Infra-red Spectroscopy of Brain Tissue. A Lipid Fraction in Normal and Irradiated Adult and Fetal Rats.* (19659)

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Previous studies have demonstrated that brain tissue shows characteristic but species unspecific infrared absorption spectra. The present paper reports: 1. Differences in qualitative and quantitative spectroscopic findings in the brain of adult and developing rats. 2. Tentative assignment of brain tissue spectra. 3. Spectroscopic studies of an effect of ionizing irradiation on developing and adult brain.

Material and methods. Female albino rats of about 200 g were used for the experiments in adult brain and pregnant rats of between 2-3 weeks gestation for the studies in fetal brain. The animals were killed by decapitation. The brain was removed quickly, and frozen and dried sections through brain stem, hypothalamus, midbrain, cerebellum and forebrain of adults and through the whole brain of fetuses were prepared for spectroscopy. Histologic studies were made from sections cut from the block remaining after sections for spectroscopy were taken. The technic of preparation of sections and infrared spectroscopy of tissues was essentially the same as described previously(1). The irradiation experiments comprised administering total body irradiation from 500-800 r in non-pregnant and from 150-400 r in pregnant animals. Adult non-pregnant animals were studied one or 2 days after the irradiation. Fetuses were removed for spectroscopic and pathologic studies 24 hours after exposure of the mothers to x-rays. In a few instances studies were carried out in animals born from 1-2 days after irradiation of the mothers. Results obtained in those animals were compared with controls of the same age. Irradiation was delivered from a Villard Westinghouse machine at 200 KW, 20 milliamperes with 1 mm aluminum and ½ mm copper filters. The rats were irradiated in thin-walled plastic or glass containers. The target distance was 52 cm. The x-ray dosage was given in a single exposure at the rate of 50 r per minute. The amount of irradiation administered under those circumstances was checked at intervals with a Victoreen symbol ionization chamber placed in the empty containers. According to those measurements, variations of selected dosage were not more than a few r from experiment to experiment.

Results of qualitative infrared spectroscopy of adult and developing brain tissue. Two different types of absorption bands were found within the different wavelength regions. 1. In the high frequency region, below 8.1 μ adult animal, fetus and newborn alike showed a number of more or less strong bands at locations almost similar to known vibrations (3.04 μ NH stretching, 3.4 μ CH bending, 6 μ CO stretching, the deformation vibration of NH at 6.44 μ, and the deformation vibration of CH at 6.88 μ found in proteins and polypeptides (Sutherland)(2). Additional weak or medium strong bands occurred at 7.20 μ (methyl vibration), and at 7.70 μ, 8.10 μ, and 8.53 μ (unassigned). 2. In the low frequency region, *e.g.*, between 8.1 μ and 11 μ, the spectra of adult and fetal brain tissue showed significant differences. In adult rats rather strong bands at about 9.26 μ and weaker bands at about 10.28 μ were found. In fetuses and newborn, weaker bands occurred regularly at 9.23 μ, 9.56-9.65 μ (a shoulder like absorption band) and at 10.30 μ.

Fig. 1 and 2 show transmittancy curves which were obtained from sections of about the same thickness (50 μ) cut through adult and fetal brain. The great differences between the transmittancies of adult and fetal brain tissue are apparent. The transmittancy curve

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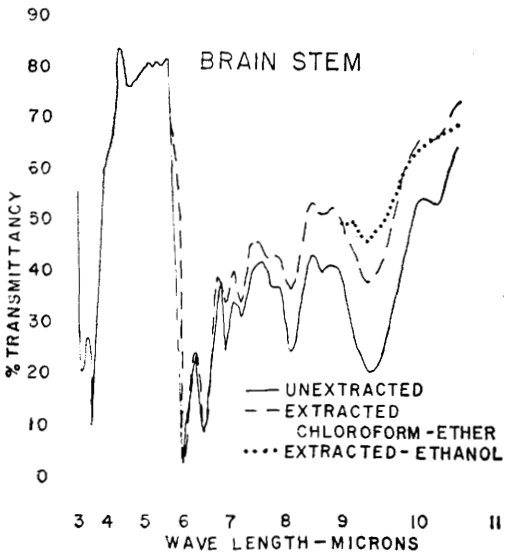

FIG. 1.

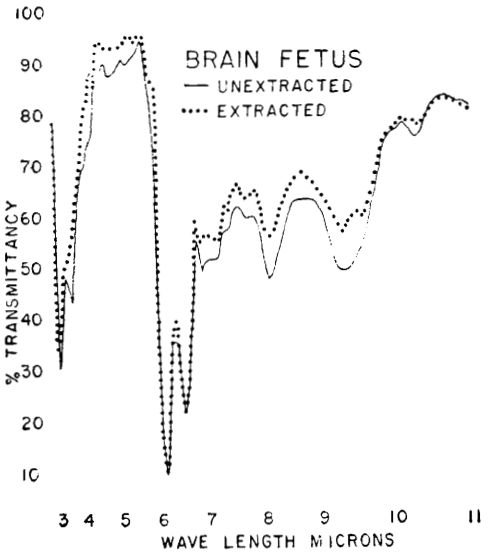

FIG. 2.

of the fetal brain (solid line in Fig. 2) closely resembles the curve of extracted adult brain tissue (interrupted and dotted line, Fig. 1).

Tentative assignments of brain tissue spectra. Lipids are contained in neural tissue in so high concentration that it was reasonable to investigate their role in producing the characteristic spectra of brain tissue. For this purpose infrared spectra were recorded from brain tissue sections before and after extraction with various lipid solvents, from evaporated films obtained from some of those extracts, and from pure compounds related to lipid metabolism. The mounted frozen and dried brain sections were extracted with mixtures of solvents such as chloroform:ether (1:1), or petroleum ether:methanol (9:1), or simple solvents (ethanol, benzene, and petroleum ether) at 4°C. The time of extraction varied from 15 to 50 hours until spectroscopic examination revealed no further effect of the extraction. The effect of chloroform:ether and ethanol extractions, which alone will be discussed here are shown in Fig. 1, and can be briefly summarized as follows: Ether:chloroform extraction greatly reduces the characteristic absorption bands of brain tissue in the 9-11 μ region. This extraction does not affect the protein amide vibrations such as the deformation vibration of NH at 6.44 μ, in the high frequency region. Ethanol extraction has even greater effect in reducing the characteristic absorption spectra and in addition sometimes measurably decreases the strength of the amide vibrations (deformation vibration of NH at 6.44 μ). The chloroform:ether extracts remaining after extraction of the brain sections were concentrated in vacuum and finally evaporated in specially designed silver chloride wells of about 15 mm diameter and 1/10 mm depth. The films, thus obtained, showed spectra which were related to the characteristic bands of brain tissue and in addition a number of bands which were not visible in the spectrum of the tissue itself, when overshadowed by absorptions of the background.

Fig. 3 shows a transmittancy curve of one of those films. Films obtained from numerous brain sections gave, except for slight variations, similar spectra. The general pattern of these spectra comprises, a strong band at about 5.70 μ, a strong triplet at about 7.8 μ, 8 μ, and 8.30 μ, a weak band at 8.5 μ, strong bands at about 8.80 μ and 9.20 μ, and weaker and broader bands near 9.80 μ and 10.30 μ. These spectra show some very characteristic groups of bands which closely resemble those of glycerides of long chain saturated and unsaturated fatty acids (Shreve *et al.*(3)) and in part the few spectra of cephalins studied by Baer(4). The strong band at 5.7 μ indicates

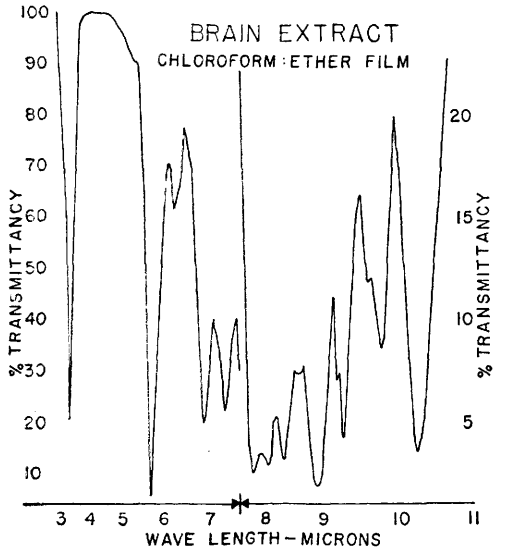

FIG. 3.

the presence of esters and the triplet below 9 μ corresponds to vibrations of fatty acid glycerides. The strong band near 9.20 μ, and the weak bands at about 9.80 μ and 10.30 μ could not be assigned so easily. It was assumed tentatively that the strong band at 9.20 μ might be produced by C-O or C-O-P vibrations contained in phospholipids and glycolipids, and that the weak band at about 10.30 μ would be, at least in part, produced by the specific vibration (at 10.36 μ) of the trans double bond found in trans unsaturated compounds and their esters. The band at about 9.80 μ remains unassigned. In reviewing the results of our studies of brain extracts, it should be stated that some of the assignments must be considered with caution. The fact that constant and characteristic spectra of a brain lipid fraction can be obtained from such small amounts of tissue suggests that spectroscopic examination of brain extracts are valuable for investigation of lipids in the brain.

Many pure compounds were studied to confirm the tentative assignments. These included unsaturated and saturated fatty acids, phospholipids (cephalin, L-α-cephalin, lecithin free of cephalin, and sphingomyelin), glycolipids (cerebrosides, kerasine, and phrenosine), sphingosine sulfate, sodium glycerophosphate, cholesterol, and cholesterol acetate. Most substances were obtained from commercial sources, L-α-cephalin was kindly supplied by Dr. E. Baer of Toronto and sphingomyelin by Dr. S. Tannhauser of Boston. Study of the spectra of these compounds, which will be reported in detail elsewhere showed the correctness of some of our assumptions. All the glycolipids, phospholipids, as well as glycerophosphate showed rather strong bands at 9.20-9.30 μ, very close to the location of the strong characteristic band found in intact brain tissue and in the chloroform:ether extract. The spectra of glycolipids annd sphingomyelin, however, differed significantly from those of the brain extract in the 6-7 μ region.

In glycolipids and sphingomyelin the strong ester band of the extract at 5.7 μ was replaced by an amide C-O vibration at about 6.10 μ and an additional NH vibration at 6.46 μ which were never seen in any chloroform:ether extract. This finding, which could have been predicted from the amide structure of those compounds, proved that not glycolipids or sphingomyelin but amide-free phospholipids such as lecithins, cephalins and other lipid-like esters were the main constituents of the chloroform:ether fraction. Cholesterol esters are found in too small quantities in normal adult and developing brain tissue to make any considerable contribution to the absorption spectrum of the lipid fraction. Also the strongest bands of cholesterol esters and free cholesterol are located at distinctly higher wave lengths than the strong bands of intact brain tissue and brain extracts in the 9-10 μ region. Measurements at 9.50 or 9.60 μ may include whatever amounts of free cholesterol (5) or cholesterol esters are present. Bands at about 10.30 μ, the location of the second characteristic band in brain tissue, were seen in many of the pure compounds but cannot be assigned more definitely at present. In reviewing the assignment work it can be stated that studies of more pure compounds and further fractionation of the chloroform:ether fraction may lead to final identification of the spectroscopically well defined amide-free lipid fraction.

Quantitative infrared spectroscopy. For quantitative estimation of the amide-free lipid fraction, spectra are recorded from mounted brain sections before and after extraction with

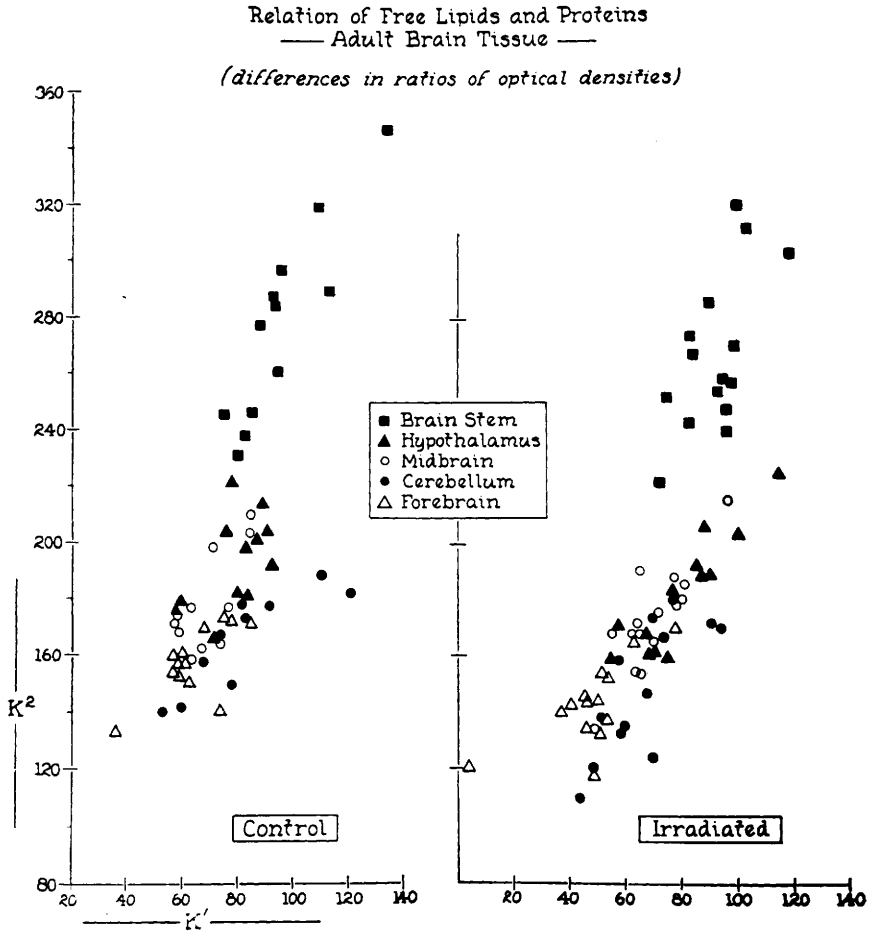

FIG. 4.

chloroform:ether. Ratios of optical densities between the characteristic bands of adult or fetal brain tissue in the 9-11 μ region and the amide bands at 6.44 μ are determined. These ratios of optical densities are independent of thickness of sections according to Beer's law. The differences of ratios of optical densities before and after the extraction are a relative measure of the relation of the amide-free lipids to the amides in the brain. Since the amount of amides from compounds other than proteins are relatively small, these differences of ratios of optical densities actually represent the ratio of the amide-free lipid fraction to proteins of the brain. Fig. 4 and 5 show the results in adult and fetal controls and the effect of ionizing irradiation. For the graphic presentation in Fig. 4 (adult brain), the differences of ratios were determined at 10.30 μ and 9.30 μ. The values obtained multiplied by 1000 were called K 1 and K 2 respectively. K 1 was plotted as abscissa and K 2 as ordinate. For the graph in Fig. 5, the differences of ratios of optical densities were measured at 10.30 μ, 9.50 μ, and 9.23 μ. The figures obtained multiplied by 100 were called K 1, K 2, and K 3, respectively. The K values were plotted as abscissa and the numbers of fetuses or newborn as ordinate.

Amide-free lipid fraction in adult and developing brain. Fig. 4 clearly demonstrates that a definite pattern of the ratios of amide-free lipids to proteins exists in adult brain with brain stem showing the highest and forebrain the lowest figures and hypothalamus, mid-

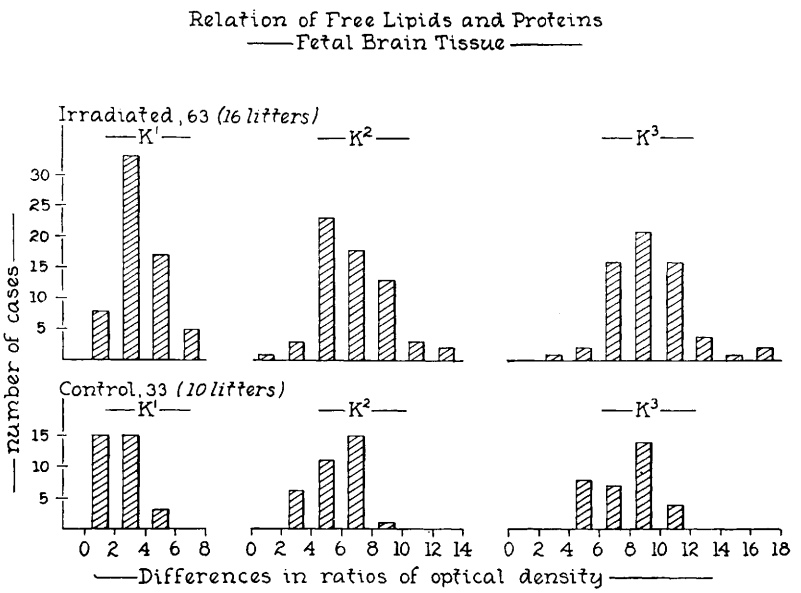

FIG. 5.

brain, and cerebellum between the 2 extremes. The differences between these ratios are so great and the change in the "protein amide" values in the dry tissue relatively so small that variations in the amounts of amide-free lipids rather than proteins are responsible for the pattern described. Comparison of the figures shown in this graph and in Fig. 5 indicates that the amount of amide-free lipids is significantly smaller in fetal brain. This finding explains in part the different appearance of spectra of adult and fetal brain (Fig. 1 and 2).

Effect of ionizing irradiation on the amide-free lipid fraction. As indicated in Fig. 4, ionizing irradiation with doses of from 500-800 r produces within 1-2 days a small but significant decrease of the amide-free lipid fraction. The effect is rather infrequent in animals subjected to irradiation with 500 r (half lethal dose) and increases, though not regularly, in frequency and intensity with higher dosage. The animals show no objective signs of abnormality of the central nervous system and routine histologic studies revealed no significant pathologic alterations. Total body irradiation of pregnant animals at 150-400 r causes an increase of the amide-free lipid to protein ratio in fetuses removed after one day and animals born 1-2 days after the irradiation of the mothers (Fig. 5). The effect occurred more frequently after irradiation at 300 r and above. Histologic studies revealed changes of the fetal brain similar to those described by Hicks(6). The ultimate effect upon the nervous system of irradiation *in utero* is the subject of further studies.

Discussion. The present studies demonstrate that the characteristic bands of the infrared spectrum of the brain are mainly produced by lipids. The amide-free lipid fraction can be completely extracted so easily from mounted brain sections (after 15 hours at 4°C) that it is very probable that this fraction is only loosely bonded or not bound to protein at all.

The variation of this lipid fraction in different parts of the brain suggests a possible relation of the concentration to the degree of neural activity since the largest amounts were found in areas of vital functions—the medulla —and the smallest in the forebrain, which in the rat has a relatively low functional development.

The concentration of the lipid fraction found spectroscopically in fetal brain may not be an entirely valid reflection of the total amount in developing brain tissue. Limitations of infrared spectroscopy employed in this investigation preclude quantitative studies of tissue sections below 3 x 3 mm. For

this reason, the fetal brain was examined as a whole. As a result, the sections prepared for spectroscopy included mainly the immature forebrain structures which even in the adult state show the lowest concentration of the lipid fraction.

The increase of the fraction in the fetus following maternal irradiation may be related to the destructive effect of x-rays upon the germinal areas of the forebrain and reflect the results of a chemical breakdown of embryonic cell elements. The formation of peroxides in the skin lipids(7) of x-ray irradiated animals and x-ray irradiated linoleic acid *in vitro*(8), as it might affect the bonding of lipids to protein, may prove a fruitful avenue for further study of the mechanism of the results obtained.

It is expected that these results may contribute to the elucidation of the greater radiosensitivity of the developing brain recently demonstrated by Hicks and confirmed by our own histologic studies. It is also felt that infrared spectroscopy may be valuable for investigation of other complex problems involving lipids in the brain.

Summary. 1. Frozen and dried sections of brain tissue, brain extracts, and pure compounds related to lipid metabolism are studied by infrared spectroscopy. 2. The characteristic infrared absorption bands are tentatively assigned to certain lipid structures. 3. An amide-free lipid fraction is studied in adult and developing brain. 4. Adult and developing brain tissues show different infrared spectra. These differences are in part caused by different content of amide-free lipids. 5. X-ray irradiation decreases the amide-free lipid fraction in the adult and increases that fraction in developing brain.

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Correction of Anemia Following Hypophysectomy by Administration of Cobalt.* (19660)

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Polycythemia can be produced in normal rats by placing the animals in an environment of reduced oxygen tension(1) or by the injection of cobalt(2). Because cobalt is thought to produce polycythemia by interference with oxidative mechanisms(3,4) and because hypophysectomy interferes with the usual increase in red cells and hemoglobin in response to an environment of reduced oxygen tension(5,6) it was thought of interest to see if hypophysectomized rats could respond to cobalt with the production of an increased circulating red cell volume.†

It has been previously shown that 0.05 mg of cobalt and 0.1 mg of iron per 100 g of body weight per day for 60 days is sufficient to produce a 50% increase in the total circulating red cell volume of normal rats(7).

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† An abstract has recently appeared by Crafts(11) in which he reports that administration of cobalt repairs anemia of hypophysectomy.