divided into two approximately equal groups, one of which (N) continued on the experimental diet, while the other (R) was placed on the normal control diet. From day 210 until termination of the experiment on day 270, the three groups continued on their respective regimens.

The composition of the diets has been described in detail in earlier publications from our laboratory (25,39). Briefly, rats were fed, ad lib, an artificial diet (ICN Nutritional Biochemicals, Cleveland, OH) in pelleted form. A Ch-free vitamin mix was used as a base in each diet with either Ch or NADe (30 mmol/kg^{-1}) added separately. Highly purified soya oil (Central Soya, Bellevue, OH) was used in all diets.

Choline and NADe

Free and lipid-bound choline and NADe were measured in erythrocytes (RBC) and plasma after 210 days on the dietary regimen and at 7, 14, 30, and 60 days after reversal (217, 224, 240, and 270 days on the dietary regimen). Bound Ch and NADe were determined in tissues after 270 days. The analytical procedure has been described in detail by Knüsel et al. (25).

Receptor Binding

Muscarinic receptors in cortex, striatum, and hippocampus were estimated using the quinuclidinyl benzilate (QNB) procedure as described by Yamamura and Snyder (46).

Enzyme Assays

Activity of the ACh synthesizing enzyme, ChAT, was measured in cortex, hippocampus, and striatum using the radiometric method described by Fonnum (17). ChE activities were assayed in the same tissues and in erythrocytes and plasma by the spectrophotometric method of Ellman et al. (13). Acetylthiocholine served as the substrate for ChE. Protein was measured by the method of Lowry et al. (27) using a Beckman DU30 spectrophotometer with bovine serum albumin as the standard.

Studies on Liver

Free Ch in the plasma is supplied by the diet and is released from phosphatidylcholine formed by transmethylation of phosphatidylethanolamine, primarily in the liver. Dietary Ch deficiency may lead to an increase in liver weight and lipid content (4). These considerations led to the inclusion of three measures in the present experiments: total liver weight, liver weight as a function of body weight, and an assay for lipids. Livers were removed immediately after sacrifice and frozen until analysis. Total lipids were analyzed by the method described by Folch et al. (16).

Body Weight

Body weights were recorded periodically from the first day after weaning until the animals were sacrificed at the end of the experiment, using a Sartorius High Capacity Balance Model 1404 MP8 and a Sartorius Model 7279 printer. The measures served as indices of possible differences in general health.

Brain Weight

Animals from all groups were sacrificed by decapitation at the end of the reversal period (day 276). Brains were removed and weighed on an analytical balance.

Core Body Temperature

Core body temperature was measured using a YSI Series 500 probe and a YSI Model 49TA digital thermometer, with an accuracy within the range of temperatures recorded of ± 0.05°C. Probes were inserted into the rectum to a depth of 6 cm. Measures were taken immediately before testing at 210, 217, 224, 240, and 270 days.

Nociception (Algesia)

Nociceptive thresholds were determined by the up-and-down procedure developed in our laboratory (7). Measurement of flinch and jump thresholds for foot-shock involved placing the animal in a test chamber, the floor consisting of stainless steel rods through which electric shocks of varying intensity could be delivered. Shock intensities were available from 0.05–4.0 mA in 20 steps arranged in a geometric series. Use of the full range of intensities was never necessary in determining thresholds. Each shock pulse (60 Hz) had a duration of 0.5 s and shocks were delivered at approximately 10-s intervals. Shock levels at the start of an up-and-down series

were set at midpoints of the ranges within which preliminary experiments had shown the thresholds were likely to lie. The experimenter then adjusted the intensity in accordance with the animal's response on each particular trial, that is, raised 0.1 $\log_{10}$ unit when no response occurred and lowered 0.1 $\log_{10}$ unit when a response was made. A "flinch" was defined as elevation of one paw from the grid floor and "jump" as rapid movement of three or more paws involving withdrawal from the floor. Thresholds were measured 30 min after the recording of spontaneous activity at 210, 217, 224, 240, and 270 days. Higher thresholds indicate hypoalgesia and lower thresholds hyperalgesia.

Spontaneous Activity

Spontaneous activity was measured in circular open-field chambers with diameters of 60 cm using the apparatus and procedure described by Silverman et al. (43). Briefly, the interior walls of the chambers were fitted with two sets of infrared-sensitive photocells and infrared-emitting LED's. One set, 4 cm above the floor, measured horizontal locomotor activity, while the second set, located 12 cm from the floor, concurrently measured vertical rearing activity. The chambers were interfaced with a TRS-80 Model III microcomputer that automatically recorded all light beam breaks and, at the end of each animal's daily 20-min session, printed the results in terms of horizontal and vertical activity during each 2-min interval. Activity was defined as the total beam breaks during a 20-min session and was measured immediately after temperature was measured in the morning at 210, 217, 224, 240, and 270 days.

Intersession Habituation

Habituation, defined as a "primitive form of learning," may be observed as a decrement in behavioral responding ". . . when an animal is exposed repeatedly to a novel stimulus without an accompanying biologically relevant consequence such as food or shock" (20). The present experiments provided opportunities to observe intersession habituation as decreases in levels of activity during the repeated 20-min assays.

Inhibited (Passive) Avoidance

Inhibited (passive) avoidance was measured in a "step-through" apparatus similar to that used by McGaugh (28). The apparatus consisted of 1) a small compartment made of white plastic, 2) a larger, dark compartment of stainless steel, and 3) a shock delivery unit with a set duration (0.5 s) and adjustable intensity (mA) of the mild electric shock used as an aversive stimulus. The procedure involved two types of trials. During a single training trial, the animal was placed in the white compartment; entry into the dark compartment led immediately to the closing of a guillotine door and the administration of a 0.30-mA footshock for 0.5 s. Similar trials without shock were given periodically during the reversal period, the measure taken being time to enter the dark compartment after release from the white. The times for animals not entering within 15 min were recorded as 900 s.

Conditioned Avoidance Response

Effects on performance of a discrete trial one-way conditioned avoidance response (CAR) were observed using the apparatus and general procedure described by Russell and Macri (40). The animal was required to traverse an alley from a start to a goal compartment. The raising of a door to the start compartment activated a buzzer, the conditioned stimulus (CS), opened the alley for the animal's response, and started a timer. If a response was not made within 10 s thereafter, an electric shock, the unconditioned stimulus (UCS), was automatically delivered. The shock was terminated and the timer stopped when the animal interrupted a light beam in the goal compartment. Thus, two responses could be studied: an innate escape response stimulated by the UCS, Re, and a learned avoidance response to the CS, Ra. The standard shock intensity for animals was fixed at 1.0 mA. Learning consisted of trials to the criterion of 7 Ra in 10 consecutive trials or to a total of 30 trials., whichever came first. Memory was measured by tests of five trials each at key times during the reversal period, the number of trials being limited to avoid overlearning (21). During learning, the procedure provided five measures of performance: time to make an avoidance response (Ra) or an escape response (Re); the numbers of escape and avoidance responses (NRe and NRa); and the number of trials to reach the criterion of learning, Nc. During the five-trial assays for memory only, the first four of these could be measured, but because NRa and NRe were linearly dependent only the former was analyzed.

Statistical Analyses

The resulting data were analyzed for two general purposes using SAS procedures (41) (SAS Institute, Inc., Box 8000, Cary, NC 27511-8000). Whenever appropriate, differences between treatment groups were tested using parametric statistics [analysis of variance (ANOVA) and Student's t]. When group differences were significant by ANOVA, Scheffé's method (42) for judging contrasts among group means was applied to clarify the contrasts mainly responsible for the significance. Two-way ANOVA's provided information about changes during the overall reversal period and the interaction factor indicated whether the group trends differed significantly as a function of duration of reversal. When measures were truncated by the nature of the measuring instrument and when data were significantly skewed, nonparametric methods of analysis were used. In situations where a two-way nonparametric analysis was required, ranks were converted to normal-order statistics (15,18) followed by an ANOVA. In the case of ties, the mean of the corresponding normal-order statistics was used. "Significance" is defined in terms of the 5.0×10^{-2} level of confidence and exact p values are given. For most purposes, effects of treatment and time on diet are included in the text only when statistically significant. Measures of central tendency are reported as mean ± SEM (sample size).

RESULTS

The results of the experiment are reported in three major sections: biochemical, physiological, and behavioral. In general, results of all analyses are presented in the following order: analyses of group means displaying the effects on the three treatment groups of time after restoration of the normal diet and of their interactions; cross-sectional one-way ANOVA's by day and/or by group; and, when significant differences are found, posthoc analyses of contrasts among the means using Scheffé's method or regression on time.

Biochemical

NADe and Ch levels in blood. At the beginning of the reversal period (day 210), groups N and R had been treated identically and no significant difference between them would

be expected. Student's t-tests showed this to be the case for total free or bound precursor and for the ratio NADe/total in both plasma and erythrocytes. Highly significant differences in all four ratios were found between the combined N + R groups and group C. As shown in Table 1, a striking reduction in total plasma phospholipid-bound precursor was seen in group N (to 37% of group C), but no comparable reduction was found in erythrocyte levels. In contrast, levels of total free precursor were higher in group N than in group C at the beginning of the reversal period in both plasma and erythrocytes, although the difference was significant only in erythrocytes, $t(10) = 0.40$, $p = 7.10 \times 10^{-1}$; $t(10) = 3.07$, $p = 1.2 \times 10^{-2}$, respectively.

Figure 1 summarizes the changes in free and bound NADe, expressed as a proportion of total precursor (Ch + NADe), in plasma (A and B), and in erythrocytes (C and D) during the reversal period. As previously reported (25,39), bound NADe was about 70% of total precursor at the beginning of the reversal period in both erythrocytes and plasma, while free NADe was 80–90%. It is clear that NADe was eliminated rapidly from all four compartments when the normal diet was resumed and that the elimination is virtually complete in 2 mo. In contrast, the proportions in groups C and N changed relatively little. Statistically, these visually obvious conclusions were highly significant. Two-way ANOVA on the ratio of NADe to total precursor in these four compartments showed significant ($p < 10^{-4}$) differences among groups, differences among time points, and interactions between the two factors. The Scheffé test applied to cross-sectional ANOVA at each time point indicated that, for all four parameters, the ratio for group N was significantly greater than control (group C) ($p < 10^{-4}$) on all days. No NADe was detected at any time in any animal in group C. Group R was significantly ($p < 10^{-4}$) lower than the N group at all time points except the beginning of the reversal period (210 days), and significantly ($p < 10^{-4}$) greater than group C (zero) for all time points except 240 an 270 days. Similar results were obtained for tissue levels of free and bound NADe, expressed as a proportion of total precursor, in cerebral cortex, kidney, heart, liver, and ileum (data not shown). Longitudinal ANOVA showed that during the experiment ratios changed little in N and C animals, as we have previously reported (25).

Total NADe and Ch. In contrast with the ratios given above, total NADe and Ch did not change substantially during the reversal period (Table 1), although some of the changes were statistically significant. Scheffé posthoc contrasts provide further details. Total bound phospholipids in plasma differed in all treatment groups: N < R < C. For RBC, treatment effects were significant between groups R and N, but not between these and group C. Total plasma-bound levels increased consistently as the duration of the reversal period increased; however, the increase was only significant between days 210 and 270. Scheffé contrasts showed significant differences between all groups in their levels of total free RBC phospholipids in the order: C < N < R. Significant changes occurred in plasma and RBC totals as the duration of the reversal period increased. There were no significant interactions, indicating similar trends for all groups.

Enzymes. Brain ChAT and ChE activities and the B_{max} for QNB binding are summarized in Table 2 for three brain regions: cerebral cortex, corpus striatum, and hippocampus. Two-way ANOVA showed, as expected, that differences among brain regions were highly significant ($p \leq 1.2 \times 10^{-3}$) for all three markers, while differences among treatment groups appeared only in ChAT, $F(2,78) = 3.44$, $p = 9.9 \times 10^{-3}$, and QNB binding, $F(2,30) = 4.32$, $p = 2.2 \times 10^{-2}$. No significant interactions were found in any of the variables, indicating that the patterns of change were not different for different brain regions. Cross-sectional ANOVA for each brain region separately showed significant differences among groups only for ChAT in hippocampus, $F(2,25) = 3.84$, $p = 3.5 \times 10^{-2}$, and for QNB binding in cortex, $F(2,15) = 3.99$, $p = 4.1 \times 10^{-2}$, but the means for both of these markers were smaller for group N than for group C in all three brain regions, a highly suggestive trend ($p = 3.1 \times 10^{-2}$, two-tailed binomial test). There were no significant changes in ChE in any brain region or in blood (data not shown).

Physiological

Liver weight and lipid content. These results are summarized in Table 3. Although total liver weight did not differ among the three treatment groups, differences in the ratio of liver weight to body weight and the percent lipids were highly

TABLE 1

MEAN CONCENTRATIONS OF TOTAL FREE AND PHOSPHOLIPID-BOUND PRECURSUR (NADe + Ch) IN PLASMA AND ERYTHROCYTES

	Plasma			Erythrocyte		
Day	C	R	N	C	R	N
Total free NADe + Ch (μM)						
210	40.68 ± 4.65 (6)		43.58 ± 5.40 (6)	24.38 ± 1.25 (6)		34.99 ± 3.23 (6)
217	37.45 ± 6.32 (3)	42.34 ± 3.12 (3)	37.35 ± 1.11 (3)	23.42 ± 2.89 (3)	40.45 ± 0.89 (3)	24.16 ± 9.75 (3)
224	31.58 ± 3.00 (3)	40.47 ± 7.75 (3)	27.70 ± 2.76 (3)	21.66 ± 3.12 (3)	29.75 ± 1.18 (3)	33.06 ± 6.89 (3)
240	33.51 ± 3.81 (6)	27.15 ± 3.19 (3)	36.15 ± 4.29 (6)	26.08 ± 2.70 (6)	30.72 ± 5.20 (3)	28.25 ± 1.44 (6)
270	36.06 ± 7.49 (6)	30.43 ± 2.40 (4)	21.40 ± 1.51 (6)	21.75 ± 1.56 (6)	28.24 ± 1.82 (4)	26.37 ± 3.44 (6)
Total phospholipid-bound NADe + Ch (μM)						
210	1.755 ± 0.212 (6)		0.655 ± 0.078 (6)	3.166 ± 0.162 (6)		3.060 ± 0.318 (6)
217	1.806 ± 0.133 (3)	1.523 ± 0.113 (3)	0.740 ± 0.011 (3)	2.004 ± 0.285 (3)	2.577 ± 0.216 (3)	2.385 ± 0.086 (3)
224	1.974 ± 0.114 (3)	1.798 ± 0.136 (3)	0.756 ± 0.017 (3)	2.779 ± 0.098 (3)	3.155 ± 0.070 (3)	2.367 ± 0.410 (3)
240	1.963 ± 0.034 (6)	1.577 ± 0.030 (3)	0.786 ± 0.039 (6)	3.009 ± 0.209 (6)	2.755 ± 0.121 (3)	2.648 ± 0.145 (6)
270	2.168 ± 0.111 (6)	2.136 ± 0.170 (4)	1.436 ± 0.473 (6)	2.496 ± 0.238 (6)	3.662 ± 0.391 (4)	2.249 ± 0.385 (6)

By group (C, R, and N) and experimental day (210, 217, 224, 240, and 270).

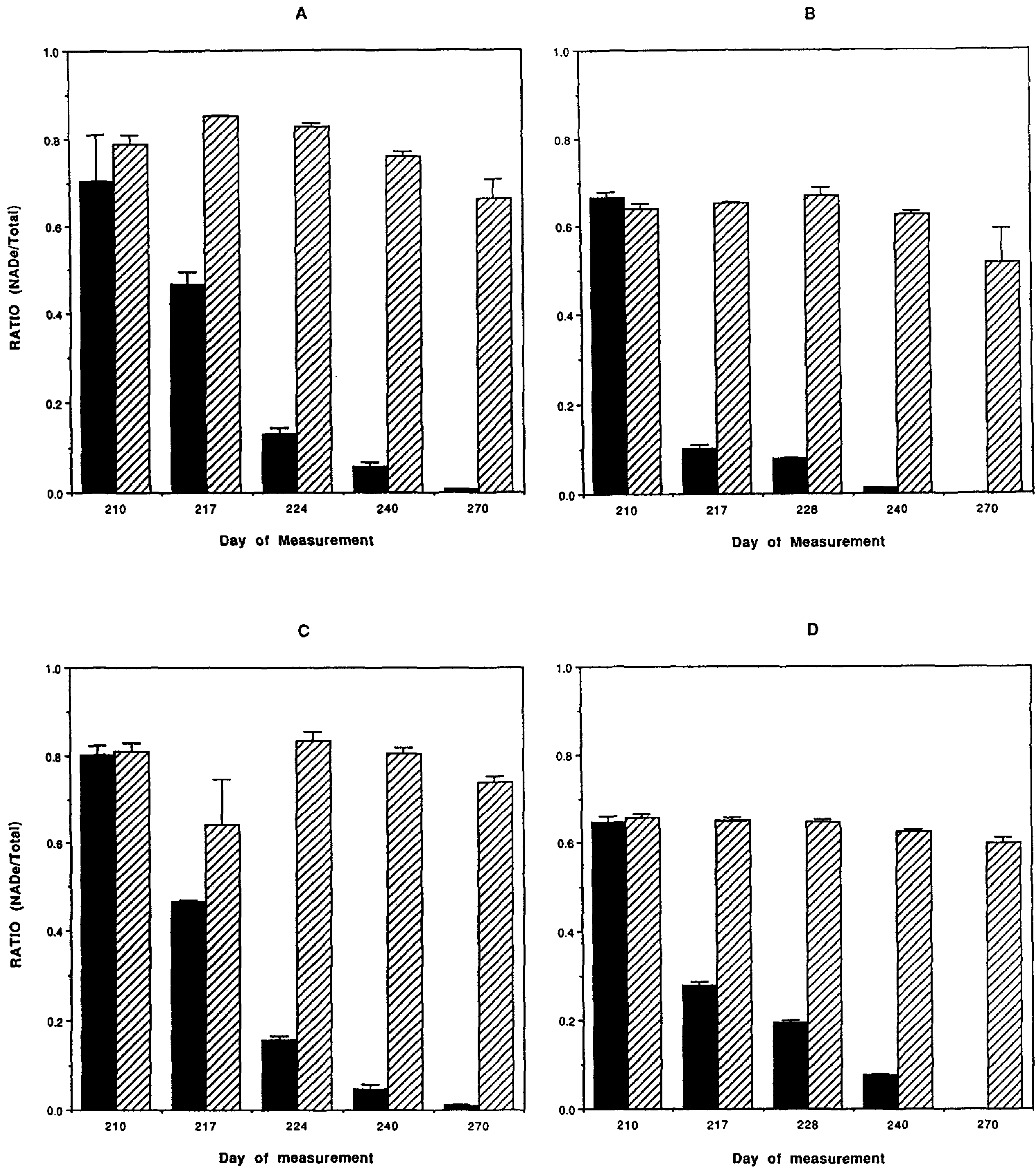

FIG. 1. Changes in free and bound NADe as proportions of total precursor (NADe and Ch). Panel A shows the changes in plasma-free precursor ratios and Panel B plasma-bound precursor ratios. Panels C and D present corresponding data for free and bound precursor ratios in erythrocytes. Solid bars represent group R and cross-hatched bars depict group N.

TABLE 2

MEANS OF SOME MACROMOLECULAR PARAMETERS OF CHOLINERGIC FUNCTION

Variable	Group	Brain Region		
		Cortex	Striatum	Hippocampus
ChE (μmol/mg^{-1}/h^{1})	C	4.02 ± 0.37 (12)	6.08 ± 0.18 (12)	26.00 ± 1.25 (12)
	R	3.99 ± 0.56 (9)	6.04 ± 0.40 (8)	24.19 ± 1.80 (9)
	N	4.26 ± 0.45 (9)	5.51 ± 0.28 (9)	24.55 ± 1.03 (9)
ChAT (nmol/mg^{-1}/h^{1})	C	37.1 ± 2.21 (12)	101.6 ± 7.48 (12)	50.08 ± 3.06 (12)
	R	34.1 ± 1.49 (8)	103.3 ± 7.76 (9)	49.04 ± 4.24 (8)
	N	35.4 ± 1.51 (9)	85.5 ± 3.43 (9)	40.75 ± 3.65 (8)
QNB binding (fmol/mg^{-1})	C	607.9 ± 20.0 (7)	770.9 ± 54.3 (4)	732.3 ± 16.4 (4)
	R	666.9 ± 87.6 (6)	736.7 ± 84.4 (3)	707.1 ± 63.0 (3)
	N	555.9 ± 20.7 (5)	661.1 ± 18.0 (3)	638.9 ± 30.8 (4)

By treatment group and brain region. Activities are expressed per mg protein.

significant. Scheffé contrasts showed that the liver as a proportion of body weight differed significantly among all three groups in the order: C < R < N. Similar analyses indicated that animals in group N had significantly greater lipid content than those in groups C and R, the latter not differing from each other. In both the last two variables, the effect of restoring the diet to normal was to change the variable in the direction of normality.

Body weight. The mean body weight for each group and day during the reversal period is summarized in Table 4. Student's *t*-tests showed that at the beginning of the period groups R and N did not differ from each other, but the combined N + R groups differed significantly from group C. Cross-sectional ANOVA indicated that the differences among the three treatment groups were significant at every time point ($p < 10^{-5}$). Two-way ANOVA confirmed a significant difference among treatment groups, $F(2,27) = 34.03$, $p = 4.2 \times 10^{-8}$), a significant effect of time, $F(4,108) = 44.66$, $p < 10^{-10}$, and a significant interaction between the two primary factors, $F(8,108) = 6.56$, $p = 5.9 \times 10^{-7}$, indicating that the patterns of change with time were different for the three groups. Changes from baseline (210 days) were greatest for groups C and R.

Scheffé posthoc contrasts showed that throughout the 60 days of reversal animals in group C were heavier than those in the other two groups. Body weights of animals R and N did not differ significantly until 270 days (60 days after the start of the reversal period), when the former were heavier than the latter and were approaching control levels.

Brain protein levels and weight. Brain protein levels did not differ among treatment groups in any of the regions studied. Brain weight at the time of sacrifice (67 days of reversal) is summarized in Table 5. ANOVA established that highly significant differences existed among the three treatment groups, $F(2,24) = 49.70$, $p = 1.3 \times 10^{-9}$. Scheffé posthoc contrasts showed that the differences between all group means were significant. The rank order, C > R > N, was the same as the rank order for body weights.

TABLE 4

MEAN BODY WEIGHT BY GROUP AND EXPERIMENTAL DAY

Day	Group		
	C (n = 12)	R (n = 9)	N (n = 9)
210	596.6 ± 19.77	413.2 ± 21.76	413.4 ± 29.65
217	616.1 ± 19.66	438.7 ± 19.29	409.7 ± 12.42
224	619.9 ± 23.50	447.0 ± 19.95	414.6 ± 12.52
240	637.0 ± 21.86	467.3 ± 21.88	420.0 ± 15.11
270	674.4 ± 22.97	520.0 ± 22.44	427.3 ± 13.38

Earlier research has raised questions about possible relations between body and brain weight (10,11,19). Table 5 records body weights at time of sacrifice and summarizes results of analyses of the ratios, brain weight/body weight, for each of the three treatment groups. The mean ratios are in a rank order exactly the reverse of the total body and brain weights. ANOVA provided a significant F value for differences among treatment groups, $F(2,24) = 7.64$, $p = 2.7 \times 10^{-3}$. Subsequent Scheffé contrasts established that this was due to the difference between groups N and C, while subjects in group R were intermediate and not significantly different from either of the other two groups.

Core body temperature. As in previous studies on NADe, core body temperature did not differ significantly among the groups throughout the reversal period (data not shown). Because core body temperature was measured at several times

TABLE 3

LIVER WEIGHT AND LIPID CONTENT AT TIME OF SACRIFICE

Group	n	Liver Weight (g)	% Body Weight	% Lipids
C	12	17.61 ± 0.718	2.58 ± 0.052	7.42 ± 0.592
R	8	16.34 ± 0.614	2.99 ± 0.038	6.27 ± 0.558
N	9	15.65 ± 0.860	3.54 ± 0.160	12.03 ± 0.806

TABLE 5

BODY AND BRAIN WEIGHTS AT TIME OF SACRIFICE

Group	n	Body Weight (g)	Brain Weight (g)	Ratio (%)
C	10	675.3 ± 25.12	2.25 ± 0.038	3.32 ± 0.141
R	8	520.0 ± 22.44	1.95 ± 0.041	3.61 ± 0.154
N	9	427.3 ± 13.38	1.78 ± 0.027	4.05 ± 0.114

TABLE 6

NOCICEPTIVE THRESHOLDS BY GROUP AND EXPERIMENTAL DAY

Day	Flinch			Jump		
	Group C (n = 12)	Group R (n = 9)	Group N (n = 9)	Group C (n = 12)	Group R (n = 9)	Group N (n = 9)
210	2.045 ± 0.035	1.792 ± 0.040	1.736 ± 0.045	2.313 ± 0.027	2.076 ± 0.052	2.048 ± 0.040
217	1.979 ± 0.034	1.880 ± 0.048	1.784 ± 0.040	2.316 ± 0.033	2.283 ± 0.043	2.050 ± 0.046
224	2.071 ± 0.042	1.926 ± 0.036	1.858 ± 0.044	2.356 ± 0.028	2.278 ± 0.045	2.204 ± 0.048
240	2.004 ± 0.037	1.971 ± 0.029	1.805 ± 0.056	2.302 ± 0.029	2.325 ± 0.041	2.106 ± 0.038
270	2.135 ± 0.028	1.700 ± 0.035	1.862 ± 0.024	2.394 ± 0.032	2.058 ± 0.033	2.109 ± 0.042

Units are $\log_{10}(\mu A)$.

during an assay period, the data could be analyzed for possible differences among treatment groups as a consequence of exposure to the "stress" created by noxious stimuli, that is, electric shock, that was an integral part of the behavioral tests. Again, no significant differences were seen among the three treatment groups.

Behavioral

Nociceptive thresholds. Mean thresholds for both flinch and jump are summarized in Table 6. At the beginning of the reversal period, N and R groups did not differ significantly, but the combined N + R groups showed highly significant decreases in threshold, that is, hyperalgesia, when compared to group C ($p \leq 10^{-5}$). Both N and R groups continued to be hyperalgesic in comparison with the control animals at all time points and for both thresholds. Results of two-way ANOVA established that the group, day, and interaction factors were significant ($p < 0.02$) for both behaviors. One-way ANOVA by days supported by Scheffé contrasts corroborated the observation that group C had significantly ($p < 0.05$) higher flinch and jump thresholds than group N throughout the reversal period, group R generally falling between the two. Longitudinal one-way ANOVA showed that during the reversal period neither flinch nor jump thresholds changed substantially in groups C and N, only the flinch threshold for group C showing marginal significance ($p = 2.9 \times 10^{-2}$), while both

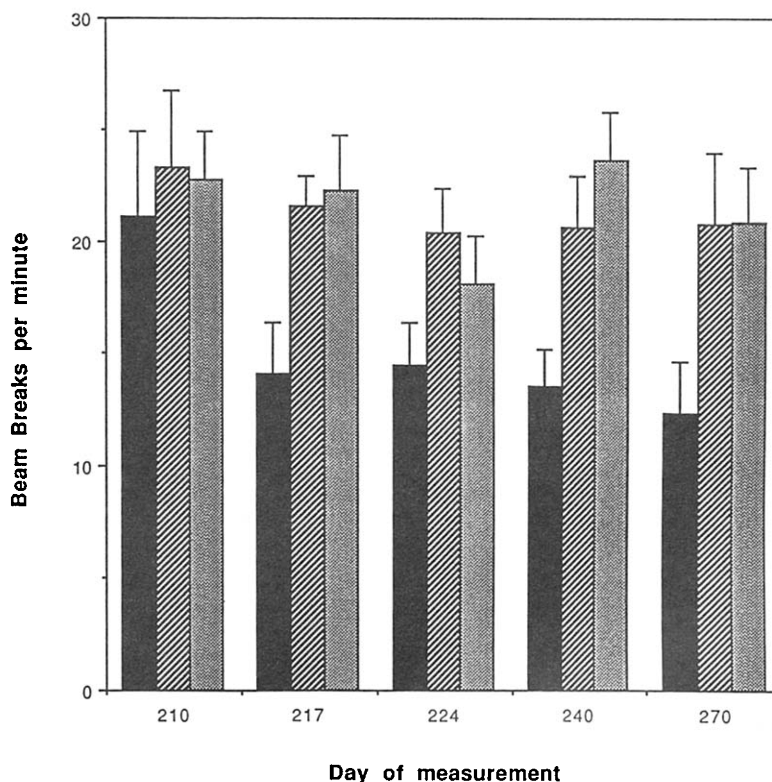

FIG. 2. Changes in spontaneous activity, that is, rearing behavior, of animals maintained on (a) the Ch diet (solid bars), (b) the NADe diet (dotted bars), and (c) those reversed from NADe to Ch diets (cross-hatched bars).

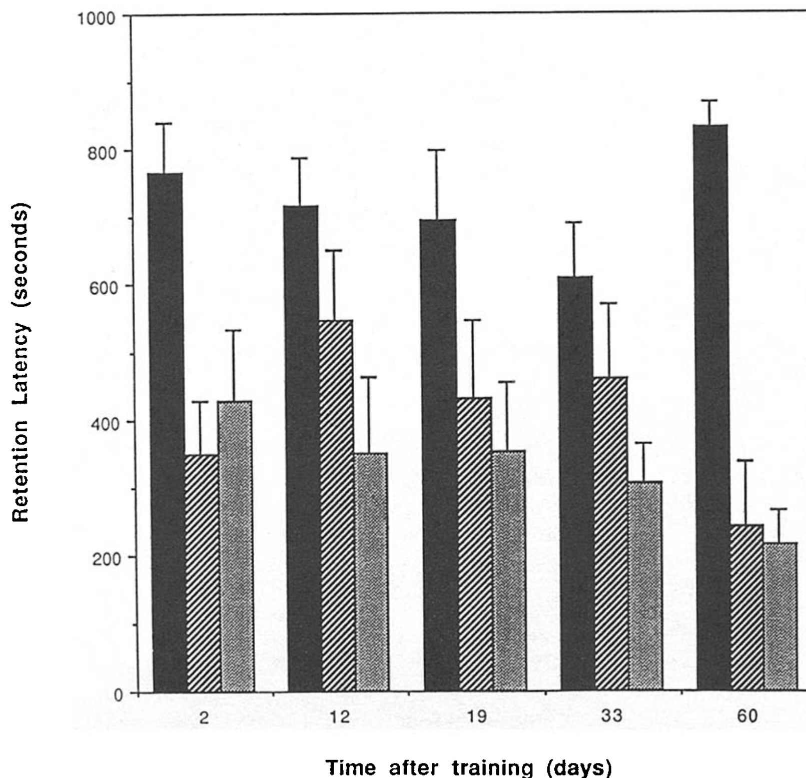

FIG. 3. Changes in memory as represented to retention latencies in the inhibited (passive) avoidance situation during the reversal period. The abscissa represents days after the training trials. Day 2 provides a baseline for performance prior to reversal, that is, day 210, which continued for 60 days. Bars in the histogram represent the same treatment groups in Fig. 2.

thresholds changed very significantly in group R ($p < 10^{-4}$). In the latter, both flinch and jump thresholds showed similar patterns of change during the 60 days of the reversal period. Thresholds were not significantly different from those of the NADe group immediately preceding the start of reversal (210 days). During the following 30 days, they fell between those of groups N and C and were not significantly different from either. At the end of the reversal period, thresholds for the R animals were not different from those of the N group.

Spontaneous activity and habituation. Inspection of Fig. 2 suggests that group N and R animals were hyperactive in rearing when compared with group C. Two-way ANOVA showed that this effect was significant for rearing, $F(2,27) = 5.07$, $p = 1.4 \times 10^{-2}$, but not for locomotion. Further examination using cross-sectional one-way ANOVA on each day followed by Scheffé contrasts established that the activities of the three treatment groups were not different when assayed immediately before reversal began, but within 1 week group C was less active than groups N and R, the latter two not differing from each other. This pattern continued throughout the reversal period. Longitudinal ANOVA on data for each group showed that only group C varied significantly ($p = 1.8 \times 10^{-2}$) during this period. A Scheffé test showed that this was entirely attributable to a decline in activity in this group between the first and all subsequent days, $F(4,44) = 3.14$, $p = 2.3 \times 10^{-2}$. This intersession habituation was notably lacking in the other two groups.

Inhibited (passive) avoidance. Figure 3 shows changes in response latencies for three treatment groups during the reversal period. Because the measured response latency was arbitrarily truncated at 900 min, nonparametric methods were used in the analyses reported below. To carry out a two-way analysis, ranks were converted to normal-order statistics (15, 18,41) followed by ANOVA. In the case of ties, the mean of the corresponding normal-order statistics was used. At the beginning of the reversal period, groups N and R did not differ significantly, but showed a significantly shorter response latency than group C, $t(27) = 3.25$, $p = 3.1 \times 10^{-3}$. Table 7 summarizes the group means by day and group. Two-way ANOVA confirmed significant differences among groups, $F(2,26) = 10.75$, $p = 4.0 \times 10^{-4}$, but differences

TABLE 7

RESPONSE LATENCIES IN INHIBITED AVOIDANCE TEST AFTER CONVERSION OF DATA TO RANKS AND NORMAL-ORDER STATISTICS

	Group		
Day	C (n = 11)	R (n = 9)	N (n = 9)
210	0.743 ± 0.209	−0.294 ± 0.232	−0.221 ± 0.349
217	0.593 ± 0.205	−0.033 ± 0.376	−0.378 ± 0.331
224	0.471 ± 0.308	−0.277 ± 0.383	−0.530 ± 0.274
240	0.406 ± 0.231	−0.085 ± 0.294	−0.434 ± 0.149
270	0.844 ± 0.122	−0.726 ± 0.287	−0.760 ± 0.211

By group and experimental day.

among days and the interaction failed to achieve significance. Scheffé analysis indicated that group C differed significantly from each of the other groups ($p < 10^{-2}$), while the latter were not different. These results indicate that memory for the single training (shock) trial was significantly more persistent in group C than in that of the other two treatment groups, and that there was no significant difference in overall performance between the latter.

Cross-sectional ANOVA were used to analyze for differences between treatment groups during each of the 5 days when the assay was repeated. With the exception of days 217 and 224, differences between groups were statistically significant on all days. Longitudinal ANOVA were used to explore trends within each group as time of reversal increased. Throughout the reversal period, animals in group C showed much longer latencies in responding than did those in the other groups. The latencies within each group did not change significantly with time.

CAR. The experimental design for CAR was analogous to that described above for inhibited avoidance, animals receiving learning trials on day 210 prior to the reversal period. On each of days 217–270 (7, 14, 30, and 60 days after training), they were given five retention trials. Each retention trial provided three response parameters (see Method section for details) that necessarily were related and gave similar results on analysis. Because of the restriction on performance scores imposed by the limited number of trials per assay and the truncated response times, the results have been analyzed, like those of the inhibited avoidance test, using normal-order statistics.

At the time of the training trials on day 210, groups N and R had been treated identically, the reversal period having not begun. Student's t-tests on the normal scores of ranks showed that, as expected, groups N and R did not differ significantly in any of the four response parameters: Re, Ra, NRe, or Nc (trials to reach the criterion of 7 avoidance responses, Ra, in 10 consecutive trials). When groups N and R were pooled, NRe and Nc were significantly greater, $t(20) = 2.56, p = 1.9 \times 10^{-2}$, and $t(20) = 2.68, p = 1.4 \times 10^{-2}$, than those of group C, while Ra and Re were not, indicating that learning required more trials in the NADe-treated animals than in controls.

The results are summarized in Table 8. Two-way ANOVA on the three response variables from the retention trials gave significant differences among groups only for NRa, $F(2,18) = 5.88, p = 1.1 \times 10^{-2}$, while only Re showed a significant difference among days, $F(3,36) = 5.25$, $p = 4.1 \times 10^{-3}$. NRe and NRa were linearly dependent variables and therefore gave identical ANOVA results. None of the response parameters showed a significant interaction between the two primary factors. Further analysis of the data by cross-sectional ANOVA showed that the difference among groups was attributable to differences near the beginning of the reversal period, on day 217 (Ra and NRa) and day 224 (Ra only). Scheffé analysis of contrasts between group means indicated there were significant differences between groups C and R for both NRa and Ra on day 217, $F(2,18) = 6.83$, $p = 6.2 \times 10^{-3}$, and $F(2,18) = 3.78$, $p = 4.2 \times 10^{-2}$, respectively and also for Ra on day 224, $F(2,18) = 5.53, p = 1.3 \times 10^{-2}$. Groups N and R did not differ significantly in any response parameter on any day. Thus, group C had significantly more avoidance responses than group R, while groups N and R were similar. Longitudinal ANOVA on each group individually revealed no significant differences among days in any group for NRa or Ra, but a highly significant change in Re, $F(3,18) = 9.27$, $p = 6.3 \times 10^{-4}$, for group R only. This proved to be associated with a highly significant linear, $F(1,6) = 15.13$, $p = 8.1 \times 10^{-3}$, and quadratic, $F(1,6) = 6.08$, $p = 4.9 \times 10^{-2}$, regression on test days. Scheffé analyses of these data showed that Re for both days 217 and 224 was significantly longer ($p \leq 2.7 \times 10^{-2}$) than for either day 240 or 270.

DISCUSSION

The research described in the present report is part of a program designed to test the hypothesis that some forms of progressive degenerative dementias may be the result of competition for available Ch between biochemical pathways involved in the synthesis of ACh and in phospholipid metabolism (23,45). Such a competition could have many precipitating causes and would be expected to have a relatively selective effect on cholinergic neurones since these uniquely utilize both pathways. Interference with ACh synthesis would have widespread effects on both central and peripheral processes essential for an organism's adjustments to changes in its physical and psychosocial environments. Interference with phospholipid metabolism is likely to impair the mechanisms responsible for maintenance of cell membranes and intracellular organelles, leading to structural damage and eventually to cell death. We have previously shown that replacement of all dietary choline by the artificial choline isostere NADe in weanling rats leads progressively to a hypocholinergic state in which

TABLE 8

NORMAL SCORES OF RANKED RESPONSE VARIABLES IN CONDITIONED AVOIDANCE RESPONSE TEST

Variable	Group (n)	Day 217	Day 224	Day 240	Day 270
Ra	C (5)	−0.684 ± 0.311	−0.935 ± 0.202	0.012 ± 0.361	−0.647 ± 0.393
	R (9)	0.594 ± 0.319	0.432 ± 0.262	−0.071 ± 0.383	0.164 ± 0.290
	N (7)	0.191 ± 0.277	0.124 ± 0.309	−0.381 ± 0.497	0.237 ± 0.349
Re	C (2)	0.074 ± 0.572	0.513 ± 0.239	−0.056 ± 0.373	0.254 ± 0.108
	R (7)	0.847 ± 0.216	0.755 ± 0.318	−0.541 ± 0.347	−0.420 ± 0.335
	N (6)	0.439 ± 0.561	−0.020 ± 0.377	−0.884 ± 0.204	−0.546 ± 0.386
NRa	C (5)	0.544 ± 0.188	0.959 ± 0.226	0.610 ± 0.596	0.463 ± 0.165
	R (9)	−0.794 ± 0.229	−0.299 ± 0.260	0.132 ± 0.415	−0.387 ± 0.252
	N (7)	−0.279 ± 0.272	0.036 ± 0.428	0.360 ± 0.277	−0.221 ± 0.304

Grouped by day of test and treatment group.

most of the free and lipid-bound Ch is replaced by NADe, and that the latter can be utilized much less efficiently than Ch. Our earlier reports (24,25,30,31,33,39) discussed neurochemical, physiological, and behavioral abnormalities produced as the duration on the NADe diet increased. The work now described addresses the questions of whether NADe is eliminated when a normal diet is reinstituted, and if so whether the physiological and behavioral changes that developed during the period on the NADe diet disappear as NADe is eliminated.

Extending the Hypocholinergic State

Two control groups were used in these experiments: animals fed on a normal diet containing an adequate amount of Ch but no NADe (group C) and animals receiving an identical diet except that NADe was substituted for Ch in an equal amount (group N). In both cases, these diets began at weaning and continued for 270 days, including the 60 days during which a normal diet was restored to group R. This provided opportunities to observe possible effects of the two diets during a period more than twice as long in duration than that covered in our earlier reports, that is, 270 vs. 120 days.

It would be expected that animals on a nutritionally adequate diet would show changes consistent with normal aging processes. Our earlier report (39) indicated that early developmental sequences proceeded according to well-established norms. As age increased, physiological variables continued to change systematically. There was no evidence that maintenance on the nutritionally adequate Ch diet induced abnormal deviations from the norms for the species. By comparison, weaning to the NADe diet produced profound changes, the general nature of which we have reported as they appeared during a period of 120 days (25,39). The replacement of Ch with NADe served as the means for manipulating the independent variable in the experiment, that is, creating a hypocholinergic state to observe its consequent effects on biochemical-, physiological-, and behavioral-dependent variables.

By day 210, at the beginning of the reversal period, biochemical differences between groups C and N were highly significant. Bound and free Ch (both plasma and RBC) in group N was reduced to levels of 15–30% compared to group C. These levels were maintained throughout the remainder of the experiments (to day 270). NADe was never observed in blood or tissues of animals in group C. The striking reduction of total phospholipid-bound precursor in plasma in group N contrasts with the increased levels of free precursors (Table 1). We reported similar results from a shorter study (39). They may perhaps be explained most reasonably by decreased efficiency of the synthesis of phospholipid by the liver, which would tend to elevate the concentration of precursor and reduce the concentration of product.

No difference between treatment groups was found in ChE activity. ChAT activity and QNB binding were less in every brain region in group N compared with group C, although the difference was significant only in the hippocampus. It is now widely accepted that in progressive degenerative dementias such as SDAT ChAT is much reduced in certain brain regions (36,37). It is possible that if the present studies had covered a greater life span such changes might have been even more pronounced. Experiments are under way to investigate this possibility.

Groups C and N also differed behaviorally as we reported earlier in results from experiments over shorter time frames (24,31,39). Nociceptive thresholds, both sensory reflexive and sensory perceptual, were consistently lower in N than C animals, indicating hyperalgesia in the former. The N animals were hyperactive when compared to the C group. They showed poorer memories, which were particularly evident as time between training and recall increased.

These results indicate that in the model syndrome produced by NADe new steady states were achieved in a wide variety of functions, some of which interfered significantly with the normal adjustments of the animals to their environments. The findings give additional support to the conclusions reported earlier (25,39). They also provide set points for comparison with the third group (R) in the present studies, namely, animals restored to a normal diet after the NADe syndrome was fully developed.

Effects of Reversal

The primary objectives of the present research were to determine the extent and the rate at which NADe was eliminated after prolonged administration and to ascertain whether biochemical, physiological, and behavioral abnormalities induced by NADe changed concomitantly.

Elimination of NADe and its biochemical consequences. Basic to the present experiments were answers to questions about the elimination of NADe in tissues once it was replaced by Ch in the diet. Our results show that NADe was, in fact, quickly eliminated when its dietary administration was discontinued. The time constant for its elimination was about 10 days, and it was virtually undetectable in all pools studied within about 30 days. NADe was not found in autopsy samples of any tissue taken 60 days after its removal from the diet.

The elimination of NADe after restoration of a normal diet was paralleled by changes in some relevant features of liver function. The biochemical roles of the liver in Ch metabolism give this organ special relevance when considering deficits in cholinergic function. The roles may be summarized briefly as: Ch has a lipotropic action without which fat accumulates in the liver; Ch serves, after its oxidation to betaine, as a source of methyl groups that are transferred to *S*-adenosylmethionine and thence to many acceptors, including phosphatidylethanolamine; and Ch is incorporated into phosphatidylcholine, the primary constituent of all cell membranes. Abnormalities in any of these roles could produce deficits in Ch utilization and thus give rise to disturbances in cellular growth and maintenance throughout the body, which might possibly lead to degenerative diseases (5,23,45). NADe can, like Ch, serve as a source of methyl groups (30), and is certainly incorporated into phospholipids. Early studies of the effects of Ch deficiency showed a rapid accumulation of triglycerides in the liver and low levels of plasma phospholipids (4,26,35). As reported previously (39), NADe-treated animals showed significantly elevated ratios of liver weight to body weight and a significantly greater proportion of lipids in liver than those on a normal diet. These effects are consistent with the hypothesis that NADe serves as a less efficiently utilized substrate in the same pathways as Ch. Disappearance of these deficits when a normal diet was restored provides evidence of the reversibility of the pathological changes associated with them.

Physiological effects. We previously concluded (39) that behavioral changes seen in the NADe model were unlikely to be attributable to imbalances in basic homeostatic mechanisms. This conclusion was supported by data on three such physiological parameters, and was confirmed in the present experiments.

Core body temperature appeared not to have been affected, a result that might be interpreted as inconsistent with earlier results showing that body temperature is affected by cholinergic agonists and antagonists. However, these latter findings concerned effects measurable immediately after ad-

ministration of the agent and quickly disappeared, indicating that mechanisms involved in temperature regulation can adapt rapidly to changes to cholinergic interference. In the present experiments, adaptation could have occurred well within the 210 days of NADe treatment that preceded the reversal period.

Body weight was significantly different among groups immediately before the reversal period, during which all treatment groups showed an increase: Group C gained 10%, group R 26%, and group N only 3%. Group R was evidently compensating for its retarded growth in body weight during the 210 days on the NADe diet.

Brain weight per se, measured at time of sacrifice (67 days of reversal), appeared to follow the same general pattern as body weight, that is, C > R > N. Because the brain/body weight ratio decreases progressively during postnatal development in mammals (22), we have reported ratios in which the rank order is reversed. On the surface, this would seem to be paradoxical in light of the fact that ". . . the limitation of size of the mammalian brain [is determined] by the capacity of the body to supply energy . . ." (22). The paradox cannot readily be clarified in terms of the "growth spurt" hypothesis (10,11), which states that ". . . processes of development in the brain are likely to be more vulnerable to restriction and other stress at the time of their fastest rate." Plots of brain growth rates in the rat show a peak at about 10 days postnatally, reaching adult weight at approximately 30 days. Thus, brain growth was essentially complete before animals were placed on the NADe diet.

Behavioral effects. In our earlier report, we concluded that as time on the NADe diet increased, "more complex behavioral functions were affected progressively, cognitive processes (e.g., learning and memory) being most sensitive and showing the least adaptability" (39). A striking feature of the present results is that these same cognitive processes showed little or no sign of recovery during the 60 days of the reversal period, when their presumed biochemical substrates were rapidly returning to normal. Persistence of hyperactivity in group R clearly illustrates the disjunction between biochemical and behavioral functions. Hyperactivity has long been observed as a feature of an acute hypocholinergic state (38). It has been shown to disappear as cholinergic function has returned to normal. In our present experiments, this did not occur, indicating that the extended hypofunction produced effects, biochemical or histological, that persisted independently of the replacement of Ch by NADe.

Sensory-reflexive behavior as evidenced in the flinch response in our assay of nociceptive thresholds is primarily innate in nature (i.e., appears without the necessity for learning). Although voluntary behaviors (e.g., preening) may follow, the initial reaction to the shock stimulus is a stereotyped withdrawal response. By comparison, sensory-perceptual behavior demonstrated in the jump response requires the participation of the CNS as well as of sensory processes (6,12), that is, patterns of coordinated motor activity. Initially, both measures of these behaviors were not different in groups N and R. During the reversal period, the relative hyperalgesia decreased, returning parity with group N as the end of reversal approached.

Sensory-perceptual processes are also involved in locomotion and rearing activities in the open-field situation. There has long been empirical evidence that exploratory behavior in such situations is determined significantly by the search for "novel" stimulus conditions. Novel involves not only sensory processes involved in registering stimuli but also the perception of their relation to past experience (2). Normally, the magnitude of responding decreases as exposure to a new situation increases, that is, habituation takes place. This primitive form of learning was evidenced in the rearing behavior of the ChCl treatment group during early trials in the reversal period, when intervals between trials were 7 days, but not in the N or R animals. These effects continued when intervals between trials were increased. Clearly, this form of learning had been affected significantly by poorer memories in the N and R animals when the intersession interval was varied, that is, increased to 16 and to 20 days. Habituation of locomotor behavior followed a somewhat different pattern. Trends in group and day parameters did not differ during the reversal period. Such differences between the two behaviors are reminiscent of results we and others [e.g., (8)] reported in the past and suggested may be due to the involvement of more than the cholinergic neurotransmitter system in rearing behavior.

Other cognitive processes also showed disjunctions between the state of the cholinergic system and behavior. The latter included both inhibited (passive) and active avoidance responses, as measured by long-established techniques. With the exception of times of response in the active avoidance situation, all measures indicated that animals on the normal control diet had significantly better memories than did those on the NADe and reversal schedules. The latter two did not differ from each other throughout the 60 days of the recovery period.

The present results may be summarized by the following generalizations. Extending the periods of time on the ChCl and NADe diets had little effect on the variables measured, except for the treatment differences already established by the time of reversal. Returning animals to a normal diet after the NADe syndrome was fully established resulted in biochemical and physiological, but not behavioral, recovery. In particular, impaired memory persisted. The latter suggests that behavioral losses induced by NADe are mediated by persisting changes in the CNS despite essentially complete recovery of biochemical parameters. The nature of these changes remains in doubt; they may be morphological or be associated with adaptive alterations in other neurochemical events in the CNS. Poverty of synaptic contacts resulting from impaired phospholipid metabolism is a particularly attractive hypothesis. Dendritic spine counts are lower with aging in cerebral cortex (14) and this loss can be repressed by chronic dietary choline supplementation (3,29). These results were interpreted to suggest that choline supplementation may enhance the synthesis and turnover of membrane phospholipids and thus promote synaptic plasticity. The possibility that synaptic plasticity may be reduced by chronically impaired phospholipid metabolism in the *N*-aminodeanol model must be examined experimentally.

In its monograph "Losing a Million Minds," the U.S. Congress Office of Technology Assessment has commented about necrosis in the brains of persons with Alzheimer's disease: "The disruption of nerve cell circuits does not explain why the nerve cells die. Complete understanding of the etiology will thus need to elucidate the sequence of events that lead to the expression of disease, and is likely to involve many steps" (44). In the series of experiments reported here (and earlier), the primary objective has been to focus on events far back in that sequence. By so doing we have, in fact, created an animal model having many biochemical and behavioral analogies with progressive degenerative dementias.

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