Brain Cholesterol VIII: Effect of Methylphenidate (Ritalin) on the Incorporation of Specifically Labeled Acetate.* (30003)

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In order further to understand factors affecting cholesterol metabolism in adult animals, we have studied the effect of a central nervous system stimulant, methylphenidate (Ritalin), on brain cholesterol metabolism. Structurally, this compound is related to compounds having a hypocholesterolemic effect(1,2,3), and also acts as a central nervous system stimulant(4). A preliminary report of these experiments has been published (5). The present paper is a more complete study of the effect of this drug on acetate incorporation into brain cholesterol.

Experimental. Radioactive precursors. Acetate-2-^{3}H (10 mC/m-mole); acetate-1-^{14}C (2.8 mC/m-mole); acetate-2-^{14}C (1.5 mC/m-mole) were used simultaneously in these studies. Radioactive nutrients were dissolved in physiological saline, with benzyl alcohol (0.9%) added as a preservative. The resultant solutions were injected intraperitoneally.

The drug (methylphenidate) Ritalin, donated by Ciba Pharmaceutical Co. (H. Sheppard), was prepared as a 1% solution in saline. The stock solution was diluted shortly before injection.

Methylphenidate was studied at 2 dose levels, 4 mg/kg and 20 mg/kg, representing respectively 1/35 and 1/7 of the usual LD_{50}. At these drug levels, no loss of appetite for food in solid or liquid form was observed nor was loss of weight noted. During the experimental period, weight gained by animals on the drug regime was comparable to that of the control group.

Dose level and schedule of treatment, before isotope injections, were established partially from experiments previously reported (4). These prior studies showed that maximum drug action appeared after one hour and lasted for several hours. The drug was excreted rapidly; over 80% was found in the urine in approximately 8 hours. By giving the mice subcutaneous doses of 2 mg/kg or oral doses of 15 mg/kg, spontaneous movement was increased by a factor of 4 or more. When the mice were confined in a modified spring cage, and administered daily doses of 3 mg/kg, increased motility was measured within the first 10 days. High motility continued through maintenance of a daily dose of 3 mg/kg.

Experimental. Sixty male Swiss mice (5-7 weeks old), weighing 20-22 g were randomly distributed into 2 groups. Each group of 30 animals was subdivided into one control and 2 drug groups of 10 mice each. For 5 consecutive days, animals were injected either with saline or drug. A final injection was given on the sixth day, one hour prior to sacrifice. Fifteen minutes before killing the mice, the animals were injected simultaneously with acetate-2-^{3}H (400 μC/kg) and acetate-1-^{14}C (40 μC/kg) or acetate-2-^{14}C (40 μC/kg). Animals were killed by decapitation and exsanguination. Appropriate tissues were surgically removed, washed with distilled water, blotted dry, quick-frozen at $-70°$, and stored in an ice chest. Blood samples were taken but the results are not reported because of the low radioactivity found in these specimens at this time interval. Tissues taken for analysis were weighed frozen (wet weight) and homogenized in a glass grinder with acetone:alcohol:ether (4:4:1). Lipid extracts were filtered and free cholesterol precipitated by adding tomatine, as previously described(6). Radioactive purity of the sterol precipitated by tomatine had been established previously(6,7). The precipitated cholesterol was dissolved in glacial acetic acid and divided into 2 aliquots. One part was taken for colorimetric analysis; the remainder simultaneously assayed for carbon-14 and tritium in a liquid scintillation counter (6).

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TABLE I. Effect of Methylphenidate on Tissue Free Cholesterol.

Organ	Total mg			mg/g tissue (wet)		
	Control	4 mg/kg	20 mg/kg	Control	4 mg/kg	20 mg/kg
Liver	1.97 ± .08*	1.84 ± .13	1.83 ± .15	2.21 ± .10	1.96 ± .11	2.00 ± .14
Spleen	.268 ± .06	.301 ± .06	.190 ± .04	2.00 ± .25	1.94 ± .04	1.99 ± .33
Brain	3.29 ± .07	2.90 ± .08	3.04 ± .15	10.5 ± .30	8.32 ± .27	8.68 ± .38

* Mean ± SE (where n = 20).

Results. Effect of methylphenidate on tissue free cholesterol. For 6 days, 2 groups of animals were given daily injections of methylphenidate (4 mg/kg and 20 mg/kg). In general, animals treated with the drug tend to have lower tissue values for free cholesterol (Table I). This sterol lowering effect was most apparent in brain tissue. Changes in cholesterol levels of the spleen were complex and difficult to interpret. A comparison was made of lipid levels in this tissue between control and drug injected animals. Some variation was noted on a total basis; little or no variance was found on a concentration basis. Apparently these changes reflect changes in the mass of the organ rather than lipid alteration.

In contrast to slight effects on liver and spleen sterol levels, the drug had a pronounced effect on cholesterol concentration in the brain. These changes, measured in both drug groups, were statistically significant ($P<0.01$). The greatest change, both in concentration and total amount of sterol, occurred in the group receiving the smallest amount of drug.

Effect of methylphenidate on acetate incorporation. To evaluate the contribution of carbon-1 and carbon-2 of acetate to the sterol moiety after drug treatment, specifically labeled fatty acids were administered to mice receiving saline and Ritalin. The animals were divided into 2 groups, each containing 30 mice. One group simultaneously received injections of acetate-1-^{14}C and acetate-2-^{3}H; the second group simultaneously received acetate-2-^{14}C and acetate-2-^{3}H. Cholesterol isolated 15 minutes after isotope injection contained both hydrogen-3 and carbon-14. The results are discussed in terms of the effect of drug on the incorporation of these precursors into liver, spleen, and brain, free cholesterol.

Animals receiving methylphenidate incorporated less methyl labeled acetate into liver cholesterol than did the control group (Fig. 1 & 2). This effect was measured whether carbon-2 was labeled with tritium or carbon. This lowered rate of incorporation was evident even in groups receiving 4 mg/kg of methylphenidate. The incorporation of carboxyl labeled fatty acid was an exception; a decrease was measured only at the higher dose levels (Fig. 3).

Incorporation of these 3 labeled precursors into spleen cholesterol was measured. A slight increase was noted in incorporation of both methyl and carboxyl labeled acetate in animals receiving 4 mg/kg. Increasing the dosage to 20 mg/kg resulted in slightly lowered incorporation of the methyl group of acetate but not for the carboxyl carbon. Under these conditions, acetate-1-^{14}C incorporation into spleen cholesterol was increased.

Brain data revealed a complex effect of drug on precursor incorporation. At low concentrations of the drug (4 mg/kg), no significant change in incorporation into brain cholesterol could be measured. At the higher drug concentration (20 mg/kg), lower incorporation rates were indicated with either carbon-14 labeled precursors but not with acetate-2-^{3}H.

Discussion. The current series of experiments was conducted to measure the influence of a hypocholesterolemic agent on brain cholesterol metabolism. The drug chosen for these experiments was methylphenidate (alpha-phenyl-α-piperdyl-2-acetic acid methyl ester hydrochloride). Its action was reported to be between amphetamine and caffeine(4). It is a mild cortical stimulant; its use results in increased motility in the mouse. This drug was chosen for our studies because its structure is reminiscent of substituted acetic acid derivatives, compounds previously shown to

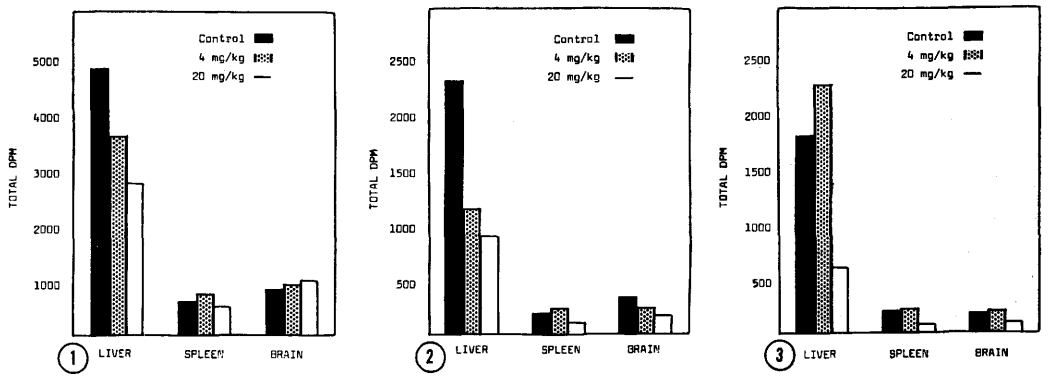

FIG. 1. Effect of Ritalin on incorporation of acetate-2-^{3}H into free cholesterol.
FIG. 2. Effect of Ritalin on incorporation of acetate-2-^{14}C into free cholesterol.
FIG. 3. Effect of Ritalin on incorporation of acetate-1-^{14}C into free cholesterol.

inhibit cholesterol synthesis(1,2,3).

Our biochemical studies showed that methylphenidate, at 2 drug levels, does not significantly lower the concentration of cholesterol in the liver.

Any effect of the drug noted for spleen cholesterol values could be blamed on a toxic reaction to the organ itself. Total amount of sterol both increased and decreased depending on the drug dose. Values for sterol concentration were similar to normal values.

Decrease in brain cholesterol after drug injection represented a loss of 10-20% of sterol during a period of 6 days.

The loss of such a large quantity of sterol from a lipid pool previously considered inert is highly significant. The loss in sterol content may be due to a decrease in newly "appearing" cholesterol, *i.e.*, sterol formed by biosynthesis or transport into the organ. An alternative explanation would mean an increase in the "disappearance" rate, *i.e.*, loss of sterol due to metabolic degradation or transport from the organ.

In an attempt to elucidate the effect of Ritalin on biosynthesis of cholesterol, initial studies were made of acetate incorporation into tissue sterol. The use of free acetate, as an indicator of cholesterol biosynthesis, is not without limitation. Inhibition or stimulation of labeled free acetate into cholesterol nucleus may reflect acetate activation, fatty acid pool size, etc.; acetate incorporation is not necessarily a reflection of the rate of sterol biosynthesis. With these limitations in mind, the data using specifically labeled acetate suggest the following conclusions.

In agreement with studies with similar compounds, Ritalin decreased the incorporation of labeled acetate into liver cholesterol at a dose of 20 mg/kg. The decrease in incorporation rates for hepatic biosynthesis from acetate, without decrease in liver level, points to some possible interference with acetate activation.

Incorporation was also decreased in spleen and brain, but not to the same significant extent as in liver. These results are in agreement with Ranney and Weiss(8), who found changes in hepatic synthesis but not into sterol of adrenal, testis and aorta.

An apparent paradox exists in incorporation values, after lower doses of methylphenidate. Decreased incorporation into liver sterol was measurable with methyl labeled acetate, but not carboxyl label fatty acid. Changes taking place in other tissues are not significant.

In summary, these studies point to interference by methylphenidate with acetate activation in the liver. These same changes in acetate incorporation were not evident for spleen and brain cholesterol values. The radioactive data do not however explain the lowering of cerebral cholesterol content at very low doses of Ritalin (4 mg/kg). A possible explanation may be that these changes are independent of acetate activation as a rate limiting step.

To elucidate further the mechanism of methylphenidate on cholesterol metabolism in the central nervous system, special attention

will be given to the incorporation of other nutrients into brain cholesterol and the schedule of drug administration.

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Alterations in the Hemodynamic Response to *E. coli* Endotoxin with Changes in the Acid-Base State.* (30004)

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Acid-base abnormalities in shock have aroused interest from both pathophysiologic and therapeutic standpoints(1-7). Alterations in vascular responsiveness related to blood pH and pCO_2 have been reported(8-13), as have been the relationships of blood pH to the release(14,15) and activity(16-19) of some of the vasoactive substances known to be involved in endotoxic shock.

Because of casual observations of unusual hemodynamic responses to endotoxin in deranged acid-base states, it was decided to undertake a systematic study of the vascular responses to endotoxin with varying pH and pCO_2. The early vascular reaction to endotoxin in the dog was found to be attenuated at low pCO_2 levels. There was, however, no loss of the usual response(20) to histamine under these conditions.

Methods. Twenty-nine adult mongrel dogs weighing 25 to 30 kg were used. Acid-base disturbances were produced in 23 dogs and 6 were used as controls. The animals were anesthetized with sodium pentobarbital (30 mg/kg) and a cuffed tracheal tube was inserted.

Metabolic acidosis was produced by slow infusion of 0.1 N hydrochloric acid *via* a femoral vein. Respiratory acidosis was attained by controlled respiration of a mixture of 90% oxygen and 10% carbon dioxide. Respiratory alkalosis was produced by overventilation with an intermittent positive pressure respirator using either 100% oxygen or compressed air. Metabolic alkalosis was brought about by infusion of 1.0 N sodium bicarbonate *via* the femoral vein. All animals, including the controls, received from 10-15 ml/kg of fluid. The control animals and those with respiratory acid-base abnormalities received normal saline. Those animals with metabolic derangements received enough normal saline to bring the total volume of fluid to 10-15 ml/kg. Only one kind of acid-base disturbance was produced in a single animal and the order of the experiments was random. Frequent anaerobic arterial blood samples were drawn from the femoral artery before and after the administration of endotoxin. The pH, pCO_2 and bicarbonate concentration of these samples were determined by the Astrup method(21).

Portal venous pressure (PVP) and femoral arterial pressure (FAP) were recorded continuously with Statham pressure transducers and an oscillograph *via* polyethylene catheters inserted into a splenic vein and a femoral artery. *E. coli* endotoxin (0111:B4-Difco) was given in a dose of 5 mg/kg. The same

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