EXPERIMENTAL PRODUCTION OF GLIOSIS *

II. REACTION OF BRAIN TISSUE TO THE LIPOID FRACTIONS AND TO THE RESIDUE OF BRAIN EXTRACTS

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The striking difference in the chemical structure of brain and spinal cord tissue and the other tissues of the body lies in the high lipin content of the brain and cord. These lipins do not include the neutral fats but consist almost entirely of the more highly complex lipoids, the most important of which are cholesterol, phospholipin and glycolipin or cerebroside. These lipoids are important constituents in all of the cells of the body and their large proportion in the nervous tissue is not due to a cellular increase but to the fact that they are the principal constituents of the medullary sheaths. This is brought out by a comparison of analysis of brain tissue taken from the white matter such as the corpus callosum and the gray matter or child's brain in which medullation is still incomplete.

It would seem possible that one or all of these lipoids might be an important factor in the production of gliosis in the degeneration of the long fiber tracts, such as the pyramidal tract, when no inflammatory process is present to provide stimuli for the multiplication of the glia cells. It is our intention, therefore, to extract brain tissue and obtain these lipins and the residue and to determine the effect of these substances upon normal brain tissue.

Dogs were given ether anesthesia, the femoral vein cannulated, the femoral artery on the same side opened and the blood washed out of the animal with physiologic saline as long as the heart continued to beat. As soon as the cardiac action ceased, the common carotid arteries were cannulated, the jugular veins opened and washing with physiologic saline by gravity was continued until the returns were clear. The brains were removed, stripped of all meninges and superficial vessels and delivered to the chemical laboratory of the hospital, to be extracted by the method given in Mathews' Physiological Chemistry.

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A precipitate is obtained by cooling a hot alcoholic extract. The ether soluble substances are then removed and the remainder consists chiefly of the cerebrosides. To the ether extract thus removed is added the cold alcoholic filtrate; acetone soluble substances are removed and the remainder is chiefly phospholipins. The acetone extract from the above is chiefly cholesterol. The residue from these extractives contains the nucleo- or phosphoproteins. The first three

TABLE 1

Composition of the Brain. Thudichum [1]

(Corrected for dried tissue)

	Corpus callosum	Gray matter
	per cent	*per cent*
Neuroplastin	30.0	53.0
Ether extract (Phospholipin and cholesterol)	38.0	18.5
Cerebrosides and myelin	23.0	2.5
Water extracts	4.7	3.3

White matter exceeds gray matter in phospholipins and cholesterol by 38 to 18.5.
White matter exceeds gray matter in cerebrosides by 23 to 2.5.

Composition of the Brain. Koch [1]

	Corpus callosum	Child's brain
	per cent	*per cent*
Protein	27.0	46.0
Extractives	4.0	12.0
Cerebrosides	18.0	7.0
Phosphatides	31.0	24.0
Cholesterol	17.0	1.8

White matter exceeds child's brain in phosphatides and cholesterol by 48 to 26.
White matter exceeds child's brain in cerebrosides by 18 to 7.

may be considered to represent roughly the materials chiefly derived from the medullary sheaths, and the fourth those from the cells.

These substances were prepared in a sterile condition by evaporating the cerebrin out of methyl alcohol, the phospholipin out of acetone, the residue out of ether and by heating the cholesterol to 120 C. for two hours. These were then preserved in sealed tubes.

Trephine openings were made over the parietal area in dogs under ether anesthesia, the meninges snipped with the scissors, a hole

made in the brain with a small trocar and a piece of the extract pushed through the hole. Five dogs were operated on, one serving as a control while each of the others received one of the extracts. The dogs were allowed to live four weeks. Pieces of the injected area were fixed in formalin, Zenker's solution and Ciaccio's solution. They were stained with Scharlach R, Ciaccio's stain, eosin-methylene blue, iron hematoxylin, phosphotungstic acid hematoxylin, neutral ethyl violet-orange G and the aniline blue connective tissue stain. The material was also studied with the polarizing microscope.

Control. No evidence of infection found. The meninges are moderately thickened at the point of incision. The hole in the brain can be readily seen.

A section through the area of the lesion shows moderate hemorrhage with phagocytes containing blood pigment. The blood vessels in and adjacent to the lesion are prominent. The endothelial cells are slightly increased in the perivascular spaces and the mesodermal fibrous tissue is slightly increased within the area as shown by the aniline blue connective tissue stain. A moderate gliosis of the spider cell type and a few cytoplastic glia cells have appeared adjacent to the lesion.

The significant reactions to this lesion were an increase in endothelial cells and fibroblasts of the blood vessels with some invasion of the area of the lesion, phagocytes full of blood cells and débris, and a moderate gliosis.

Phospholipin. The phospholipins are contained in the ether extract of the brain tissue. They are one of the major constituents of the so-called protagon of the medullary sheaths and also of all cellular protoplasm. They are characterized by Mathews [1] as being a "water-containing, semifluid, highly-reducing, auto-oxidizable, semilipoid crystalline substratum, which contributes to or makes possible the vital phenomena." They are also important in immunity and it is suggested that they are an important factor in staining of the living cell by basic dyes. They constitute 31 per cent of the dried white matter (Koch) and may thus be an important factor in producing gliosis in tract degeneration.

The important phospholipins of the brain are lecithin, cephalin, myelin, paramyelin and sphingomyelin. The chief difference in these substances is apparently in the fatty acid radicle. Lecithin has been the most studied and it is the only one whose formula is

known. On hydrolysis it yields glycerine, oleic acid, stearic acid, phosphoric acid and choline. Thudichum[1] claims that the chief properties of lecithin are due to its oleic acid. For our purposes, however, the fatty acids may prove of less interest than the phosphoric acid and choline.

Choline is a nitrogen base and has powerful physiologic action. By intravenous injections of minute quantities it increases the blood pressure (Mendel et al.[2]) and by intracerebral injection it produces convulsions and paralysis (Donath[3]). Coriat[4] speaks of it as "the most important decomposition product of lecithin which is found in the central nervous system, blood and cerebrospinal fluid in conditions where active degeneration is taking place." He claims that it is found in cerebrospinal fluid, brain and cord only with active medullary sheath degeneration which leads to decomposition of the lecithin and to a coincident increase in phosphoric acid in cerebrospinal fluid. Donath[5] claims that choline and glycero-phosphoric acid pass into the fluid on decomposition of lecithin while stearic acid combines with glycerol to form neutral fats which accumulate beneath the neurolemma.

The extract used in this work must be considered an impure mixture of the various phospholipins and specific results are not to be expected.

The trephine opening is filled with fibrous tissue and the meninges are adherent to it and the underlying brain at the point of the lesion. The meninges are thickened at this point. A vessel in the meninges apparently draining this area is very conspicuous by its size and the thickness of its wall.

On cutting the sections, the microtome knife met with considerable resistance at the point of the lesion. The knife was nicked and the tissue torn. This was apparently due to calcification of the injected phospholipin occurring within the four weeks during which it had been in the brain. Among the various theories of pathologic calcification, the best accepted is the formation of calcium-binding substances within degenerating areas, especially phosphoric acid and fatty acids (Wells[6]). It has been suggested that calcium soaps are formed from the fatty acids and are then transformed into the less soluble phosphates and carbonates. Cellular lecithin and nucleoproteins have been considered as the probable sources of phosphoric acid and fatty degeneration has been recognized as a

precursor of calcification. However, Wells questions these theories. In a chemical study of a large quantity of material he found only traces of calcium soaps in calcifying matter even in the early stages (Wells [7]). He has implanted in the abdominal cavity of rabbits various tissues that had been killed and sterilized by boiling and found that the tissues rich in nucleoproteins show no greater tendency to calcify than those poor in nucleoproteins. However, the residue used by the writer, which presumably consisted largely of nucleoproteins, also produced substances in the brain giving marked resistance to the microtome knife, though it was not nicked. None of the lesions produced by the other extracts seemed unusually hard. This seems to suggest that the phosphoric acid may play some part in calcification. The phospholipins, however, contain both phosphoric acid and the fatty acids.

Studies of the lesion produced by the phospholipin show a picture very similar to that of the control as far as the mesodermal elements are concerned. Hemorrhagic elements are present with phagocytes containing blood pigment. The blood vessels in and about the lesion are prominent because of a slight endothelial and fibroblastic increase. The area contains some fibrous tissue. However, more phagocytes occur here than in the control lesion and many of these contain a substance stained by Scharlach R. Gliosis of the fibril-forming type is more marked but still moderate.

It would appear then that except for the calcification, the hypertrophy of the meningeal vessels and the greater gliosis, the effect of introducing phospholipins into the brain was no greater than the mechanical effect of the trocar hole as seen in the control animal. Possibly if this extract were further broken down into its constituents, namely, pure lecithin or choline, phosphoric acids and the fatty acids, a more pronounced reaction would be obtained.

Cerebrin. The cerebrosides are glycolipins and phosphorus free. They are obtained from the hot alcoholic extract. On hydrolysis they yield a sugar, usually galactose, various fatty acids and a nitrogen-containing alcohol. The common ones found in the brain are phrenosin, kerasin and cerebron. They are not present in the embryonic brain but increase with medullation and constitute 18 per cent of the dried adult corpus callosum (Koch). Apparently less work has been done with the cerebrosides than with the phospholipins from either the chemical or the biologic point of view.

Ossification has occurred in the trephine opening in the animal which had received the cerebrin. The meninges are thickened at the point of the incision. The sections show a microscopic picture somewhat similar to those described above. Hemorrhagic elements are present and the small blood vessels are prominent just as they were in the control and phospholipin specimens. A little more fibrosis has occurred in the area with a slight tendency to capsule formation. There is considerably more gliosis both of the fibril-forming and the cytoplastic type adjacent to the area of the lesion. The amount of phagocytosis is about the same as in the phospholipin experiment.

The gliosis was the most characteristic feature though it was not as marked as that seen in the cholesterol experiment (to be discussed later). It was quite decided and will probably justify further experiment.

Cholesterol. Cholesterol is a sterol or solid alcohol. It constitutes 17 per cent of dried corpus callosum (Koch) but it is also an important constituent of all cell protoplasm. Its empirical formula is $C_{27}H_{45}OH$. It is an unsaturated mono-alcohol and belongs to the general group of terpenes. It is associated with numerous physiologic and pathologic phenomena. Thus it is supposed to prevent hemolysis of the erythrocytes, to check lipolytic enzymes and in this way control the metabolism of fats, to be the mother substance of the bile salts and hence regulate fat absorption and to regulate the water metabolism of cells. It is one of the constituents of gall stones and is found in sclerosis of arteries. It is present in old degenerative processes such as old infarcts, exudates and degenerating tumors in the brain or in other parts of the body. Wells [6] thinks that its action in the cell must be purely a physical one since it is chemically inert. He also speaks of it as behaving like an inert foreign body in pathologic conditions and as being removed by giant cells and foamy endothelial cells. In the previous paper of this series it was shown that an experimental hypercholesterolemia had no effect on the brain tissues of rabbits. When it was introduced subdurally by lumbar puncture, however, there was a fibrous increase in the dura and in the cellular content of the arachnoid, with phagocytosis of the cholesterol and a slight gliosis in the subjacent spinal cord tissue.

In the dog brain there is no gross evidence of infection. The meninges are slightly thickened and adherent as in the other cases. The microscopic picture of the section is striking. Polymorpho-

nuclear cells are sprinkled around a few of the blood vessels and it will therefore be necessary to repeat this work to eliminate the possibility of infection, although these cells might have been called forth by the extract itself. A definite fibrous capsule has formed around the injected substance with ingrowths of fibrous tissue toward the center. The blood vessels around the area are thickened by an increase of their endothelial cells and fibroblasts. Numerous phagocytes are present but these are outside the fibrous capsule which walls off the cholesterol and seems to be associated with the hemorrhage and tissue injury. The gliosis both of the fiber-forming and cytoplastic type is marked, the latter being very conspicuous in places. Except for the gliosis this reaction corresponds closely with that of the spinal cord of the rabbits in which a fibrous increase in the dura and a cellular increase in the arachnoid were produced. The cholesterol was taken up by the phagocytes while in the brain of the dog it was walled off by the fibrous capsule. This difference in the glia reaction may be due to a difference in the substances injected as that used in rabbits was Merck's purified cholesterol while that used in the dog was extracted from a dog's brain.

Residue. The residue is the solid substance left after the brain has been extracted with hot alcohol and ether and may be considered to be the precipitated proteins. It is probably largely nucleo- or phosphoproteins and undoubtedly many other substances. Little is yet known of the nucleic acid of the brain. It yields on hydrolysis phosphoric acid, a purine base and a hexose (Mathews [1]).

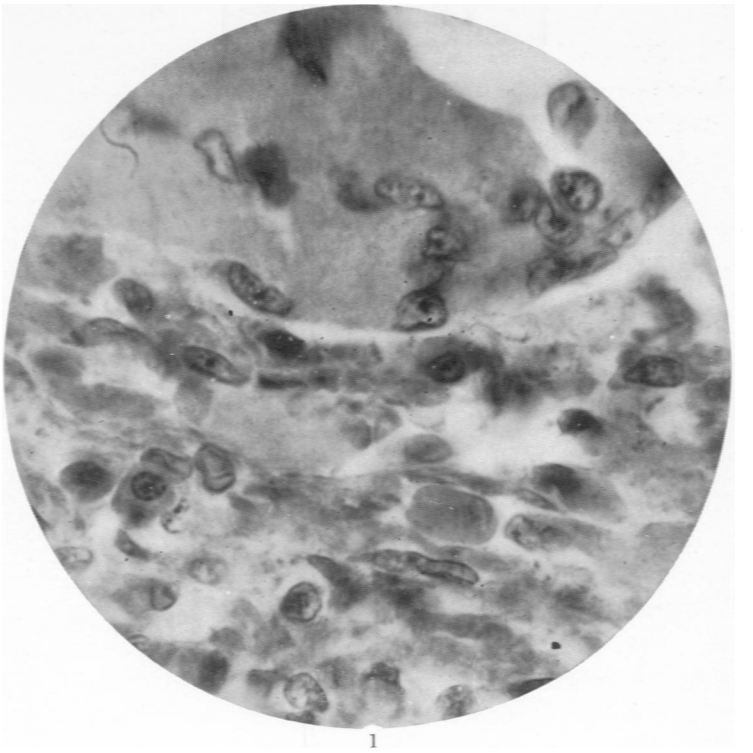
In the animal injected with this residue, the brain in gross looks like the others. Microscopically, a conspicuous fibrous capsule surrounds the injected mass like that in the cholesterol experiment. Outside of this capsule is seen some hemorrhage at one place and also a little tissue destruction. Strikingly few phagocytes occur and the gliosis of the brain tissue is less than in any of the specimens except the control. The striking part of the picture is the heavy fibrous capsule which differs from that in the cholesterol specimen in the number and form of the nuclei. These are numerous and very bizarre in their shape. In addition, a border of syncytial tissue with numerous nuclei and a frothy cytoplasm which seems to be invading the foreign substance with tongue-like projections has developed just inside the capsule. This tissue has been interpreted as a phagocytic syncytium. Such tissue formations are of very considerable

interest. The products of nuclein hydrolysis are said to stimulate cell growth (Wells,[6] and Calkins et al.[8]) and such an intense and bizarre nuclear reaction as seen here will certainly justify further studies with this substance and also further experiment to determine whether the active factor is the nucleic acid or some other ingredient.

Conclusions

One of the interesting results of these experiments is the way each picture overlaps the next. If we may consider each of the four extracts as a sort of fractional product with a predominating substance but containing lesser quantities of the others, we may explain the overlap.

The effect on the blood vessels is about the same in all of the series including the control. These vessels are more prominent in and about the area of the lesion and show an increase in the endothelial cells and the fibroblasts. Calcification was evident in the phospholipin and suggested in the phosphoprotein experiments. The phagocytic reaction varied directly as the amount of hemorrhage and tissue destruction and inversely as the amount of encapsulation. The fibrous reaction within the lesion and capsule formation were least in the phospholipin injection and a little greater in the cerebrin experiment, there being an indication of capsule formation. A definite capsule had developed in the cholesterol experiment with invasion of the mass by fibroblasts, but still numerous extracapsular phagocytes were to be found. In the residue experiment the capsule formation was complete. The rapid nuclear production was at its maximum as a reaction to the residue, though a slight suggestion of it occurred in the cholesterol experiment. On the other hand, the fibrous and invasive reactions were at the maximum with the cholesterol extract where bands of fibrous tissue invaded the mass. Gliosis was the least marked in the control and in the residue experiments. It was increased by cerebrin and was marked with phospholipin and perhaps even more so with cholesterol. The fact that purified cholesterol failed to produce any marked gliosis in the spinal cord of rabbits suggests that some substance common to the phospholipin and cholesterol extracts, which is not present in the purified material, serves as the glia stimulant.



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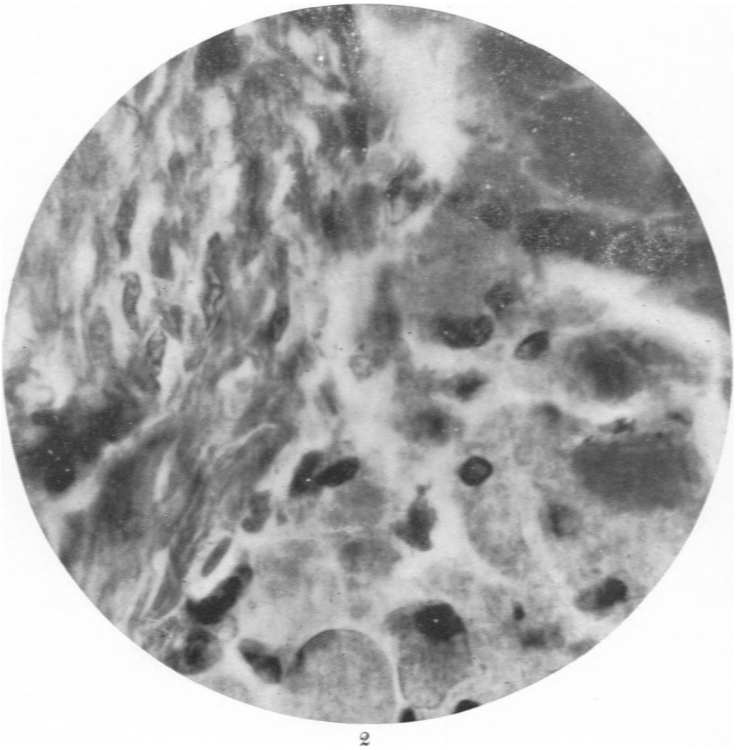

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These experiments have to some degree separated the various constituents which usually form a single picture in a lesion in the brain. To separate these constituents more completely, it will be necessary to obtain either more nearly pure extracts or to fractionate these more completely. Studies in the various stages in the development of lesions resulting from the present series of extracts might also be of value. The water-soluble extracts were not used in these experiments and these may be important factors in the tissue reactions to lesions in the central nervous system. They include alkaloids, organic and inorganic acids, carbohydrates, amino acids and other unknown substances.

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DESCRIPTION OF PLATE CIV

FIG. 1. Oil immersion photomicrograph of the area of a dog's brain injected with the brain residue or nucleoproteins. At the lower edge of the picture is the brain tissue with the cellular and fibrous tissue which has encapsulated the injected mass and above it the phagocytic syncytial tissue which is invading the mass. The bizarre nuclear forms are well shown.

FIG. 2. Oil immersion photomicrograph of the injected brain residue or nucleoprotein (in the upper right-hand sector) surrounded by the syncytial phagocytic tissue (in the lower left-hand sector) and encapsulated by a fibrous tissue (on the left).