Inhibition of Cholesterol Synthesis *in vivo* by a Convulsant, Metrazol.* (28877)

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Pentamethylene tetrazol (Metrazol), a convulsant which was used in psychotherapy prior to the introduction of electroshock, inhibits synthesis of cholesterol in isolated mouse fibroblasts(1). It also inhibits sterol synthesis in yeast(2). After an initial inhibition, the drug causes a considerable increase in the growth rate of mammalian cells but has no effect on yeast cells(3). This report is concerned with the effects of single and multiple injections of varying amounts of Metrazol on the pattern of sterol biosynthesis in intact mice. Labeled substrates were used in an attempt to establish the site of inhibition along the acetate-cholesterol pathway.

Materials and methods. Young mice, ICR strain females weighing 20-25 g, were divided into matched groups. Control groups received injections of 0.1 cc of distilled water intraperitoneally, while the other groups received single doses of Metrazol ranging from 20 to 80 mg per kg of body weight. Labeled substrates were injected simultaneously with Metrazol. Prolonged experiments were carried out for 50 days. The animals received daily injections of 16 mg of Metrazol per kg of body weight. On the 50th day labeled compounds were injected along with the drug. The animals were placed in individual metabolic chambers. A stream of CO_2-free air passed through the chambers and into a solution of barium hydroxide. Expired $C^{14}O_2$ was collected for 30 minutes. Barium carbonate precipitate was washed thoroughly with water, ethanol, and ether, and plated for assay. The animals were sacrificed by cervical fracture and bled through heart puncture. Cholesterol in the plasma was determined by a modification of the Sperry and Webb procedure(4). Excised livers were hydrolyzed with potassium hydroxide. Lipids were extracted from hydrolysates with petroleum ether and sterols precipitated with digitonin. Digitonides were decomposed with pyridine and sterols plated for assay as before(5). Samples of digitonin-precipitable sterols were purified further with bromine(6).

Metrazol was purchased from Bilhuber-Knoll Corp., Orange, N. J. Sodium acetate-1-C^{14} and mevalonic acid-2-C^{14}-DBED salt were purchased from Volk Radiochemical Co., Skokie, Ill. Biosynthetic squalene-C^{14} was isolated by us from livers of rats injected with mevalonate-2-C^{14}. Lanosterol-T was tritiated for us by New England Nuclear Corp., Boston, Mass. It was dissolved in pentane, washed thoroughly with aqueous potassium hydroxide, and recrystallized to constant specific activity from ethanol-ether, ethanol and methanol. We still have strong doubts, however, about its radiopurity and believe that results obtained with it must be treated with caution.

Results. The radioactivity present in the sterol fraction of livers of mice given single injections of Metrazol and acetate-1-C^{14} decreased with increasing amounts of the drug (Fig. 1). There was a definite though small inhibition even at subconvulsive concentrations. No break was observed in the activity-concentration curve at the point where convulsions began. Thus, the effect appears to be due to Metrazol itself rather than to convulsive seizures. These data from *in vivo* experiments agree with previous findings in yeast and mouse cells in tissue culture.

Prolonged treatment with subconvulsive doses of the drug did not visibly affect the animals. Treated mice, as well as those receiving placebo injections only, continued to gain weight and respond normally to external stimuli. No difference in food and water intake between controls and treated animals

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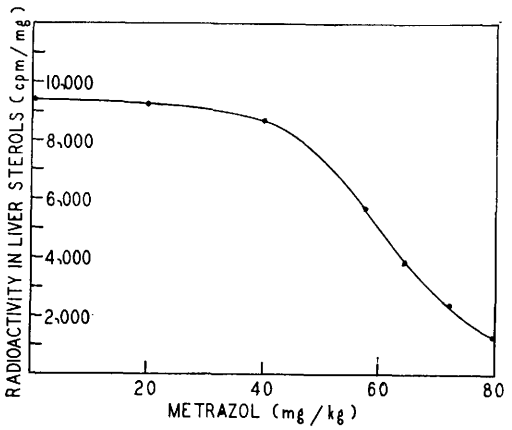

FIG. 1. Recovery of C^{14} in liver sterols after a single injection of Metrazol and acetate-1-C^{14} (10 μc/animal).

was observed. At autopsy, however, depletion of body fat deposits was observed in treated animals.

Plasma cholesterol levels were depressed almost 20% after prolonged treatment (Table I). Synthesis of labeled sterols from acetate-1-C^{14} and mevalonate-C^{14} was strongly inhibited, whereas synthesis from squalene-C^{14} and lanosterol-T was enhanced by the drug (Table II). In control animals given acetate-1-C^{14}, 71.6% of the radioactivity of the sterol fraction remained with cholesterol purified via the dibromide, as compared with 72.5% in animals treated with Metrazol. Thus, the drug did not cause an accumulation of late precursors of cholesterol. The level of radioactivity in $C^{14}O_2$ expired by mice injected with acetate-1-C^{14} was not significantly altered by administration of Metrazol (Table III). In contrast, the total amount of radioactivity expired as $C^{14}O_2$ by treated animals given mevalonate-2-C^{14} was 95% below the amount expired by controls. This almost complete inhibition of the conversion of mevalonate-2-C^{14} to $C^{14}O_2$ represents the most striking example of the effects of administration of Metrazol that we have observed.

TABLE I. Effect of Prolonged Treatment on Plasma Cholesterol.

Treatment	No. of animals	Level of cholesterol, mg/100 cc
Placebo	16	57.97 ± .69
Metrazol	16	46.30 ± .87

TABLE II. Typical Yields of Cholesterol-C^{14} from Livers of Mice After a Prolonged Treatment with Metrazol.

Substrate	Metrazol	Radioactivity in cholesterol, cpm
Acetate-1-C^{14}	—	2,184
"	+	1,570
Mevalonate-2-C^{14}	—	14,445
"	+	6,008
Squalene-C^{14}	—	597
"	+	806
Lanosterol-T	—	1,197
"	+	2,427

TABLE III. Typical Yields of $C^{14}O_2$ Expired by Mice After Prolonged Treatment with Metrazol.

Substrate	Metrazol	$C^{14}O_2$, cpm
Acetate-1-C^{14}	—	6,449
"	+	7,886
Mevalonate-2-C^{14}	—	2,292
"	+	131

Discussion. Most of the $C^{14}O_2$ obtained from acetate-1-C^{14} is derived through decarboxylation of components of the tricarboxylic acid cycle. Only a small portion of $C^{14}O_2$ released is derived from decarboxylation of C_6 precursors of sterols. Thus, inhibition of sterol synthesis need not be reflected in yields of $C^{14}O_2$ when acetate-1-C^{14} is used as substrate. Unlike acetate, mevalonate-2-C^{14} is converted entirely to sterols (except for carbon-1 which is released as CO_2). No other metabolic pathway is known for the compound. Therefore, all of carbon atom 2 of mevalonate is incorporated into squalene and lanosterol. One-sixth of it is released when lanosterol looses its 3 "extra" methyl groups, presumably by oxidation followed by decarboxylation. All $C^{14}O_2$ is derived from this decarboxylation step. Inhibition prior to demethylation of lanosterol should affect both the amount of cholesterol-C^{14} synthesized in the liver and the amount of $C^{14}O_2$ expired. Any significant difference in degrees of inhibition must be due to side effects such as retention of CO_2 in the tissues because of inhibition of carbonate hydrolase (carbonic anhydrase). Therefore, we have tested the

effect of Metrazol on purified preparations of carbonate hydrolase *in vitro* and found that Metrazol indeed depresses the activity of this enzyme. This will be reported later.

The mechanism through which lanosterol loses its 3 "extra" methyl groups is not completely known(7). Oxidation of these groups may take place when they are no longer attached to the sterol nucleus. Were the methyl groups transferred intact to an unknown acceptor, cholesterol synthesis from lanosterol could proceed even when oxidation of the 3 methyl groups and their release as CO_2 is blocked.

The significance of the increased utilization of squalene-C^{14} and lanosterol-T in the experiment described in Table II remains to be evaluated. The animal may have the means to compensate for the depression in cholesterol levels in the presence of Metrazol by stimulating the rate of conversion of squalene and lanosterol to cholesterol. This would represent a *bona fide* increase in rate of conversion of these precursors to sterols. There is a possibility, however, that the increase is an artifact. A metabolic block in the sterol pathway prior to squalene would cause a severe depletion of that substance and all those which follow it along the pathway. Labeled substrates would, therefore, be diluted with smaller unlabeled pools. Higher yields of radiocholesterol would result even without enhancement of the actual rate of sterol synthesis.

The utilization of acetate and mevalonate in sterol synthesis was depressed by the presence of Metrazol. Inhibition of mevalonate alone can account for the inhibition of acetate as well. It is likely, therefore, that the site of action of Metrazol lies beyond the point at which mevalonate enters the acetate-cholesterol pathway and prior to cyclization of squalene. The effect of the drug must be exerted either upon the phosphorylation of mevalonate or phosphomevalonate, decarboxylation of pyrophosphomevalonate, or subsequent condensation of C_5 fragments to squalene.

For clinical purposes, inhibition of cholesterol synthesis prior to formation of the first C_{27} precursor of cholesterol is more desirable than inhibition in later stages. Inhibitors of late stages, such as triparanol (MER 29), cause accumulation of steroidal precursors. Those are likely to have deleterious side effects(8). Feedback inhibition due to the presence of excess cholesterol occurs at an early stage(9). Metrazol, too, appears to inhibit at an early stage, although later than cholesterol. While the compound itself is, of course, a potent convulsant, perhaps a study of its action will yield information of potential value in controlling sterol levels through metabolic manipulations.

One is tempted to speculate that there may be more than a casual connection between inhibition of cholesterol biosynthesis and decrease in seizure thresholds caused by Metrazol. Very little is known about the role of cholesterol in the brain. Although its concentration in the human brain is higher than in any other tissue, except possibly the adrenals (10), no special function has been assigned to it except for the ephemeral role of "protective structural unit"(11). As a constituent of the myelin sheath it helps to insulate the nervous system from extraneuronal effects. Its turnover rate in adults is so low that until recently it could not be measured, and brain cholesterol was considered metabolically inert. Brain sterols labeled early in life persist for long periods. Labeled acetate injected intraperitoneally into adult animals is not incorporated into brain sterols; injected directly into the brain area, it is slowly converted to cholesterol(12). Korey and Stein have suggested that limited turnover of cholesterol "increases the vulnerability of the brain"(11). It is possible therefore for small or localized variations in sterol turnover, too small to be detected by present techniques, to be of critical importance in the control of flow of neuronal impulses. The process of induction of seizures may be intimately connected with sterol levels and turnover rate. A derangement of the sterol pattern may prove to be among the pre-conditions for loss of control over the rate of discharge of electrical impulses leading to convulsive seizures.

Summary. Pentamethylene tetrazol inhib-

ited the incorporation of acetate-1-C^{14} into sterols in intact mice even after a single injection. Prolonged treatment of mice with very low, subconvulsive, doses of the drug resulted in a 20% decrease in plasma cholesterol level, as well as a pronounced decrease in the amount of radioactivity found in liver sterols synthesized from acetate-1-C^{14} or mevalonate-2-C^{14} but not from squalene-C^{14} or lanosterol-T. Metrazol treatment also resulted in almost complete absence of radioactivity in carbon dioxide exhaled by treated animals injected with mevalonate-2-C^{14}. These findings indicate that the site of action of Metrazol along the acetate-cholesterol pathway lies between mevalonate and squalene.

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Influence of Sodium Bicarbonate Level and Culture Temperature on Anterior Pituitary Lactogen Production *in vitro*.* (28878)

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Various investigators(1,2,3) have cultured anterior pituitaries (AP) *in vitro* and obtained the production of lactogen in excess of the amount initially introduced with the tissue. Pasteels(2), using a natural medium, did not indicate the culture temperature, and Meites and co-workers(2,3), using a synthetic medium, cultured AP from various species at 35°C. In subsequent studies Meites and co-workers(4,5) cultured pituitaries at a temperature of 37°C; in these experiments, however, the culture duration was for a shorter time and no comparison could be made with the initial reports. In all the studies using a synthetic medium(1,3,4,5), bicarbonate was added to the medium at a constant level. This investigation was conducted to determine the influence of level of sodium bicarbonate and culture temperature on AP lactogen production *in vitro*.

Materials and methods. Anterior pituitaries were obtained from female, hooded Norway rats approximately 3 months of age. In Experiments 5, 6, 7, and 8 of the 2.0 ml bicarbonate group, pituitaries were from animals weighing 180-200 g, while in all other experiments, pituitaries were from animals weighing 165-175 g. Culture techniques were similar to those reported by other investigators(1). Anterior pituitaries were divided into 6 pieces and one AP was placed in each culture dish. Six to 10 dishes were cultured per experiment per group for a period of 3

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