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Brain Cholesterol X: Effect of Scheduling on the Sterol Lowering Capability of Methylphenidate (Ritalin).* (30437)

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Methylphenidate (phenyl-piperidyl-(2)-acetic acid methyl ester) is a compound which causes marked psychomotor stimulation. As a stimulant, it is classified somewhere between amphetamine and caffeine(1).

Previous reports from this laboratory(2-5) dealt with the effect of methylphenidate (Ritalin) on brain cholesterol metabolism, after chronic drug injection. The scheduling of drug administration in previous studies was not evaluated. To investigate further the problem of drug mechanism on brain cholesterol, a more systematic approach was attempted. The present report is a study of the effect of single and multiple drug injection on brain cholesterol level.

Experimental. The 295 male Swiss mice of Albino stock used in these studies were supplied by Stout Farms of Dexter, Mich. Animals representing age groups 5-6 weeks or 8-10 weeks were selected randomly within each age group, divided, and segregated into groups, each containing 5 mice. Food and water were administered *ad libitum*. Food was withheld for 24 hours prior to sacrifice. During the experiment, weights and daily intake of solid food and liquids were recorded. No significant change in these variables was measured in the drug groups.

A fresh stock saline solution of methylphenidate (1%) was made each week. This stock solution was diluted immediately before injection. Intraperitoneal injections of saline or drug were made with a volume of 0.5 ml. The smallest drug dose chosen (4 mg/kg) represents a level which significantly lowered brain cholesterol; larger doses (20 mg/kg) were not as effective. These drug levels represent respectively 1/35 and 1/7 of the usual LD_{50}.

Mice were killed by decapitation. Brain and liver were removed, washed, blotted dry, placed in test tubes, and quick frozen (acetone and dry ice). Tissues were stored in an ice chest (—30°C).

Separate tissue aliquots were taken for water and lipid content. The wet tissues were extracted with acetone:alcohol:ether (4:4:1). Solutions were filtered free of precipitated protein. Analyses of free and esterified cholesterol in the filtrate were made by precipitation with digitonin(6) or tomatine(7); by paper chromatography(8); or by gas-liquid chromatography(9). Details of precipitation methods have been reported(6,7). The chromatographic method was a modification of LaBarre *et al*(8). Strips of filter paper 1″ × 30″ were drawn through a 5% (v/v) so-

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lution of Dow Corning 1107 Silicone in cyclohexane and then dried in an oven at 100-110°C for one hour. The mobile phase was methanol:acetone:formic acid:water (50:50:5:5). The atmosphere was saturated with the solvent system one hour before running the

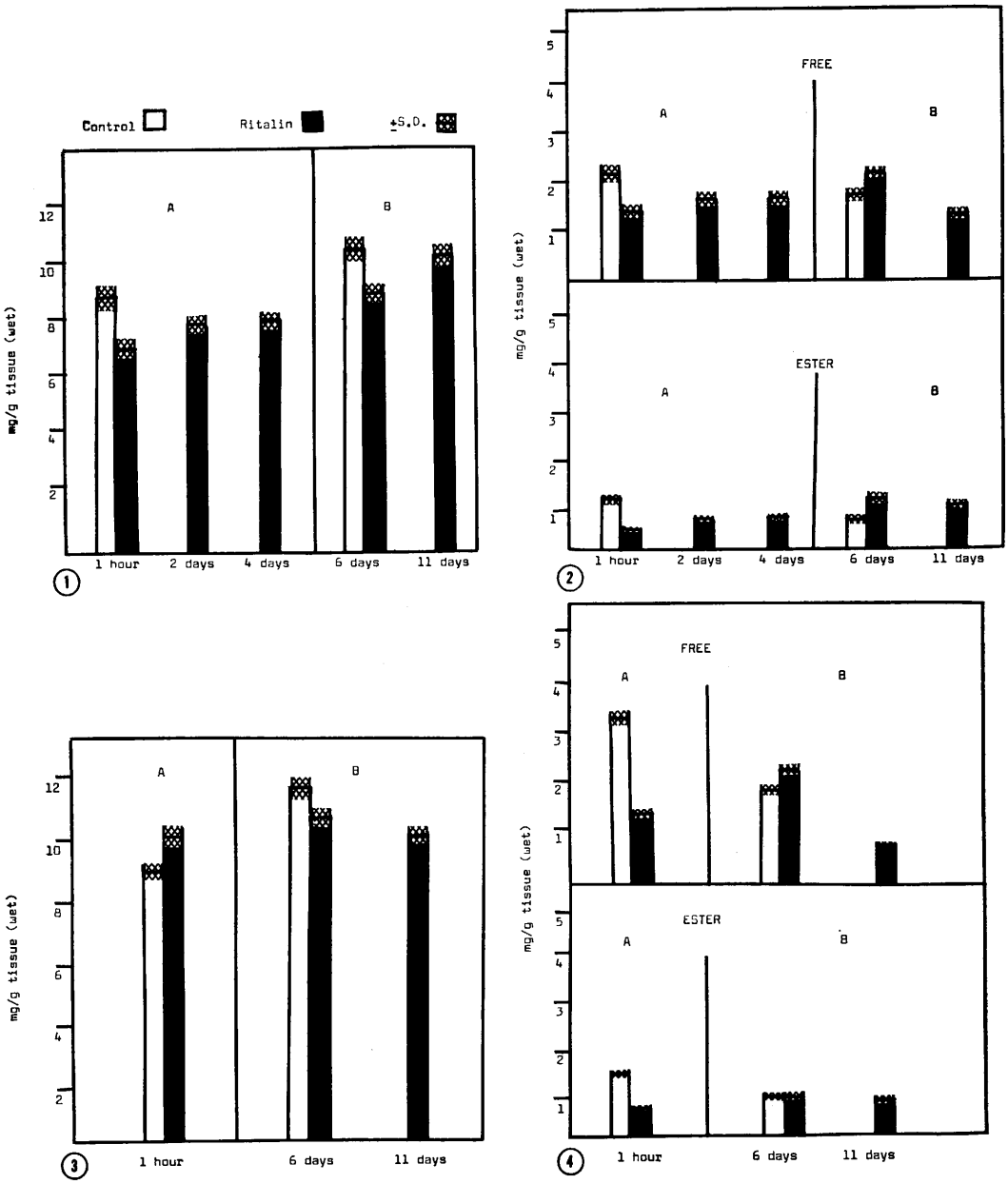

FIG. 1. Free cholesterol content of brain tissue of mice (A = 5-6 weeks old, B = 8-10 weeks old) injected with 4 mg/kg of Ritalin.

FIG. 2. Cholesterol content of liver tissue of mice (A = 5-6 weeks old, B = 8-10 weeks old) injected with 4 mg/kg of Ritalin. Control □ Ritalin ■ ± S.D. ⊕.

FIG. 3. Free cholesterol content of brain tissue of mice (A = 5-6 weeks old, B = 8-10 weeks old) injected with 20 mg/kg of Ritalin. Control □ Ritalin ■ ± S.D. ⊕ ⊕.

FIG. 4. Cholesterol content of liver tissue of mice (A = 5-6 weeks old, B = 8-10 weeks old) injected with 20 mg/kg Ritalin. Control □ Ritalin ■ ± S.D. ⊕ ⊕ ⊕.

paper strips. The separated free and ester cholesterol were eluted and quantitated by the Liebermann-Burchard reaction.

Since quantitative data obtained by (a) digitonin precipitation, (b) tomatine precipitation, and (c) paper chromatography were similar, no distinction is made in the reported values.

Gas chromatography was used as a qualitative method. Representative specimens in each drug group were examined for sterols other than cholesterol, especially desmosterol. Cholesterol was the only sterol detected in any of the experiments.

Results. As with other amphetamine-like compounds, the number of mice per cage caused significant variation in drug response (10). To control this variable, only 5 mice were housed in the same cage. H. Sheppard, Ciba Pharmaceutical Co., concurred with this observation in relationship to LD_{50} determination (personal communication). This is similar to results with amphetamine.

At the 2 dose levels studied (4 mg/kg and 20 mg/kg), injected animals (5-10 weeks old) appeared highly excited. This state lasted for hours. After initial stimulation subsided, they seemed to drowse off as though exhausted. Comparison of body weight and food intake between drug groups and saline controls showed no significant difference.

The first series of experiments used mice from 2 age groups, the first group being 5-6 weeks old and the second group 8-10 weeks old. Separate controls were used for each group. Animals were injected daily at the 4 mg/kg level and were killed after 1 hour and 2, 4, 6, and 11 days. A minimum of 10 mice was used for each time interval. Results are shown in Fig. 1 and 2.

Fig. 1 shows free cholesterol in mice brains expressed in mg/g tissue on a wet basis. Calculations on a dry basis were similar to wet weight results. Even allowing for variations in cholesterol content of brains of individual mice, there was a definite reduction of cholesterol content in brains of mice injected with the drug. The reduction seemed marked after the first dose. After 11 days, the values for sterol content gradually returned to near normal values. After 11 days, the mice appeared vigorous and healthy. Except for changes in tissue weight of the spleen, no gross tissue pathology was seen.

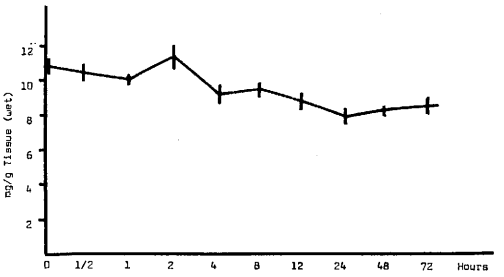

FIG. 5. Maximum effect of a single injection on mean value ($\pm$ SE, $n = 10$) of brain cholesterol. Values for control, 1 hr, and 24 hr groups represent 40, 20, and 30 animals respectively.

Fig. 2 shows cholesterol content, free and esterified, of livers of the same control and injected mice. Changes in cholesterol content, both free and ester, followed the same trend as measured in the brain. Greatest deviation from controls occurred at the 1-hour interval. Continued injection of drug resulted in near normal levels after 11 days.

In a second series of experiments, mice were injected at the 20 mg/kg level; they were sacrificed after 1 hour and 6 and 11 days. This large dose caused pronounced physiological effects. After 3 days, the animals appeared in a state of physical exhaustion compared to the controls. Cholesterol content of the brain tissue on both dry and wet basis weights showed no significant change (Fig. 3). Liver values varied (Fig. 4), but in general, indicated a lowering of normal values after prolonged treatment. Changes in liver ester values imitated changes found at the lower drug dose.

To determine whether this decrease took place earlier than 2 days, mice were injected and killed at shorter time intervals. At least 10 mice were killed at each interval. A single 4 mg/kg dose of Ritalin was given and the animals were sacrificed after ½, 1, 2, 4, 8, 24, 48, and 72 hours. The results are shown in Fig. 5. After 4 hours, a marked decrease in brain sterol was measured. This effect became maximal after 24 hours; values then tended to rise to normal levels. Liver specimens were also obtained at 1, 4, 8, 16, and 24 hours (Fig. 6). Liver values showed maxi-

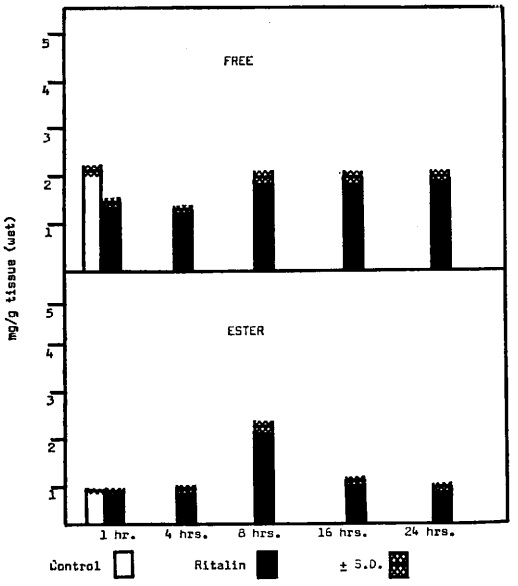

FIG. 6. Cholesterol content of liver tissue of mice (5-6 weeks old) injected with 4 mg/kg Ritalin.

mum lowering between 1 and 4 hours, and a gradual return to normal values 24 hours after drug treatment. This contrasted with brain data wherein normal values were reached in 48-72 hours.

Discussion. Excluding water, cholesterol is present in the brain in greater quantity than any other one constituent. Its function is unknown. Active metabolism is measurable in young animals with isotopic labeled acetate, but not in adult animals(11). The eventual conclusion of a small and limited metabolism for cholesterol in adult animals precludes a structural rather than metabolic role for this lipid. That this conclusion might be too limited, has been emphasized earlier(12). The present report further substantiates the hypothesis of active brain cholesterol metabolism.

Data accumulated in the present series of experiments indicated the cholesterol content in brains of young adult mice (5-10 weeks old) can be lowered by methylphenidate.

The first series of experiments dealt with single and multiple doses of methylphenidate (Ritalin); animals were injected daily up to 11 days. Data indicated (a) initial lowering of cerebral sterol and (b) gradual return to near normal level with increased drug administration. Apparently some homeostatic mechanism was involved, adjusting to the impetus of methylphenidate.

Lowering of brain cholesterol was more indicative at 4 mg/kg than at 20 mg/kg. The lack of effect on brain cholesterol at higher dosage substantiated previous reports(4,5). The effect of Ritalin on cholesterol level in the brain was more pronounced than in other tissues. Single injections of Ritalin (Fig. 5) were as effective as multiple injections (Fig. 1). Physiological effect of methylphenidate reached a peak between 1-2 hours; specific biochemical changes were greatest 24 hours after drug injection. Adjustment to normal levels was very slow in the following 48- and 72-hour period. Losses of almost 20% of brain cholesterol in a 24-hour period suggested extremely rapid metabolism of brain sterol. A loss of this magnitude in a compartment normally considered metabolically stable is unusual. Accumulation of data over a period of 4 years; experience with over 400 mice; and use of 3 different analytical procedures (1 digitonin precipitation, 2 tomatine precipitation, 3 paper chromatography) lessen the possibility of chance entering into these observations.

These studies also tend to shed light on possible biochemical explanations of the action of analeptics. Alexander and Alexander have pointed to the inhibition of cholesterol synthesis by Metrazol in fibroblasts yeast, and in liver of female mice(13). These studies, coupled with our work, suggest a nonstructural role for cell sterol. The pharmacological effects (stimulation of central nervous system) of Ritalin may well be related to brain sterol metabolism. Also, it is of interest that a tranquilizer, chlorpromazine(14), and many barbiturates(15) increase the amount of lipid in tissues. An hypothesis can be formulated: stimulation by a drug seems to result in loss of cholesterol; depression by a drug seems to result in increased cholesterol. Further work is necessary to corroborate these findings and support our hypothesis.

Summary. The amount of cholesterol in brain tissue of male mice (5-10 weeks old) can be lowered by intraperitoneal injection

of small amounts of Ritalin. Maximum effect occurs twenty-four hours after the initial dosage. Adjustment to normal levels occurs in the ensuing 48 and 72 hour periods. It is hypothesized that the stimulating effect of analeptics is related to brain sterol metabolism.

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Pathogenicity of Murine Hepatitis Virus Recovered from Infant Swiss Mice.* (30438)

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In the spring of 1963 a natural outbreak of disease with high mortality occurred in infant Swiss mice maintained at the Virus Laboratory of the Rockefeller Foundation. A filterable agent identified as a virus of the hepato-encephalitis (H-E) group of Morris *et al*(1) was isolated in our laboratory from survivors of one litter. Observations on its behavior in nurslings and weanlings were indicative of differences from other members of the group and are reported in the present paper.

Materials and methods. Three 7-day-old survivors from a litter of 9 were received and killed for autopsy. Two showed focal necrosis of the liver. One was lesion-free but proved to be an intestinal carrier of reovirus type 3. All mice used in the subsequent experiments were drawn from specific pathogen-free colonies (SPF) of the Rockefeller Institute and unless otherwise noted were random-bred Swiss. Intracerebral injections were made with a .02 to .03 ml of liver or brain supernatants. Intestinal washings were tested by intraperitoneal injection of supernatants containing 2000 units of penicillin per 2 ml of saline suspension. All other methods were described earlier(2).

Results. Peritoneal transmission. The hepatic reaction was reproduced in one-day-old nurslings by intraperitoneal injection of a liver suspension from the 2 naturally infected infants and was subsequently maintained for 40 passages. The causal agent was resistant to penicillin and was not cultivable in bacteriologic media. Its activity was retained on filtration through Millipore filters of 300 and 220 mμ but not 100 mμ. The highest dilution which consistently resulted in hepatic involvement was 5×10^{-5}. The necrotic foci in the liver resembled those produced by other members of the H-E group. The agent was regarded, hence, as a murine hepatitis virus and designated MHV (SR4).

A study of host susceptibility was carried out with 3 different age groups of nurslings and with weanlings of nearly uniform age.

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