Detection of monohydroxy "bile" acids in the brains of guinea pigs afflicted with experimental allergic encephalomyelitis

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ABSTRACT 3α-Hydroxy-5β-cholanoic acid (lithocholic acid) and some unidentified acids (one of them perhaps 3β-hydroxycholest-5-en-26-oic acid) have been detected in the brains of guinea pigs afflicted with experimental "allergic" encephalomyelitis. None of the acids could be identified in comparable amounts of brain tissue from normal guinea pigs. Thin-layer chromatography of the free acids, and thin-layer and gas–liquid chromatography combined with mass spectrometry of the methyl esters were used for identification.

The occurrence of these acids may possibly be the result of cholesterol oxidation in the brain. The acids, or compounds related to them, may play a role in development of demyelination in experimental allergic encephalomyelitis.

SUPPLEMENTARY KEY WORDS demyelination · cholesterol oxidation · lithocholic acid · thin-layer chromatography · gas–liquid chromatography · mass spectrometry

Several studies during the past 10 yr have suggested that cholesterol in the central nervous system (CNS) does turn over slowly, even in the adult animal (1–3). The turnover has been measured by changes in specific activity of cholesterol-^{14}C formed within the brain after intraperitoneal administration of acetate-^{14}C or cholesterol-^{14}C to young animals. If a small amount of cholesterol is "lost" in this process of turnover, then the form in which the cholesterol leaves the CNS becomes of interest. It is possible that the sterol is released as unmetabolized cholesterol. For reasons previously discussed (4), the bile acid route also seems possible, and this is the subject of the present manuscript. Preliminary investigation indicated that compounds resembling the monohydroxy bile acids are present in immature rat brains, but the compounds were not positively identified (4). Attention was next directed to adult brain tissue, with particular interest in animals afflicted with experimental "allergic" encephalomyelitis (EAE). The rationale for investigating EAE brain tissue was based upon the demyelinating effect of cholic acid when injected intracerebrally (5) and upon recent experience (unpublished observations) with several monohydroxy bile acids, including lithocholic, which are mildly demyelinating and paralytogenic when injected in the same manner.

This manuscript describes the detection of monohydroxy "bile" acids in the brains of guinea pigs afflicted with EAE.

MATERIALS AND METHODS

Animals

Hartley strain male guinea pigs weighing about 500 g were obtained from the Animal Farm Division, Department of the Army, Ft. Dietrick, Frederick, Md., and guinea pigs of an unidentified strain were obtained from a local animal supply house. During the experimental period they were maintained on a standard diet of Purina guinea pig chow occasionally supplemented with fresh lettuce.

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Abbreviations: CNS, central nervous system; EAE, experimental allergic encephalomyelitis; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GC–MS, gas chromatography–mass spectrometry.

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Inoculation and Collection of Tissue

EAE was produced in the animals by inoculating them with a mixture of Freund's adjuvant and spinal cord prepared as follows. 25 g of fresh bovine spinal cord in 100 ml of 0.15 M NaCl was homogenized in a Virtis homogenizer. 1 ml of the homogenate was emulsified with 3 ml of Freund's complete adjuvant by repeated aspiration and ejection through a syringe. Each animal was inoculated with 0.2 ml of this emulsion by injection into the foot pad or by five intradermal injections over the sternum. In one experiment (series II) the Freund's adjuvant was replaced by a second preparation: [light mineral oil (Drakeol 6-VR)]–[emulsifier (Arlacel A)]–[H-37 RV mycobacteria], 9 ml:1 ml:10 mg.

Series I. Exploratory work was performed on locally obtained guinea pigs inoculated with bovine spinal cord–Freund's complete adjuvant by injection into the foot pad. These animals developed the typical hind leg paralysis characteristic of EAE (6) within 3 wk of inoculation. However, the CNS tissue was not examined for any histological changes and the diagnosis of EAE was based on visible symptoms only. The animals (nine normal controls and nine with EAE symptoms) were killed by decapitation under ether anesthesia. Brains of all animals were crudely separated into white and gray matter and the respective fractions were pooled; these fractions will be referred to as normal gray, normal white, EAE gray, and EAE white. The fractions were processed according to the general method outlined in Fig. 1.

Series II. 30 Hartley strain guinea pigs were inoculated with bovine spinal cord emulsified with the second adjuvant preparation (replacing Freund's adjuvant) described above by intradermal injections over the sternum. Of these animals, 23 developed hind leg paralysis and other symptoms characteristic of EAE (6) within 20 days. The presence of typical EAE lesions was confirmed by histological examination of pieces of spinal cord from nine randomly selected, diseased animals. Each was decapitated under ether anesthesia. Blood was collected and extracted three times with excess hot ethanol, followed by three extractions with chloroform–methanol 2:1. The combined extracts were distilled to dryness. The residue thus obtained and the brains (not separated into white and gray matter) and spinal cords were processed as shown in Fig. 1.

The adjuvant preparation (adjuvant only, no spinal cord tissue) was injected into 30 additional guinea pigs in the same manner. 28 of these (no symptoms) and 30 normal Hartley guinea pigs were killed after 20 days, and the blood, brain, and spinal cords from each group of animals were respectively combined and processed as for the EAE animals. The tissue preparations will be referred to as adjuvant controls and normal controls, respectively.

In the hope of making a quantitative comparison between normal and EAE CNS tissue, we dried the tissues from the different groups of series II animals under reduced pressure over anhydrous $CaCl_2$ to constant weight. The weights of the dried brain tissue from the three groups of animals were: normal controls (30 animals), 15.80 g; adjuvant controls (28 animals), 15.03 g; and EAE (23 animals), 10.39 g.[1]

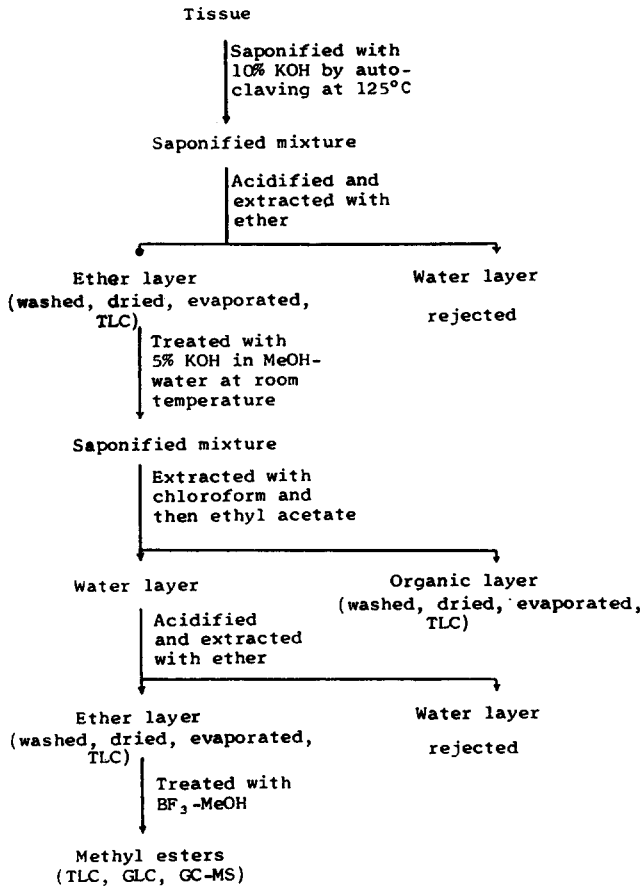

FIG. 1. Procedure for extraction of the saponifiable and nonsaponifiable material from CNS tissue.

Series III. Four Hartley strain guinea pigs were inoculated with Freund's complete adjuvant–bovine spinal cord by intradermal injections into several sites over the sternum. At the onset of neurological symptoms (hind leg paralysis) three animals were killed. The brains were transferred to a flask and minced by cutting with a pair of scissors. An ethanolic solution of 3.0 μc of cholesterol-4-^{14}C was added and the material was saponified and processed as for the other series of animals (Fig. 1).

Thin-Layer Chromatography (TLC)

TLC was performed on Silica Gel G (Merck) plates (20 × 20 cm; 0.25 mm thick). Several solvent systems

[1] Calculated weight of dried tissue for 28 normal brains is 14.72 g and for 23 brains, 12.12. Since the animals used were of approximately the same weight to start with the noticeably lower observed weight of the 23 EAE brains (10.39 g) may be significant.

were tried, but the results described here were obtained in trimethyl pentane–ethyl acetate–acetic acid 75:45:1 for free monohydroxy bile acids, and the same solvents in the ratio 60:20:0.4 for the methylated products. For isolation and subsequent manipulation the material to be examined was subjected to preparative TLC with known compounds as controls on one side of the plate. These were made visible by spraying with 50% H_2SO_4 (v/v in water). The unsprayed area of the developed chromatogram was marked into sections and each section was scraped off the plate and eluted by shaking with chloroform–methanol 1:1. The adsorbent was removed by centrifugation and the eluates were evaporated to dryness for further examination.

Gas–Liquid Chromatography (GLC)

GLC was performed in a Barber–Colman (model 5000) gas chromatograph. A 2 m glass column (I.D. 4 mm) packed with 3% QF-1 (methyl fluoroalkyl silicone) on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories Inc., State College, Pa.) was used for the separation of methyl esters of monohydroxy bile acids. The samples were chromatographed with the column at 220°C, the detector at 235°C, and the flash heater at 295°C; the carrier gas was argon with a flow rate of 60 ml/min. The samples were dissolved in benzene or acetonitrile and injected by means of a Hamilton microliter syringe equipped with a Chaney adapter.

GLC–Mass Spectrometry (GC–MS)

GC-MS was performed on an LKB (model 9000) gas chromatograph–mass spectrometer. A coiled glass column (length 2 m; I.D. 4 mm) filled with 3% QF-1 on Gas-Chrom Q (100–120 mesh) was used. The column temperature was 215°C, the flash heater 235°C, the molecule separator 225°C, and the flow rate of helium gas 25 ml/min. The filament temperature was kept at 290°C, the accelerating voltage was 3.5 kv, and the energy of the ionizing beam 70 ev. The samples were analyzed as the methyl esters. Background spectra were recorded and subtracted from the sample spectra.

Reference Compounds

3α-Hydroxy-5β-cholanoic acid, 3α-hydroxy-5α-cholanoic acid, 3β-hydroxy-chol-5-enoic acid, and 3-keto-5β-cholanoic acid were obtained commercially or from Dr. W. H. Elliott of the Department of Biochemistry, St. Louis University School of Medicine. They were further purified by preparative TLC and recrystallized from ethyl acetate. 3β-Hydroxy-cholest-5-en-26-oic acid (mp 175–177°C) was prepared by the method of Kucera and Sorm (7). For this purpose a generous gift of 3β-acetoxy-norcholest-5-en-25-one was supplied by Dr. G. A. D. Haslewood of the Department of Biochemistry, Guy's Hospital Medical School, London. 3β-Hydroxy-5α-cholanoic acid (mp 218°C) was synthesized by hydrogenation of the Δ^5-compound, and 3β-hydroxy-5β-cholanoic acid (mp 175–177°C) was prepared by catalytic reduction of 3-keto-5β-cholanoic acid. The methyl esters of the acids were prepared by treating the free acid with freshly prepared diazomethane or BF_3–methanol reagent (Applied Science Laboratories).

RESULTS

Series I

About one tenth of the acidic fraction obtained by saponification of the separate brain fractions (control white, control gray, EAE white, EAE gray) and spinal cord was subjected to TLC, and the developed chromatogram was sprayed with H_2SO_4. Several spots could be seen in the fatty acid region but none in the more "polar" region. Increasingly larger aliquots were then examined until the complete fraction had been utilized; with concentrated material faint spots in the bile acid region could be seen. The acidic fraction from EAE white and EAE gray had sufficient material to show spots in the lithocholic acid region. This material, when separated by preparative TLC and methylated, moved with the R_f of methyl lithocholate. No further evaluation was possible because of the scarcity of material.

Series II

No compounds corresponding in R_f to monohydroxy bile acids could be identified in the brains, spinal cord, or blood of the normal and adjuvant controls or in the blood of the EAE animals. EAE spinal cord showed traces of material corresponding in R_f on TLC and retention time of the methyl ester on GLC to that of lithocholic acid, but the EAE brain provided sufficient material for more positive identification; the following description will illustrate the general method used.

Fig. 2 shows a thin-layer chromatogram of some known sterols and bile acids, and the acidic fractions from EAE, adjuvant control, and normal control brains. EAE brain showed spots in the monohydroxy bile acid region; the presence of more polar acidic material was also indicated. The precise nature of these acids has yet to be investigated. The brain acidic fractions remaining after this preliminary survey were methylated and the methyl ester preparation was subjected to TLC; only the material from EAE brain showed spots in the methylated monohydroxy bile acid region (Fig. 3) that had the same R_f values as some standards. The methylated material was subjected to preparative TLC. The area of the chromatogram corresponding to methyl lithocholate plus methyl 3β-hydroxy-cholest-5-en-26-oate (fraction I), and the

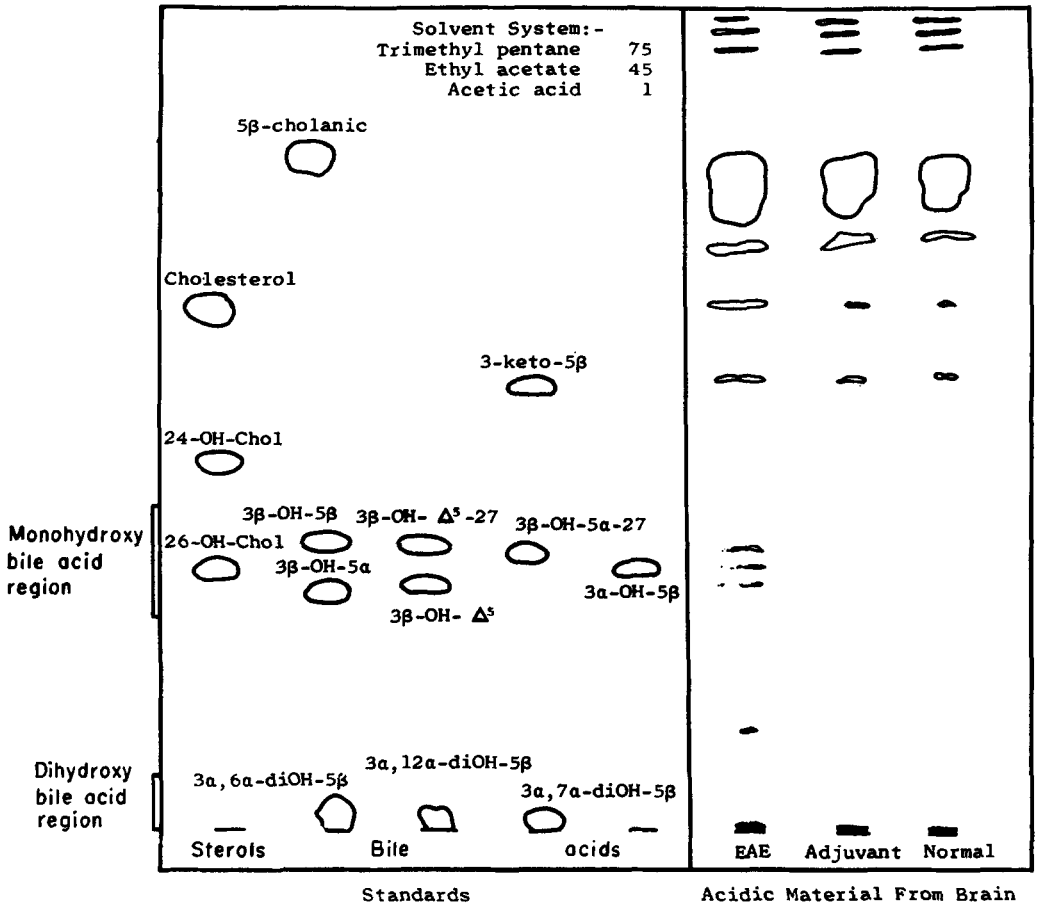

FIG. 2. TLC of some standard sterols and "bile" acids (3β-OH-Δ5-27 and 3β-OH-5α-27 refer to substituted 26-COOH cholestanoic acids and the others are substituted cholanoic acids), and of the saponifiable fraction from guinea pig brain.

area corresponding to methyl 3β-hydroxy-chol-5-enoate (fraction II), were scraped separately and eluted.

Fraction I showed spots with the same R_f as methyl lithocholate and methyl 3β-hydroxy-cholest-5-en-26-oate on TLC in two solvent systems; examination of this fraction by GLC showed two peaks with the same retention times as the two standard compounds (Fig. 4). The two compounds were separated by preparative TLC and again subjected to GLC. Both fractions gave single peaks, but the peak obtained from material suggested to be methyl 3β-hydroxycholest-5-en-26-oate was very small and was not sufficient for any further examination. Material from the GLC peak corresponding to methyl lithocholate was subjected to GC–MS. The fragmentation pattern of this material was similar to that of the authentic standard (Fig. 5) and also to the published mass spectrum of methyl lithocholate (8).

Fraction II showed a peak on GLC that had a retention time of 1.121 (relative to methyl lithocholate), which is not quite the same as that of methyl 3β-hydroxychol-5-enoate (relative retention time, 1.0). However, the mass spectrum of this material showed a mass peak at m/e 388 and other ion fragments at m/e 370 (M–18), 355 (M–18 + 15), 277, 255 (M–18 + side chain), 213 (M–18 + side chain + ring D), etc. The TLC, GLC, mass spectrometry, and other data suggest that the compound is a C_{24}, unsaturated, monohydroxy steroid acid.

Series III

The saponifiable fraction obtained from these brains did not show any radioactivity. The material was methylated and then subjected to preparative TLC; the area of the chromatogram corresponding to monohydroxy bile acids was scraped and eluted. GLC of the residue showed the same pattern as shown in Fig. 4.

DISCUSSION

The presence of monohydroxy "bile" acids in brain tissue, suggested by preliminary investigations (4), has now been confirmed. The exact source of the acids, however, has yet to be ascertained. Although none was found in the blood of guinea pigs used for this investigation, lithocholic acid has been detected in human blood serum (9). On

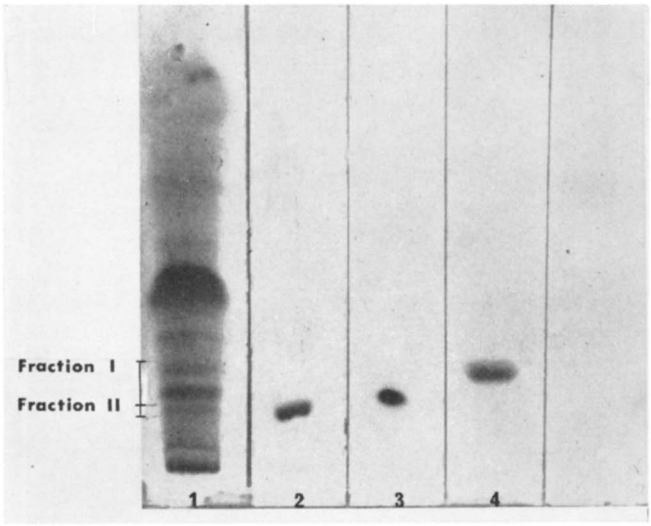

FIG. 3. TLC of standards and the methylated sample of the acidic fraction from EAE brain. *1*, Material from brain; *2*, methyl 3β-hydroxy-chol-5-enoate; *3*, methyl lithocholate; and *4*, methyl 3β-hydroxy-cholest-5-en-26-oate.

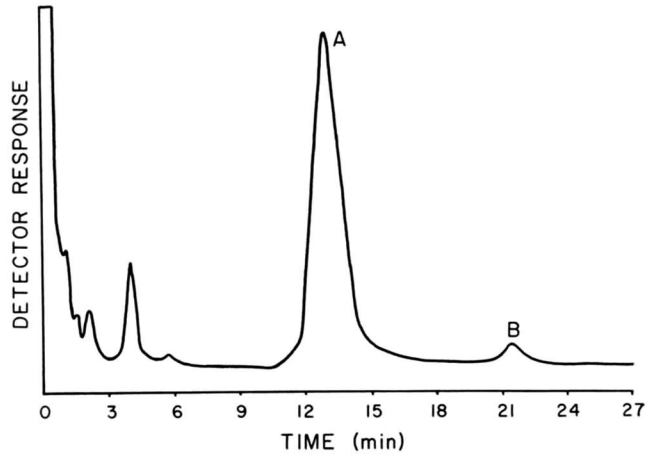

FIG. 4. GLC: Fraction I from the preparative TLC of the methylated material from EAE brain. *A*, peak identified as methyl lithocholate. *B*, peak with the retention time of methyl 3β-hydroxy-cholest-5-en-26-oate.

this account more extensive investigation is warranted. Danielsson (10) reported the formation of some acidic autoxidation products of cholesterol. We found none under the conditions of our experiments in control animals; series III confirmed that the acidic products obtained from the EAE brain are not artifacts but are present in the EAE brain. The fact that the amounts of the acids detected are so small is understandable in view of our unpublished observations with intracerebral injections of sodium lithocholate-24-^{14}C, most of the radioactivity from which was found in feces within 72 hr.

We believe that the detection of monohydroxy steroid acids in the brains of animals afflicted with EAE is biologically significant. The experiments, difficult to place

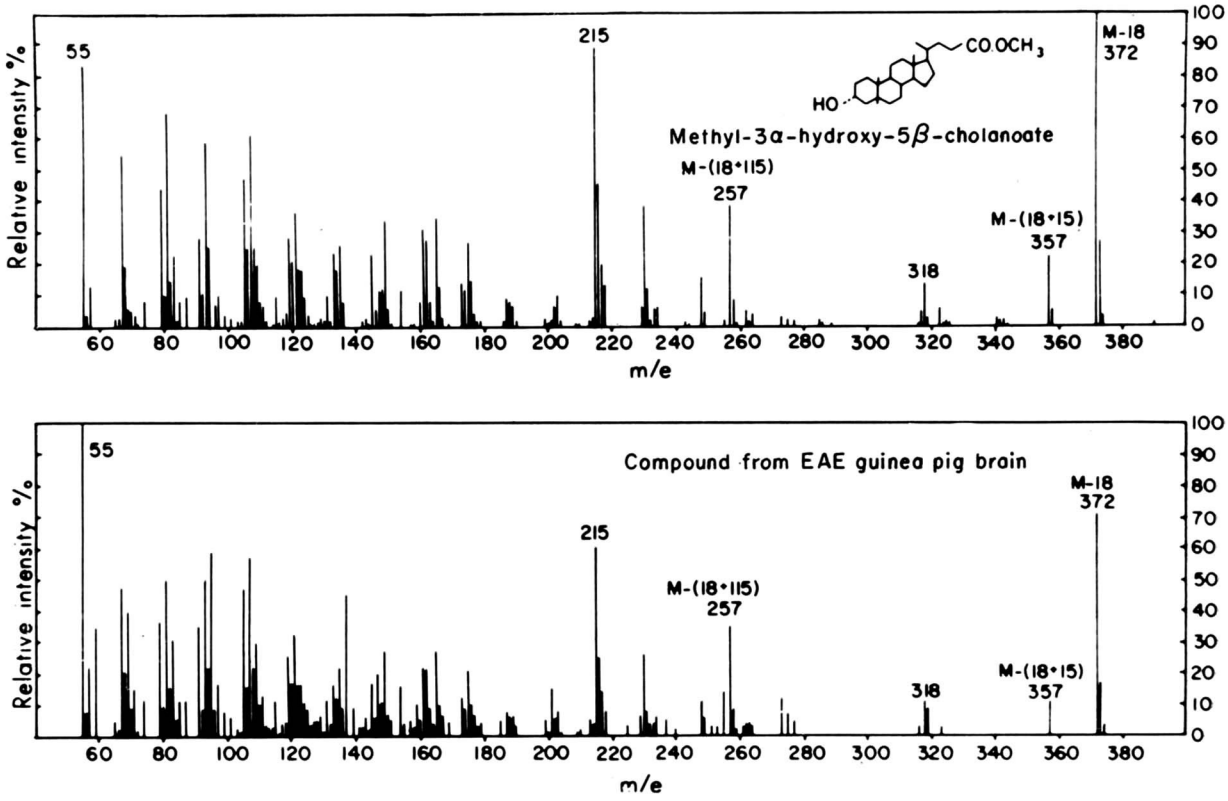

FIG. 5. Mass spectra of standard methyl lithocholate and of the compound from EAE brain.

on a quantitative basis, do not eliminate the possibility of the acids being present in normal brain tissue. However, since the same methods were applied to both types of tissue there is, at the very least, a marked difference in quantity between normal and EAE brain tissue.

It is not clear at the moment whether the presence of bile acids in the EAE brain is due to increased cholesterol metabolism under some antigenic or other stimulation, or is due to a lack of a mechanism for "detoxification" and excretion. Whatever the cause of their presence in the EAE brain, these bile acids could be the direct cause of demyelination. We have previously expressed the opinion that cholic acid would be unlikely to be found in CNS tissue (5), since the ability to oxidize the C-12 position on the steroid nucleus seems limited to a few tissues, of which CNS tissue did not seem to be one. Recently, however, corticosteroids have been isolated from human brain tissue (11) and the ability of brain to oxidize the steroid C-11 position in vitro has been demonstrated (12, 13). On this account a search for a more potent demyelinating bile acid than the monohydroxy bile acids detected in brain thus far might be well justified.

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References

1. Davison, A. N., J. Dobbing, R. S. Morgan, and G. Payling Wright. 1959. *Lancet*. **i:** 658.
2. Kabara, J. J., and G. T. Okita. 1961. *J. Neurochem*. **7:** 298.
3. Kabara, J. J. 1967. *Advan. Lipid Res*. **5:** 279.
4. Nicholas, H. J. 1965. *J. Amer. Oil Chem. Soc*. **42:** 1008.
5. Nicholas, H. J., and B. Herndon. 1964. *Neurology*. **14:** 549.
6. Alvord, E. C., Jr., and M. W. Kies. 1959. *J. Neuropath. Exp. Neurol*. **18:** 477.
7. Kucera, J., and F. Sorm. 1958. *Collection Czech. Chem. Commun*. **23:** 116.
8. Ryhage, R., and E. Stenhagen. 1960. *J. Lipid Res*. **1:** 361.
9. Carey, J. B., Jr., and G. Williams. 1966. *Science*. **150:** 620.
10. Danielsson, H. 1960. *Acta Chem. Scand*. **14:** 846.
11. Touchstone, J. C., M. Kasparow, P. A. Hughes, and M. R. Horwitz. 1966. *Steroids*. **7:** 205.
12. Peterson, N. A., I. L. Chaikoff, and C. Jones. 1965. *J. Neurochem*. **12:** 273.
13. Grosser, B. I., and E. L. Bliss. 1966. *Steroids*. **8:** 915.