be resistant to compositional changes resulting from 24, 48, and 72 hr of fasting. The incorporation of isotopes, however, was shown to vary in proportion to the fasting state, changes in incorporation being significant. Failure to demonstrate greater compositional changes in the rest of the central nervous system, especially with respect to cholesterol, may be due to the fact that in the adult central nervous system, the newly synthesized lipid is a small but important proportion of the total amount of lipid present.

The above data, integrated with the idea that labeled cholesterol persists in the mammalian central nervous system for over a year (31,32,38), seems to suggest a series of sterol compartments in brain tissues, each with individual turnover rates. The idea of several sterol compartments, each with a different metabolic rate, has been given support by Davison and Dobbing (42), Pritchard (43), and Radin (44). The size and relative importance of each compartment would vary with age and physiological condition.

ACKNOWLEDGMENT

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Cholesterol Turnover in the Central Nervous System

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Abstract

Immature rat brains were examined for a metabolite of cholesterol which could conceivably form in the central nervous system as a result of cholesterol turnover. A basic assumption was made that an acidic product would be formed, preferentially by oxidative degradation of the cholesterol side chain. Chemical fractionation of brain tissue and thin-layer chromatography of an appropriate acidic fraction indicated the presence of a monohydroxy steroidal acid(s) which remains to be positively identified. Preliminary tracer experiments did not clarify the origin of the unidentified metabolite.

THE CLASSICAL WORK of Waelsch, Sperry and Stoyanoff (1) twenty-five years ago set the foundation for our present basic knowledge of cholesterol metabolism in the central nervous system (CNS). According to this concept cholesterol biosynthesis occurs extensively in the immature brain and spinal cord, especially during the period of active myelination. The cholesterol content reaches a fixed level as the animal approaches maturity and then remains essentially undisturbed for the rest of the individual's life.

Within the past eight years a new facet has been added to this concept, a facet which we believe gives considerable impetus to further attempts to extend our knowledge of brain and spinal cord cholesterol metabolism. Experiments of varied nature from several laboratories have all supported the fact that the cholesterol concentration in the CNS remains unchanged as maturity is reached, but cholesterol *biosynthesis* can still take place in the adult. In other words the cholesterol already present may be metabolically inert but *the capacity* to synthesize the sterol still exists in the mature CNS. Exactly where in the CNS new sterol can be synthesized and deposited has not been determined, but this lack of knowledge does not influence the basic premise. This new concept has been suggested by in vivo studies in which C^{14}-labeled precursors were given parenterally (2–4), by intracisternal (5) and intracerebral (6,7) injection, and by in vitro studies (8–10). We believe the isolation of squalene from adult brain (11) also supports this concept. Why else should squalene be present in adult nervous tissue? Unfortunately the most simple experiment to demonstrate the ability of adult nervous tissue to synthesize cholesterol, namely: cholesterol biosynthesis in isolated tissue from C^{14}-la-

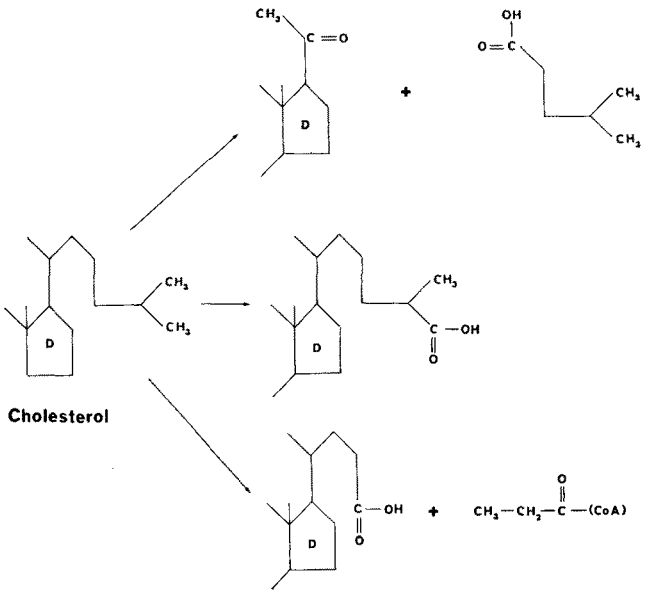

FIG. 1. Degradation of the cholesterol side chain by various mammalian tissues.

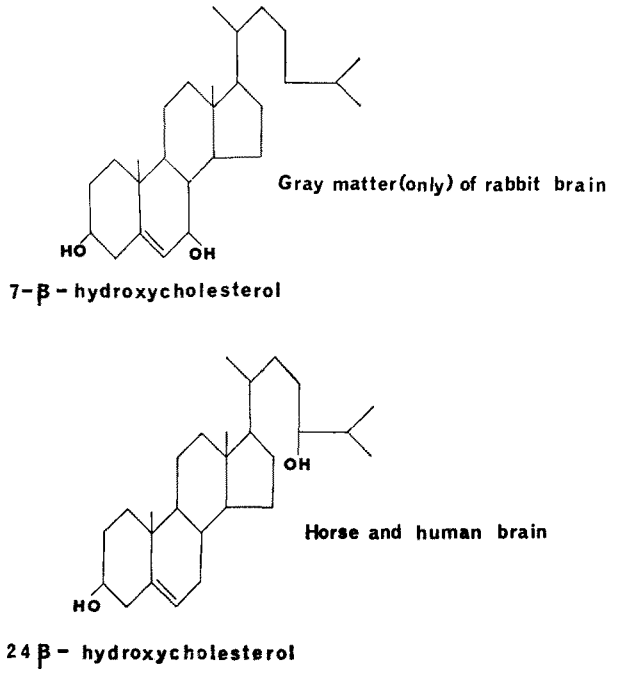

FIG. 2. Oxygenated cholesterol derivatives isolated from brain.

beled precursors, is not impressive. Only a small amount of precursor appears to be incorporated, and even in vivo under the most favorable of circumstances the amount incorporated seems small. This apparent amount is deceptive, however. Recently Kabara (12) has partially clarified this problem by considering the brain cholesterol pool size of animals receiving C^{14}-labeled cholesterol precursors. On this basis older animals receiving glucose-U-C^{14} incorporate as much or more radioactivity into brain cholesterol than do young animals.

Why the adult CNS should maintain the capacity to synthesize cholesterol while the sterol level remains unchanged is a question of possible import. One guess is that this capacity remains in order to replace a minute amount of sterol lost in a slow but contined turnover which has not been detected as yet. Information has been presented by Davison (13) suggesting that cholesterol "turnover" is more rapid in some areas of the brain than others, but neither the nature of the turnover mechanism nor the specific areas have been defined, except to indicate that myelin constituents turn over less rapidly than the same constituents in other areas (29) of the CNS.

We should like to introduce a new aspect into the overall problem of CNS cholesterol metabolism by posing the question: Does any cholesterol degradation (or loss) ever occur in the CNS either by removal of cholesterol as such or by conversion to another compound with subsequent elimination of this compound from the area undergoing metabolism? The term "turnover" implies a synthetic phase (anabolism) as well as a degradative phase (catabolism). In regard to the CNS the only information bearing on this problem is a number of observations that loss of cholesterol from the brain to the general circulation occurs in the degenerative diseases (14) and following experimental disturbances in cerebral circulation (15).

We have approached this problem—admittedly speculative—by assuming that if some degree of cholesterol degradation occurs in the brain, it will ultimately result in formation of a product with partial degradation of the side chain (Fig. 1). Cholesterol as a precursor of the steroid hormones is cleaved to isocaproic acid and pregnenolone. Since this reaction is limited to certain endocrine tissues, this type or extent of degradation seems most improbable. In the formation of the bile acids, cholesterol is oxidized at one of the terminal side chain methyl groups with the formation of a cholestanoic acid (16) and more extensively by the removal of a propionyl residue (17) leaving the characteristic bile acid side chain. Unfortunately these reactions, which have only been studied in liver tissue, must be preceded by hydroxylations of the sterol nucleus at positions -7 and -12. Now it would seem most unlikely that brain tissue would contain a 12-hydroxylase enzymatic mechanism, but a 7-hydroxylase system appears to be present (Fig. 2). Both 7-hydroxycholesterol (18) and 24-hydroxycholesterol (19) have been isolated from brain tissue. We have utilized these findings as an impetus for further investigation since both the -7 and -24 positions are key ones for cholesterol side chain degradation. 7-Ketocholesterol and 25-hydroxycholesterol have also been isolated from central nervous tissue (20), but the 25-hydroxy derivative appears to be an artifact resulting from oxidating during the isolation procedure. "Oxidation products" with double

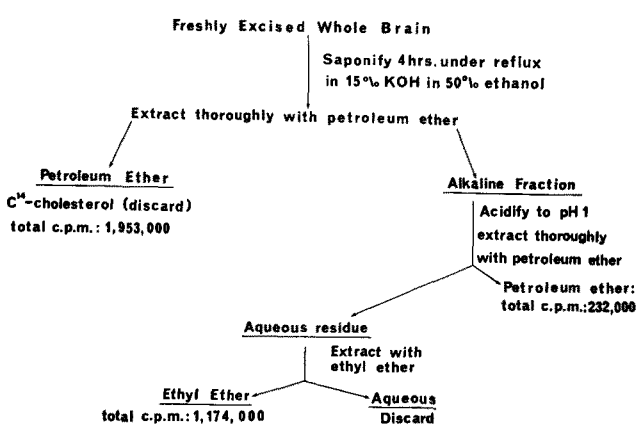

FIG. 3. Fractionation of rat brain tissue for isolation of "acidic cholesterol metabolites."

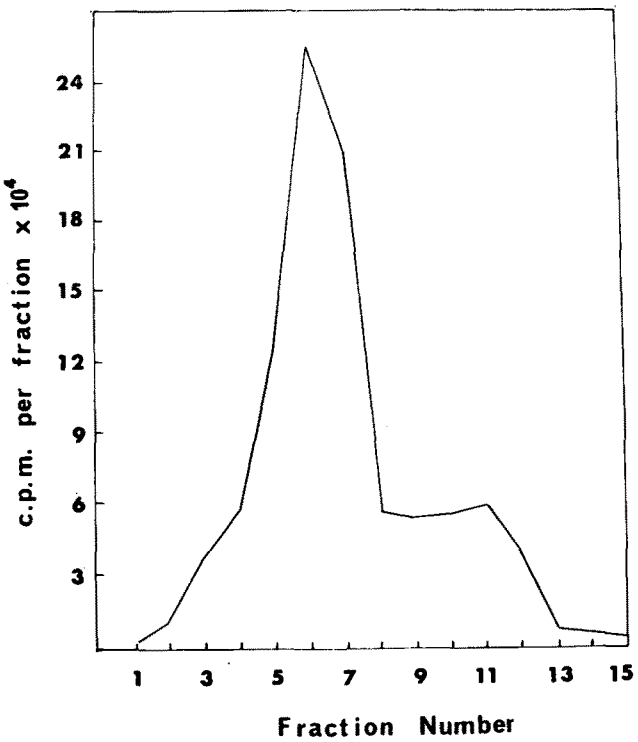

FIG. 4. Chromatography of C^{14}-labeled ether-soluble acids from rat brains.

bonds in ring B have been reported present in brain tissue(21) but these appear less germane to our problem because of their lack of identity

Following is a description of some experiments performed with the objective of finding an "acidic degradation product" of cholesterol in the brain. We hope it is evident that this search has been made with little precedent with which to work, despite extensive studies that have been conducted on this problem with liver. Your attention in this regard is directed to some interesting comments on possible "extrahepatic cholesterol turnover" that were made in association with a review on bile acid metabolism (22).

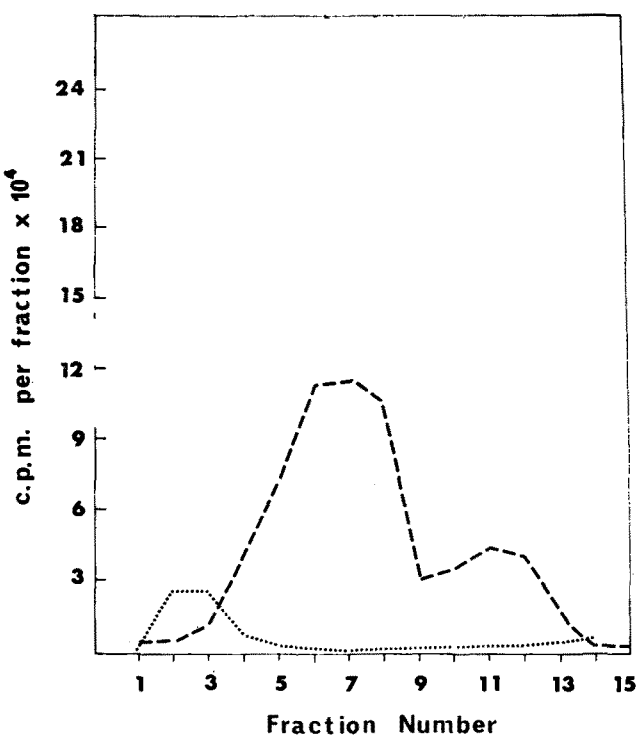

FIG. 5. Chromatography of ether-soluble rat brain acids on a reversed phase partition column. The column was hydrophobic SuperCel, the stationary phase 1:1 chloroform-heptane, the mobile phase 80% methanol. Upper curve: free acids; lower curve: methyl esters.

In the experiments to be described either sodium acetate-1-C^{14} or -2-C^{14} was injected intraperitoneally into immature rats. A basic assumption was made that cholesterol in the brain would become labeled under these circumstances (23), be metabolized (degraded) to some extent within a given period, and thereby permit identification of a labeled degradation product. Without any precise knowledge of what we were looking for, of pool sizes or of degradation, some of our assumptions may be open to misinterpatation. They have provided, however, a starting place for more detailed study. Tissues for examination were removed from decapitated rats, carefully washed and then saponified (Fig. 3). The alkaline mixtures were fractionated as indicated on this figure. The counts shown for the various fractions show the distribution of C^{14} in crude brain fractions in a typical experiment. Cholesterol was removed by thorough extraction with petroleum ether. The alkaline solution was then acidified and extracted thoroughly with petroleum ether to remove fatty acids. The aqueous phase remaining was extracted with ethyl ether. This fraction yielded material which we shall refer to as "ether-soluble brain acids," believed to contain the expected degradation products. In model experiments lithocholic and deoxycholic acids were recovered in large part in this fraction by similar fractionation.

In order to gain some idea of the nature of this acidic fraction two 22-day-old rats were each injected with 20 μc of sodium acetate-2-C^{14}. Brains and livers, respectively, were combined after 3 days and fractionated to obtain brain and liver acids as described. Each fraction, weighing about 50 mg, was chromatographed on 8 g Celite 545 columns prepared as described by Matschiner et al. (24). The stationary phase was 70% acetic acid, the mobile phase was Skellysolve B with increasing volumes of benzene. Fractions (5 ml) were collected with an automatic fraction collector. When chromatographed in this manner most of the counts were found in fractions 1 to 10, which fractions correspond (as nearly as we can determine) to fractions 0–1 of the St. Louis University Biochemistry Group (24) and contain monohydroxycholanic acids (for bile, at least). With both liver and brain a small percentage of counts representing more polar material are evident in fractions eluted by increasing amounts of benzene; these have not yet been investigated.

An additional batch of labeled brain acids was chromatographed in this manner, giving the curve shown in Fig. 4. To provide additional assurance that fractionation was thorough, combined material represented on this curve by fractions 1 to 10 were distributed between 5% KOH and ethyl ether, and the acids were recovered from alkali. These were subjected to chromatography on a reversed phase column prepared according to Ray et al. (25) (Fig. 5). Here the column consisted of 4.5 g of hydrophobic SuperCel, with the stationary phase 1:1 chloroform:heptane and the mobile phase 80% methanol; 10 ml fractions were collected. The C^{14} content of the chromatographed fractions are shown here as the upper curve. Fractions 1 to 10 again represent the area in which monohydroxycholanic acids occur. Fractions 1 to 10 from this curve were combined and again distributed be-

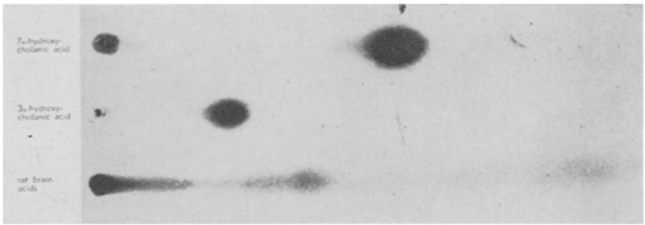

FIG. 6. Thin-layer chromatograph of methyl ester peak (fractions 1 to 5) from Figure 5. Silica gel G was the support, with the developing solvent (ascending) benzene-acetone 93:7. Plate sprayed with phosphomolybdic acid and heated for visualization of spots. (Solvent front, not visible, at the right.)

tween alkali and ethyl ether, and the acids were recovered from the alkaline fraction. By this time it was felt that the alkaline fraction was quite free of neutral material, and was also found to be free of organic phosphorus (26). Finally, this combined acid fraction was methylated and rechromatographed on hydrophobic Hyflo SuperCel column as previously described. Considerable loss in C^{14} activity is indicated (lower curve, Fig. 5), with a peak in the area in which methyl esters of monohydroxycholanic acids are recovered. This combined ester peak was chromatographed on silica gel G thin-layer plates, with the developing solvent benzene:acetone 93:7 (Fig. 6). When sprayed with phosphomolybdic acid and heated, several spots appeared, as indicated. The large spot shown between 7α-hydroxycholanic acid and 3α-hydroxycholanic acid (lithocholic acid) was eluted from other plates before spraying with the detecting agent and examined by infrared spectroscopy. Absorption peaks were found at 3,333 cm^{-1} (hydroxyl band) and at 1,748 cm^{-1}, indicating the presence of a methyl ester. This gave us some additional assurance that we were working with a carboxylic acid. Absorption in the region 1,667–1.161 cm^{-1} also suggested the presence of unsaturation, which had been indicated by fairly intense fluorescence under ultraviolet light. Two crude methylated acid fractions obtained as previously described were chromatographed in a similar manner on thin-layer plates (Fig. 7) but stained with 10% antimony pentachloride in chloroform, which is more specific for steroids. From top to bottom the spots are:

3α-hydroxycholanic (lithocholic) acid → 3β-hydroxycholanic acid → Unknown 20–140 → Unknown 20–144 → 3-ketocholanic acid → 3β-hydroxy-Δ^5-cholenic acid → 7α-hydroxycholanic acid → 12α-hydroxycholanic acid → cholesterol
(all acids as the methyl esters)

The ringed areas are spots which are visible under ultraviolet light without staining. The nearest approximation of an unknown spot, both in color and position, is the second spot from the bottom in Unknown 20–140, and has the polarity of 3β-hydroxycholanic acid. It will be noted that at least seven compounds are indicated in Unknown 20–140.

Because of the low radioactive content of methylated preparations, which may indicate that the rationale for our turnover concept is incorrect, it was decided to try a different approach.

A 250 mg sample of ether-soluble acids was obtained from the brains of eleven 13-day-old rats which had received a total of 1 mc of acetate-1-C^{14} intraperitoneally, followed by dilution, prior to saponification, with 91 nonradioactive rat brains of the same age. The crude fraction was chromatographed on a reversed phase column prepared according to Norman and Palmer (27). The column consisted of 30 g of hydrophobic SuperCel; the stationary phase was chloroform:heptane 45:5 and the moving phase was methanol:water 180:120. When fractions of 10ml obtained from this column were titrated with 0.02n NaOH, two peaks were obtained (Fig. 8), very similar to a curve obtained by Norman and Palmer in studying the metabolism of lithocholic acid, which appeared in their column in a second peak corresponding to our fractions 20 and 24. Only traces of radioactivity were found in the fractions shown here, with the greatest amount—a total of somewhat more than 1000 cpm—in fractions 20 to 24. Combined material from these two peaks were obtained (Fig. 8), very similar to a gel G plates developed with the system trimethylpentane 50, ethyl acetate 10, acetic acid 0.25 (27). This is a Xerox copy (Fig. 9) of a plate sprayed with

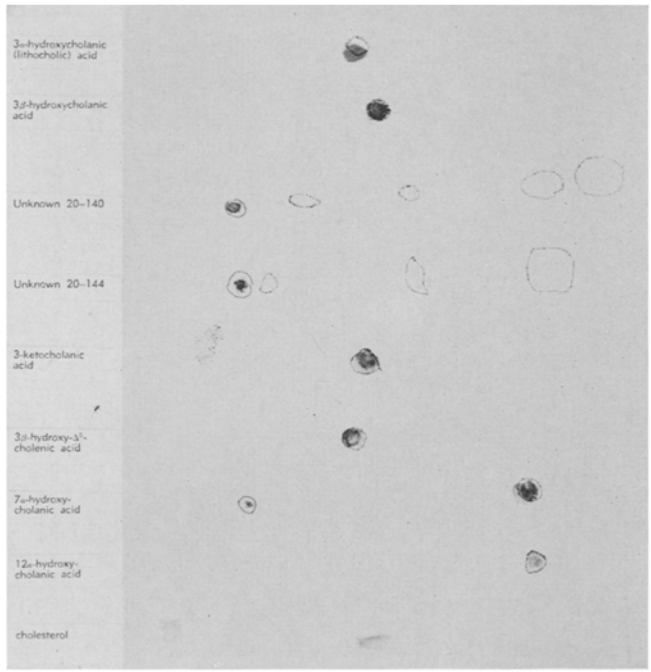

FIG. 7. Thin-layer chromatograph of ether-soluble rat brain acid methyl esters and controls. Experimental conditions the same as for Figure 6 but visualization of spots was made by spraying with 10% $SbCl_5$ in $CHCl_3$ followed by heating. (Solvent front, not visible, at the right.)

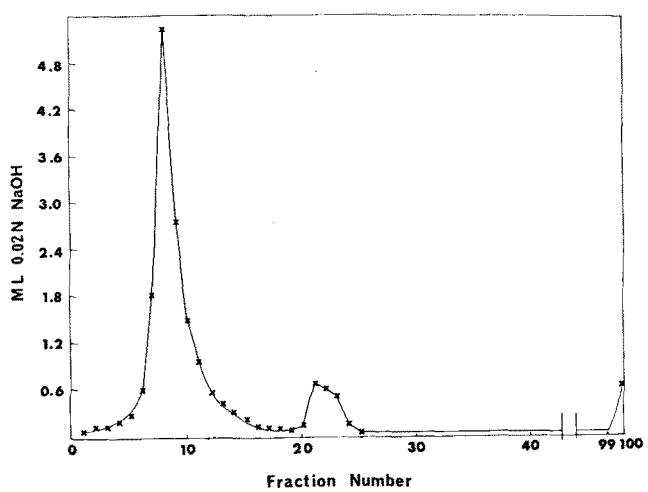

FIG. 8. Reversed phase partition chromatography of crude ether-soluble rat brain acids. The column contained hydrophobic SuperCel; the stationary phase was chloroform: heptane 45:5, and the moving phase was methanol:water, 180:120.

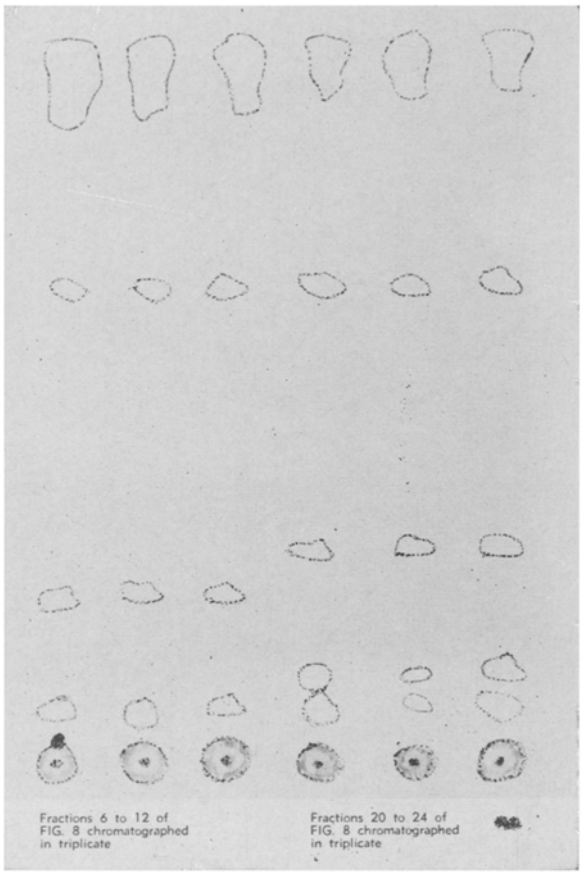

FIG. 9. Thin-layer chromatograph of the ether-soluble rat brain acids. The supporting layer was silica gel G, the developing solvent (ascending) was trimethylpentane:ethyl acetate 10:acetic acid 50:10:0.25, and visualization of the spots was made by spraying with phosphomolybdic acid followed by heating.

phosphymolybdic acid and heated. At least six compounds are shown in fractions 20 to 24. In an additional chromatograph prepared in the same manner but sprayed with antimony pentachloride (Fig. 10) and heated, the following spots were obtained. From top to bottom they are:

3α-hydroxycholanic acid → 3β-hydroxycholanic acid → 3-ketocholanic acid → 3β-hydroxy-Δ^5-cholenic acid → 6β-hydroxycholanic acid → 12α-hydroxycholanic acid → cholanic acid → Unknown 20–160 Fractions 20–24 → 6α-hydroxycholanic acid

The ringed areas again indicate spots which are visible under ultraviolet light. As can be seen we have not satisfactorily separated the control spots, and in polarity and staining to at least three of the spots they are shown here only to illustrate the similarity in the unknown brain acid mixture. Again, one of the spots in unknown fractions 20 to 24 has the polarity and stains in a manner similar to 3β-hydroxycholanic acid.

Some preliminary evidence has been presented supporting the conclusion that a steroid with a carboxyl group is present in rat brain tissue. Our data do not permit specific identification of any of the compounds indicated. By inference the carboxyl group should be on the (cholesterol) side chain. It is hoped that with a wider selection of reference compounds and a greater amount of material for examination, positive identification of one or more of the compounds in the brain acidic fraction can be identified. Our C^{14} studies have not permitted us to relate the unidentified metabolites to cholesterol turnover, and this must be an area for future work. In another investi-

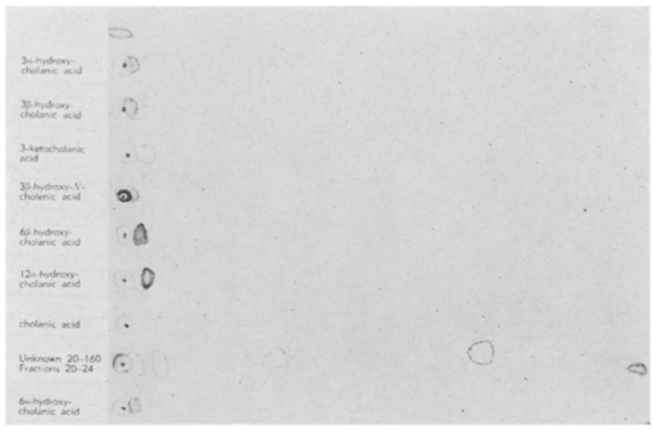

FIG. 10. Thin-layer chromatograph of ether-soluble rat brain acids (free) with control spots. The experimental conditions were the same as for Figure 9 but the spots were visualized by spraying with 10% $SbCl_5$ in $CHCl_3$ followed by heating. (Solvent front, not visible, at the right.)

gation (28) it was shown that cholic acid is markedly demyelinating in vivo. The probability of this substance being formed in nervous tissue by degradation of cholesterol seems biochemically remote. If less polar degradation products such as we have indicated in the present work were formed, however, they too could have mild demyelinating properties which would make them formidable and insidious agents if produced in excess in specific areas of the central nervous system in certain diseases. We feel this possibility alone makes further investigation well worth while.

ACKNOWLEDGMENTS

Lithocholic and 3β-hydroxy Δ^5-cholenic acids were obtained from commercial sources. Dr. W. H. Elliott and his associates provided a generous supply of the other free bile acids and methyl esters with the technical assistance of Sharon Pendleton, Susan Roeder and Raymond Aexel.

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