A STUDY OF THE CHEMICAL NATURE OF COMPONENTS OF BOVINE WHITE MATTER EFFECTIVE IN PRODUCING ALLERGIC ENCEPHALOMYELITIS IN THE RABBIT*‡

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Experimental allergic encephalomyelitis (EAE) is a well defined, reproducible entity based on sensitivity to autologous tissue antigen (for recent reviews, see references 1–3). Its importance as a model for certain forms of demyelinating disease in man has made it a subject of interest to investigators from a number of unrelated disciplines. However, immunologic and other investigations of the disease mechanism have been seriously handicapped by lack of information regarding the exact chemical nature of the encephalitogenic antigen or antigens in nervous tissue. The present paper presents for the first time precise chemical data regarding fractionated materials which show antigenic activity.

The tissues of the central nervous system contain several distinct substances with the capacity to induce circulating antibody formation (4). Many of these substances, like the antigen(s) responsible for the production of EAE, are organ- rather than species-specific. Their exact chemical nature, however, even after 30 years of investigation, remains obscure. A few simple lipides have been reported to possess antigenicity even when produced synthetically. It is not known if any of these substances are encephalitogenic when administered with adjuvants, although some negative data have been obtained on this point (5).

The earliest studies of EAE indicated that the encephalitogenic activity of nervous tissue is largely confined to those parts that are rich in white matter (6, 7), and is absent from fetal or unmyelinated brain (7, 8). As nervous tissue of foreign species is effective in producing EAE (7–11) the antigenicity is said to be organ-specific rather than species-specific. It has been reported that activity is associated with

material sedimenting at 8000 G and is non-dialyzable (12, 13). It resists autolysis, boiling, autoclaving, and ultrasound (8, 11), fixation with formalin (8, 9, 11), and the lecithinases of *Clostridium welchii* alpha toxin (9) and cobra venom (14). Simple extraction of nervous tissue with ethanol or acetone (8, 13) or with other common organic solvents (14) destroys its antigenicity.

In recent years there have been several attempts to find and characterize the antigen responsible for producing EAE, and these have yielded somewhat conflicting results. Alvord studied the production of EAE in guinea pigs with lipide fractions prepared from bovine and human brain (15). He reported success with fractions containing phosphatides and also with a pure bovine lipide preparation,[1] when these were used at doses corresponding to 30 or more times the threshold dose of whole brain. On the other hand, Lumsden (11) found activity associated with fractions containing cerebrosides and sphingomyelin. Hottle *et al.* (16) reported that a dialyzable, biuret-negative substance, extracted from rabbit brain by M/10 calcium acetate after preliminary benzene and ether extraction, produced EAE in guinea pigs, while the residue was inactive. This finding was confirmed in some laboratories (17) but not in others (18). The nature of the antigenic substance was not investigated further.

The use of modern techniques of fractionation was initiated by Tal and Olitsky (19, 20). They employed methods developed in this laboratory (21) to separate mouse brain into several fractions (see Fig. 1). Of these, only the fraction known as "fluff," which contains one or more proteolipides plus certain uncombined lipides, produced EAE in mice. This fraction appeared, on the basis of a titration at the 50 per cent level, to have essentially all the activity of the original brain (22), while other fractions were negative at levels of 100 to 2400 times the threshold dose (based on original weight of brain). Antigenic activity was destroyed by drying the "fluff" in the presence of water, a procedure which splits protein from lipide (20). These authors were also able to produce EAE with bovine proteolipide A, used at about 300 times the threshold level (19). Following Tal and Olitsky's work, preliminary reports appeared simultaneously from this laboratory, on the use of bovine white matter fractions in rabbits (23), and from the Mayo Clinic (24), on the use of fractions of guinea pig brain in guinea pigs (this group has subsequently published its data *in extenso* (25)). These reports indicated that antigenic activity was present not only in the fluff but in the lower phase as well. A large part of this activity was traceable to the ether-soluble fraction of the lower phase, a material containing very little protein and consisting largely of phosphatides (21), in particular acetal phosphatide, which is largely absent from gray matter and fetal brain.

Thus in three different laboratories, fractions prepared by the Folch and Lees method of fractionation from different tissue sources have been tested in 3 species of test animals. All have reported that some of the antigenic activity of whole brain or white matter is accounted for in the proteolipides of the "fluff"

[1] This material, which was provided by one of us, was prepared by the colloidal iron method' discussed in this paper; *i.e.* it presumably contained less than 0.2 per cent of protein but was not carbohydrate-free. Only 1 of 5 animals injected twice with large doses of this material developed obvious signs of paralysis, and no histologic data are reported for this one animal.

(Fig. 1). Two of these groups agree on the importance of the ether-soluble fraction of "lower phase," rich in acetal phosphatide and poor in protein. None of the data published thus far, however, have included analytical determinations of the actual amounts of protein or acetal phosphatide in the various fractions tested.

The present paper summarizes the results of one phase of an investigation, covering several years, directed at isolating a purified antigen or antigens from bovine white matter. Findings with the Folch and Lees fractionation technique are presented as well as analytical data on the protein and acetal phosphatide contents of antigenically active fractions. Also included are titration data permitting a comparison of the activity of these materials as antigens.

Materials and Methods

Analytical Methods.—Most of the methods used here have been described elsewhere (21, 26).

Protein was determined by estimating the concentration of α-amino groups (R—CH—

$$|$$

$$NH_2$$

COOH) (27) in the test material before and after hydrolysis by 6 N HCl at 100°C. for 16 hours. The value for α-NH$_2$ in amino acids after hydrolysis minus that obtained before hydrolysis corresponds to the amount of amino acids liberated by the hydrolysis of protein present. This value multiplied by 12 gives a figure representing the amount of protein in the material. The factor 12 is derived empirically from the study of isolated proteolipide protein (21). The accuracy of the procedure is limited by the relatively small error of each of the two estimations involved. The results obtained are accurate within 1 per cent when the amount of protein present is 5 per cent or greater. They become increasingly inaccurate as the concentration of protein decreases. Values of the order of 0.2 per cent or less, express only maximal possible concentration of protein.

Acetal phosphatides were estimated by an adaptation of the method of Feulgen and Grünberg (28).[2] The acetal phosphatides are hydrolyzed with mercuric chloride, and the liberated fatty aldehydes are estimated by their reaction with acid fuchsin, followed by spectrophotometric reading of the color developed.

Preparation of Fractions from Brain White Matter.—White matter was obtained by dissection from the brain stem and/or the centrum semiovale of slaughter house cattle brains. Usually 1 to 2 hours elapsed between the death of the animals and the beginning of the fractionation procedure. The preparation described as "old" or "aged" white matter was stored at −15°C. for 15 months before use. It was thawed on several occasions. Tissue was handled in one of three different ways.

(a) *Fractionation by the method of Folch and Lees (21).*—The method is given in outline in Fig. 1. The procedure was carried out exactly as described elsewhere (21) except for the following details. The insoluble tissue residue from chloroform-methanol extraction is contaminated with lipides and proteolipides present in the aliquot of solvent with which it is

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[2] Our thanks are due to Dr. G. Schmidt, of the Department of Biochemistry, Tufts College Medical School, Boston, who has kindly allowed us access to his unpublished procedure for this determination. The method has been standardized by Dr. Schmidt with pure brain acetal phosphatide prepared by Thannhauser *et al.* (29).

BRAIN WHITE MATTER

Homogenized with chloroform-methanol

TISSUE RESIDUE   CHLOROFORM-METHANOL EXTRACT

Equilibrated with water

UPPER PHASE   INTERFACIAL FLUFF   LOWER PHASE

Dissolved in chloroform-methanol; placed at −10°C.

Dried and extracted with acetone

Precipitate
PROTEOLIPIDE A   Filtrate

Treated with an equal volume of acetone; placed at 4°C.

Acetone
insoluble   ACETONE
                             SOLUBLE

Extracted with ether

Precipitate
PROTEOLIPIDE B   ACETONE
                      FILTRATE
                      FROM
                      FLUFF

Acetone and
ether insoluble   ETHER
                       SOLUBLE

Extracted with ethanol

Residue   ETHANOL
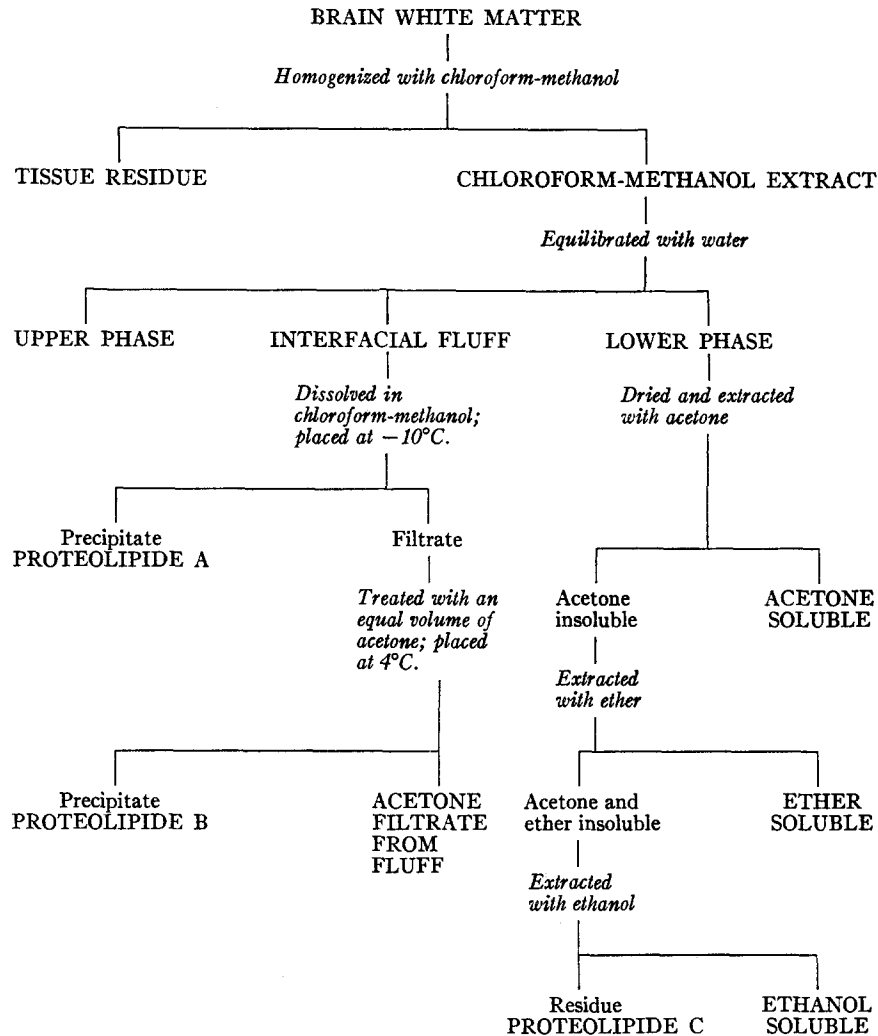PROTEOLIPIDE C   SOLUBLE

FIG. 1. Preparation of white matter fractions by the method of Folch and Lees (21).

wet. In order to remove the last traces of this contaminating material, the tissue residue was extracted with boiling chloroform-methanol under reflux for 1 hour. Proteolipide protein, obtained, as directed in the method, by drying the chloroform-methanol extract under a vacuum and extracting the resulting residue with chloroform-methanol, was freed of the last traces of lipides and proteolipides in the same manner. Finally, proteolipides A and B were precipitated as a single fraction by adding to a solution of interfacial fluff in chloroform-methanol an equal volume of acetone and placing the mixed solution at 4°C.

(b) *Preparation of tissue total lipides and proteolipides in the solid state, and of total lipides with different amounts of proteolipides.*—In the original method of Folch and Lees, it is im-

possible to obtain all the tissue lipides and proteolipides in the solid state by simply taking the chloroform-methanol extract to dryness, since this step results in splitting the bond between lipide and protein in proteolipides. This difficulty is avoided by the following procedure, which was developed in the course of other work (30): To a chloroform-methanol extract of white matter is added exactly one-fifth its volume of water, and the liquids are mixed thoroughly. On centrifugation, the system separates into two phases without formation of interfacial fluff. One phase consists mainly of water and methanol containing all the non-lipide contaminants from the original extract and about 0.2 per cent of the tissue lipides. The other consists mainly of chloroform containing essentially all the tissue lipides and proteolipides. When the chloroform phase is dried by vacuum distillation of the solvents, no splitting of proteolipides occurs.

From a preparation of total lipides and proteolipides obtained in this way, samples containing total lipides but varying amounts of proteolipides were prepared as follows: The material was dissolved in 2:1 chloroform-methanol and 5 per cent water was added. This solution was taken to dryness by vacuum distillation of the solvents. The resulting residue was dissolved in chloroform-methanol and insoluble material removed by filtration. On analysis, the filtrate was found to contain considerably less protein than the total lipide extract. The protein content of this material could be reduced further by adding 5 per cent water to the filtrate, and repeating the drying and extraction. Carrying out the procedure twice usually yields a material with about 0.5 per cent protein. When it is repeated a third time, the material becomes essentially free of protein (<0.2 per cent).

These findings require modification of our earlier observations (21), which seemed to show that the process of taking to dryness of white matter chloroform-methanol extracts (which contain about 4 per cent water contributed by the tissue) results in complete destruction of proteolipides. This had been established by extracting the dried residue with hot chloroform-methanol, and analyzing the solution for protein; none had been found. It now appears that while a single drying of the tissue extract does in fact destroy the bulk of proteolipides, a small proportion (between $<\frac{1}{20}$th and $\frac{1}{4}$) remains intact. Attempts to determine the factor(s) responsible for this wide variation have failed. The discrepancy between the earlier and the present observations may be due, at least in part, to two factors. In the first place, in the original work, the conditions for analysis were such that protein concentrations below 1 per cent could not be detected with certainty. In the second place, in the recent work, the dried residue from the original tissue extract was treated exhaustively with chloroform-methanol. In the earlier work less care may have been exercised on this particular point, with the possible result that some intact proteolipide could have remained unextracted.

(c) *Preparation of tissue lipides by the colloidal iron method.*—In this method (31), the tissue is homogenized with water, and the proteins and lipides in the homogenate are precipitated by addition of colloidal iron and aqueous $MgSO_4$. The precipitate is washed repeatedly with dilute aqueous $MgSO_4$ to remove all water-soluble extractives and then with water to remove the bulk of $MgSO_4$. Finally, lipides in the precipitate are extracted with ethanol-ethyl ether 1:1 mixture, by volume. In the actual preparation, 70 gm. of minced white matter were homogenized with 700 ml. of water. To the homogenate was added with mixing 100 ml. of Merck's "dialyzed iron" (liquid, 5 per cent Fe_2O_3) followed by 52 ml. of saturated aqueous solution of $MgSO_4$. The suspension was centrifuged at 2500 R.P.M. for 10 minutes and the clear supernatant discarded. The precipitate was washed with three 600 ml. portions of a 1 to 8 dilution of saturated aqueous $MgSO_4$, and with one of water. It was then transferred to a one liter glass-stoppered cylinder with the help of several portions of ethanol-ethyl ether 1:1. The resulting suspension was made up to volume with the same solvent, thoroughly shaken, and filtered. The filtrate was evaporated to dryness *in vacuo*. The lipide obtained amounted to 154 mg. per gm. of tissue and contained <0.2 per cent protein.

The lipide remaining in the alcohol-ether–insoluble part of the colloidal iron precipitate, which had been collected on the filter paper, was recovered by washing this precipitate three times with 400 ml. each time of the solvent mixture to which 15 per cent water had been added. The washed precipitate was extracted with 2 liters of chloroform-methanol 2:1, by volume, to which 5 per cent water had been added. By filtration, a clear extract was obtained. It was taken to dryness. The lipide residue amounted to 13 mg. per gm. of fresh tissue.

An aliquot of the same starting tissue mince was found to contain 178 mg. of lipides (exclusive of protein) per gm. of fresh tissue. By comparison, the colloidal iron procedure yielded in the two successive extracts 154 and 13 mg. of pure lipides, a total of 167 mg. per gm. of fresh tissue, i.e., over 93 per cent of the tissue lipides. The first of the two consecutive preparations obtained contained no phosphatidyl-serine, and the second contained a small amount. Thus it appears that the 7 per cent of lipides not extracted by this method are mostly, if not exclusively, phosphatidyl serine.

Preparation and Injection of Antigenic Mixtures.—Fractions were incorporated into antigenic mixtures by grinding in a mortar with bayol F containing 3 mg./ml. heat-killed, virulent human type tubercle bacilli of the Jamaica 22 strain.[3] No water was included. Most fractions gave homogeneous emulsions, when handled in this manner; but certain non-lipide materials required the addition of a small amount of falba, as an emulsifying agent, to produce a mixture suitable for injection. At the standard level, an amount of each substance equivalent to 2 gm. wet weight of white matter was combined with 3 ml. of the bayol F containing tubercle bacilli. The final volume differed only slightly from 3 ml. In titration experiments directed at determining the activity of certain fractions, greater or lesser amounts were similarly combined with 3 ml. of oil and bacilli. In all instances, healthy 2.5 to 3.5 kg. female rabbits of a mixed albino strain were given single simultaneous injections in the 4 footpads of 0.05 to 0.1 ml. of a particular mixture. The approximate total was 0.3 ml., containing an amount of fraction derived from 0.2 gm. fresh white matter (at the standard level). Each preparation was injected into a group of 4 or 5 rabbits; and in most instances, other preparations of the same fraction were later tested at the same or other dose levels.

Evaluation of Disease.—All rabbits were examined daily and sacrificed either after the appearance of EAE or at the end of an observation period of 6 weeks. The following observed variables lent themselves to evaluation of the severity of the disease response.

(a) *Gross EAE.*—The obvious signs of illness were graded according to criteria laid down in an earlier publication (18), with ++++ representing severe paralysis, ataxia, or death within 5 days of onset; +++, the same in more than 5 days; ++, intermediate degrees of disease; and +, minimal signs. As the actual location of lesions in the central nervous system rather than their number or severity may determine the gravity of the manifest illness, it was felt that histologic severity was a better measure of the disease process. The rapidity of progression of the disease, which is also taken into account in the above grading, is more precisely measured by the day of onset discussed below. The cerebrospinal fluid changes represent another observation which might well be suited for comparing disease severity in different rabbits. However, fluids were not available from all our animals. There is also an arbitrary element involved in selecting the days on which fluids are to be taken.

(b) *Histologic EAE.*—The histologic estimate of disease severity usually agreed quite well with that derived from the gross manifestations. It was based on the degree of dissemination of the lesions seen in hematoxylin and eosin–stained sections and on their extent. Sections were made as routine through the spinal cord at 3 levels, the brain stem in the region of the pons, and the cortex. Nerve roots, spinal ganglia, and optic nerves were frequently included. The disease in many instances appeared to involve one part of the nervous system more than

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[3] Obtained through the courtesy of Dr. Jules Freund of The Public Health Research Institute of the City of New York, Inc.

others. Since, however, the factors determining distribution remain undetermined, no effort was made to include this characteristic of the disease in the histologic appraisal. The rare instances in which definite signs of EAE were present but no lesions found post mortem may be attributed to our failure to examine spinal ganglia as a routine, since in some instances, the disease seemed limited to these. Among the 298 rabbits forming the subject of the present study, 15 showed mild histologic lesions in the absence of gross manifestations and 9 showed gross signs for which no histologic basis was found.

(c) *Proportion of animals with disease.*—This figure was always based on the animals showing histologic lesions, but included the occasional rabbit with clear cut signs of disease in which nothing was found post mortem.

(d) *Onset.*—Figures for onset are employed only when a definite time could be assigned on the basis of daily observation. If there were questionable signs of disease for 2 or 3 days before its presence was certain, the 1st day on which such signs occurred was taken as the onset.

(e) *Death.*—Actual spontaneous death occurred in a small proportion of animals. Sick rabbits that received much individual nursing care were less likely to die than those that did not. Thus death from disease was an unsatisfactory variable. The duration of disease till death was unsuitable for the same reasons, quite aside from the difficulties and expense involved in maintaining large numbers of sick rabbits over long periods of time. Most rabbits in the present study were killed within a week or two of the onset of disease.

Skin Tests and Serologic Studies.—The technique and results of these studies will be published in detail elsewhere. We will only indicate that all animals were bled on the 14th day and that the sera were tested for conventional complement-fixing antibody with a variety of fractions. At the same time almost all animals were skin-tested with several fractions, and the reactions were evaluated at 48 and 72 hours. The reactions were of the tuberculin type (18).

RESULTS

Antigenic Potency and Chemical Composition of Fractions Obtained from Bovine Brain White Matter by the Method of Folch and Lees.—The analytical data obtained with the various Folch and Lees fractions and their ability to produce EAE when injected at the standard dose level are shown in Table I.

It can be seen that the chloroform-methanol extract is active antigenically, while the denatured tissue residue and water washings are not. The antigenic activity can be traced in part to proteolipides A and B and in part to the lower phase, more specifically to its ether-soluble fraction.

A correlation of the activity of fractions and their chemical composition brings out the following points: Proteolipides A and B are antigenic, whereas proteolipide C, accounting for about half the tissue proteolipide, is inactive. Thus if the tissue contains an effective antigen which is proteolipide in nature, it must be a specific one, not all of the tissue proteolipides. Such a specific proteolipide may account for all observed antigenic activity. On the other hand, the fact that the antigenically active ether-soluble fraction is poor in proteolipide raises the question whether it may contain a non-proteolipide antigen.

The outstanding chemical difference between the ether-soluble fraction and other fractions of the lower phase is its high content of acetal phosphatide. Thus the possibility is raised that acetal phosphatide may be the hypothetical

TABLE I

Antigenic Potency and Chemical Composition of Bovine Brain White Matter and of Fractions Prepared Therefrom by the Method of Folch and Lees

Material injected in rabbits at standard dose (20-fold threshold)	Rabbits with EAE / Rabbits injected	Composition of fractions			Amounts in dose injected (from 200 mg. fresh white matter)			Effectiveness at other dose levels
		Main components	Proteolipide protein	Acetal phosphatide	Total solids	Proteolipide protein	Acetal phosphatide	
			per cent	*per cent*	*mg.*	*mg.*	*mg.*	
White matter	7/7	—	—	—	—	—	—	Effective at 1/20th standard dose
Solutes from washed chloroform-methanol extract*	15/19	Total lipides and proteo-lipides	9.65	6.5	34.4	3.32	2.24	Effective at 1/25th standard dose
Water washing from lipide extraction	0/6	Small acid-soluble components	—	—	—	—	—	—
Tissue residue	0/4	Balance of components	—	—	—	—	—	—
Proteolipides A and B	5/12	Proteolipides,	24.5	0.8	10.4	2.5	0.083	—
Proteolipide A	1/4	cerebrosides, phosphatides	15.1	0.5	6.4	0.97	0.032	Similar results at 10 times std. dose
Proteolipid B	2/7		26.2	0.6	0.81	0.214	0.004	Similar results at 5 times std. dose
Solutes from acetone supernatant from fluff	0/4	Mixed lipides	1.44	6.8	27.2	0.40	1.88	Ineffective at 20 times standard dose
Solutes from lower phase	9/9	Proteolipides, phosphatides, cerebrosides	8.00	10.0	21.55	1.75	2.15	Effective at 1/20th standard dose
Fractions from lower phase:								
Acetone soluble	0/4	Cholesterol	0.5	0.3	6.6	0.033	0.020	—
Ether soluble	5/7	Lecithin, cephalin, acetal phosphatide	3.87	16.5	12.1	0.47	2.00	Effective at 1/5th standard dose
Ethanol soluble	0/4	Sphingomyelin, cerebrosides	8.4	6.6	4.36	0.37	0.29	—
Proteolipide C	0/8	Proteolipide	70.0	1.1	2.3	1.7	0.025	—

* Prepared according to (*b*) under Preparation of Fractions.

non-proteolipide antigen. This idea is made more plausible by the parallel finding that gray matter and unmyelinated brain, both devoid of antigenicity, contain acetal phosphatide at a much smaller concentration than does white matter. However, the data in Tables I and II render this hypothesis untenable for several reasons. Certain active fractions, such as proteolipide B, contain vanishingly small amounts of acetal phosphatide, while the inactive acetone

supernatant from proteolipide B contains a great deal. In addition, when the ether-soluble fraction of the lower phase is processed for further purification of the acetalphosphatides, its antigenic activity diminishes markedly. Finally a number of the inactive, protein-free preparations (Table II) discussed in the next paragraphs have very high contents of acetal phosphatide.

In order to evaluate the hypothesis that proteolipide may be responsible for all the antigenic activity observed, it became necessary to determine in detail the distribution of compounds of this type in fractions obtained by the Folch and Lees technique. In the original paper on proteolipides, the emphasis was placed on establishing the presence of this new group of compounds in brain tissue and on the description of methods adequate for their isolation (21). Therefore little detailed information was made available regarding their presence in the more predominantly lipide fractions. Proceeding on the assumption that all the protein in a given fraction is proteolipide protein, the protein content of various fractions was determined in a number of different preparations. It was found that the yield of some of these fractions as well as their protein content varied within wide limits. Thus no generally valid balance sheet for the distribution of proteolipides can be drawn up. It is necessary to do actual protein determinations each time fractionation is carried out. Such determinations are presented in Tables I and II. It is proper to summarize here the results of these and other determinations, though the values given are approximations which can only serve for purposes of orientation. About one-tenth of the solutes in washed chloroform-methanol extracts of white matter are proteolipide protein. What this represents in terms of fresh tissue depends on the particular area in brain from which the white matter is obtained. An average figure would be 19 mg. proteolipide protein gm. fresh tissue. Of this proteolipide protein 80 per cent or more is found in proteolipides A, B, and C. The balance is found in the other fractions. Of these, the acetone supernatant from proteolipide B and the acetone extract from the lower phase are usually poorest in protein, containing about 1 per cent. The ether-soluble fraction from the lower phase is richer in protein, containing between 2 and 4 per cent. The alcohol-soluble fraction from lower phase appears to be the most variable in protein content, values obtained ranging from traces to 9 per cent. Thus it is clear that proteolipide might well be responsible for the antigenicity of the ether-soluble fraction (Table I).

As a conclusive test of the hypothesis that all the antigenic activity of our total lipide preparations can be attributed to proteolipide, the following experiments were carried out. A series of total tissue lipide preparations, containing varying amounts of intact proteolipides, was prepared by the methods described under Preparation of Fractions, paragraph (b). Their antigenic activity and chemical composition are given in Table II. It can be seen that the antigenic potency of these preparations parallels the proteolipide content. To

meet the valid objection that the procedure for the destruction of proteolipide might result in the elimination of some unrecognized non-proteolipide antigen, lipides were isolated from brain by the colloidal iron method, de-

TABLE II

Antigenic Potency and Chemical Composition of White Matter Lipide Preparations Obtained by Different Methods

Lipide preparations		Dose*	Rabbits with EAE / Rabbits injected	Yield	Components in preparation		Amounts of components in dose injected		
					Proteo-lipide protein	Acetal phos-pha-tide	Total solids	Proteo-lipide protein	Acetal phos-pha-tide
				mg./gm. fresh tissue	per cent	per cent	mg.	mg.	mg.
White matter (for reference)		1	7/7						
		1/5	4/7						
		1/20	3/6						
All of the lipides and proteolipides		1	8/9	172	9.65	6.5	34.4	3.32	2.24
		1/5	4/5		9.65	6.5	6.84	0.65	0.46
		1/25	2/5		9.65	6.5	1.38	0.132	0.09
All lipides plus	preparation I	1	5/6	158	1.42	7.7	31.7	0.46	2.46
different	preparation II	1	3/6	144	1.55	7.4	28.8	0.45	2.13
amounts of	preparation III	1	0/4	144	0.55	6.7	28.8	0.16	1.92
proteolipides	preparation IV	1	0/5	140	<0.16	6.5	28.0	<0.046	1.82
	preparation IV	5	0/5	140	<0.16	6.5	140.0	<0.230	9.10
Proteolipide protein		1	0/7	28	92.0	—	5.6	5.2	—
Lipides extracted by the colloidal iron method		1	0/5	158	<0.2	10.0	31.6	<0.063	3.16
		5	0/5		<0.2	10.0	158.0	<0.316	15.80
Lipides extracted with CHCl₃-CH₃OH from tissue residue of preceding preparation		5	0/5	12	<0.2	19.0	12.0	<0.024	2.28

* A dose of 1 means that the amount injected represents the amount of that particular fraction in 200 mg. of fresh white matter.

scribed in Preparation of Fractions under heading (c). As discussed in detail there, the two successive preparations obtained by this method yield all white matter lipides, free of proteolipides and of phosphatidyl serine. Their activity is reported in Table II. Both these preparations are completely inactive.

Titration Data and Their Quantitative Significance.—In Table III are summarized the manifestations of disease and histologic observations in animals

injected with actively antigenic fractions at several dose levels. In the last column, average values of the graded score discussed in the Appendix are included. The first point which stands out in this table is that all the measured variables vary with the dose of inoculum. There are irregularities apparent.

TABLE III

Disease Manifestations and Histologic Data Obtained with Active Fractions at Several Dose Levels

Preparation	Dose level*	EAE‡				Average graded score§
		No. of rabbits	Average severity	Average day of onset	Average histologic severity	
Fresh white matter	1	7/7	3.3	10	2.7	5.4
	1/5	4/7	2.3	13	2.3	2.6
	1/20	4/7	3.5	16	1.6	1.4
Old white matter	1	8/10	2.5	13	1.6	3.2
Total lipides and proteo-lipides	5	5/5	3.0	14	1.5	3.0
	1	15/19	2.1	15	1.7	2.5
	1/5	4/5	2.2	16	1.1	1.8
	1/25	2/5	0.5	?	0.7	0.3
Lower phase	1	9/9	1.9	14	1.8	3.1
	1/5	3/4	3.0	16	1.8	1.3
	1/20	1/4	0.5	22	1.0	0.3
Ether-soluble lower phase	1	5/7	1.8	13	2.0	3.0
	1/5	5/7	0.7	16	0.7	1.0
	1/20	0/7	—	—	—	0
Proteolipides A + B	1	5/12	1.2	14	1.1	0.8

* The dose level designated as 1 represents an inoculation of each animal with an amount of fraction derived from 200 mg. of whole white matter.

‡ Averaged only for positive animals. Maximum severity of disease manifestations is 4.0; maximum histologic severity is 3.0.

§ Averaged for entire group. Maximum score is 6.0.

In the case of gross severity of disease, these irregularities are so great as to render this measurement useless for comparisons, aside from the objections considered under Methods. The remaining variables which may be regarded as more or less reliable are the proportion of rabbits developing disease, the day of onset, and the histologic severity. The mathematical treatment of these measurements will be considered in the Appendix. For our purposes here, however, it is clear, simply from looking at the data, that the disease produced by aged white matter or by any of the fractions is less severe at

all dose levels than that produced by fresh white matter, as shown both by the day of onset and by the histologic severity. However, increasing the dose to five times the standard level, as was done with total lipide extract, does not increase the severity of the disease beyond a certain maximum, well below the maximum reached with fresh white matter. This difference between fresh white matter and old white matter or the fractions is not reflected in the proportion of animals developing disease. Thus it would seem that some factor other than the actual amount of antigen present must be responsible for the clear cut difference in disease response. It cannot be due to the absence of water and protein in mixtures made up with the fractions, as the 19 rabbits which received total lipide extract at the standard level include groups of 5 which received antigen mixtures to which water or water and bovine serum albumin were added in proportions corresponding to those found in whole white matter. Actually the severity observed with a given dose of old white matter or the fractions corresponds roughly to that produced by fresh white matter at one-fifth that dose level. On the other hand, if one compares old white matter with the total lipides and proteolipides, lower phase, and ether-soluble lower phase, it is clear that no major loss of antigenic activity has occurred in this series of fractionation steps.

DISCUSSION

The evidence reported in this paper was obtained in two different stages. In the first stage of the work, bovine brain white matter was fractionated by the method of Folch and Lees, and the different fractions obtained tested for their ability to produce EAE in rabbits. As described in detail above, antigenic activity was found only in the chloroform-methanol extract, the balance of tissue material (largely denatured protein) being inactive. The activity of the lipide extract could be traced, to a small extent, to proteolipides A and B, but to a much greater extent to the lower phase, and thence to the ether-soluble fraction therefrom. When these observations were interpreted in terms of the knowledge available at the time on the chemical composition of the different fractions they suggested the presence in the total lipide extract of two different antigens, one present in proteolipides A and B and presumably a proteolipide, and a second one concentrated in the ether-soluble lower phase and most probably not a proteolipide. These results and this interpretation were reported at that time in preliminary form (23).

The second stage in the work began with the study of the chemical nature of the hypothetical non-proteolipide antigen presumably present in the ether-soluble fraction. The analysis of different fractions (Table I) showed, on the one hand, that the distribution of acetal phosphatides among the lower phase fractions paralleled the distribution of antigenic activity and thus suggested that acetal phosphatides might be the hypothetical antigen, a surmise which was disproved

by further work. On the other hand, this chemical work showed that all fractions, active or inactive, contained proteolipides. This second finding, and a thorough consideration of all other evidence available, brought out the point that all our results could be explained by the assumption that the effective antigen was a small specific fraction of proteolipides. Experiments designed to prove or disprove the new hypothesis were carried out with the results reported in Table II. The results obtained showed that white matter total lipides are effective antigens only when they contain proteolipides. The complete destruction of the latter yields preparations of white matter total lipides (free of proteolipides) which are antigenically inactive. Of course this loss of antigenic activity could be explained by the assumption that the procedure for destruction of proteolipide resulted in the incidental removal of non-proteolipide antigen. This is unlikely since white matter lipide preparations obtained by the colloidal iron method (which yields lipides free of proteolipides on direct extraction of the tissue) are antigenically inactive.

The foregoing clearly shows that under our conditions of operations, total white matter lipides free of proteolipides are non-antigenic; it suggests strongly that the encephalitogenic antigen is a proteolipide; it proves that a large part of the proteolipides are non-antigenic and warrants the corollary that the effective proteolipide antigen constitutes only a small specific fraction of tissue proteolipides. The logical reasoning at the basis of the last statement is as follows: Among the terminal fractions obtained, the antigen is present only in proteolipides A and B, and in the ether-soluble fraction. If it is assumed that the antigen is the same in all three, it is obvious that proteolipide in the ether-soluble fraction is a much more active antigen than proteolipide in proteolipides A and B; *i.e.*, it is necessary to conclude that only part, and possibly a small part of proteolipide in the latter fractions is antigenically active. Thus the active antigen would represent at the most, the proteolipide in the ether-soluble fraction, and a smaller amount in proteolipides A and B. Since proteolipide in the ether-soluble fraction amounts to about $\frac{1}{10}$ of total tissue proteolipides, the active proteolipide antigen can be computed to amount to not much more than that, and to account only for about 1 per cent of total tissue chloroform-methanol extractives. It must be emphasized that our results do not establish conclusively that the antigen is a small specific proteolipide fraction. In fact, the very finding that the antigen probably represents a quantitatively unimportant fraction of total chloroform-methanol extractives of the tissue, suggests the alternative possibility that the antigen might be some as yet undetected "trace" component, the nature of which could only be a matter of speculation. Finally, another possibility to be considered is that EAE is not produced by any one definite chemical compound, but that it is produced, instead, by a "total" effect of the whole lipide-proteolipide mixture in the sense that certain spatial relationships of the components of the mixture would

be required for its acting as antigen. Such spatial relationships would require, for their preservation, the presence of proteolipides, which, on account of their molecular size, would be apt to act as a spatial framework.

It is pertinent to compare our results with the earlier ones of Tal and Olitsky and with those obtained independently by Goldstein *et al.*, the two groups of workers who have used our method of tissue fractionation. Tal and Olitsky, using homologous brain in mice, found antigenic activity only in proteolipides A and B, the other tissue fractions being inactive. By implication, the equivalent, in their work of our ether-soluble fraction was inactive. This discrepancy between their observations and ours may very well correspond to a species difference between mice and rabbits. However, before the occurrence of a species difference is accepted, a much simpler explanation for the discrepancy should be investigated. This is that there are marked quantitative chemical differences between mouse brain and cattle white matter; *e.g.*, the latter contains two-fold the amount of lipides and ten-fold the amount of proteolipides of the former. Thus it is very likely that when our method of fractionation is applied to mouse brain it yields fractions that are not identical with the fractions it yields when applied to cattle white matter. Anyway, the discrepancy in observations is not very significant, since our conclusions bear out completely the conclusions reached by Tal and Olitsky.

Goldstein *et al.* used homologous brain in guinea pigs. Their results are almost identical with those reported in Table I. The only differences between their results and ours are that these authors find some antigenic activity in the tissue residue from the chloroform-methanol extraction, and also in the isolated proteolipide protein. The activity they observed in both of these fractions can easily be explained by the fact that Goldstein *et al.* did not reextract these materials sufficiently thoroughly with boiling chloroform-methanol. Presumably, enough intact proteolipides were left in them to account for the slight antigenic activity reported. Goldstein *et al.* interpreted their results as we interpreted ours in our preliminary note; namely, they felt two different antigens are present in the tissue. Clearly, our later study applies equally well to our results and to theirs, and their observations are compatible, as are ours, with the hypothesis that the effective antigen is a small specific fraction of proteolipides.

Some light is shed on the question of whether two different antigens are present in the total lipide extract by the results of skin tests (these will be reported in detail elsewhere). It was found, with regard to the 3 preparations of interest, proteolipides A and B and the ether-soluble lower phase, that inoculation with one produced skin reactivity to each of the others and that this reactivity was considerably greater in rabbits which developed EAE than in those which did not (18). No such cross-reactivity was observed with the other more purified fractions nor did these elicit skin reactions correlated with disease. On the other hand, with the complement fixation test, there appeared to be a broad

overlapping of antigenic specificity among all the fractions tested. The presence in nervous tissue of multiple antigens, at least as far as the production of circulating antibody is concerned, has been known for some time (4). The skin test findings are compatible with the hypothesis that a single effective antigen is present in proteolipides A and B and the ether-soluble lower phase.

None of the foregoing evidence should be interpreted as showing that the chloroform-methanol extractives from the tissue account for all of the EAE-producing potency of the intact tissue. In fact our titration data indicate that an important change in antigenicity occurs when white matter is allowed to age or when it is extracted with chloroform-methanol (Table III). In spite of the fact that at corresponding dose levels the same proportion of animals develops disease as with fresh white matter, this disease is of later onset, milder and less severe histologically. This difference cannot be attributed to loss of antigenicity, since injection of five times the standard dose of total lipide extract does not result in increased severity of disease over that obtained with the standard dose. In other words, the disease response reaches a different asymptotic maximum from that obtainable with whole fresh white matter (see Appendix for graphic formulation of these findings). Three types of events may be responsible for the observed changes in antigenic behavior. First, there may be a change in the physical state of the antigenic material which influences the inflammatory response at the inoculation site and through it the speed and intensity of sensitization. While this possibility could apply to the material extracted by chloroform-methanol, it is hard to see how it could be true of white matter aged by storage at −15°C. for a few months. Second, there may be some alteration of the antigenic molecule, so that the antigen in old white matter or any of the extracts is qualitatively different from that in fresh white matter. We have no data bearing directly on this possibility. The third possibility is that there is a second antigen, which deteriorates with aging in whole white matter and which is not extracted by chloroform-methanol. The fact that antigenicity was not observed in the reextracted residue from chloroform-methanol extraction does not weigh strongly against such an idea. Simple proteins in this material are completely denatured and the lipoproteins are split with removal of their lipide moiety. In actual fact the residue may show considerable antigenic activity before reextraction and some activity even after several reextractions (25). Antigenic activity has been observed in water-soluble materials derived from nervous tissue by several observers (16, 17, 32). Thus we must recognize the very clear possibility that a second type of antigen may exist in the non-lipide constituents of myelin.

SUMMARY AND CONCLUSIONS

Fractions of bovine white matter, prepared by the methods of Folch and Lees, were studied for chemical composition and for their ability to produce experimental allergic encephalomyelitis in rabbits. Evaluation of the disease

and of the lesions in animals injected with the more active fractions at several dose levels permitted comparison of the antigenic activity of these materials.

When tissue was fractionated by the methods of Folch and Lees, antigenic activity was found in the chloroform-methanol extract but not in the denatured tissue residue. This activity was traced to proteolipides A and B and to the lower phase, more specifically the ether-soluble fraction of the lower phase. Proteolipide C was inactive.

Correlation of the chemistry of fractions with their antigenic activity suggested two possibilities: (a) that there might be two antigens, one proteolipide and the other non-proteolipide; or (b) that a small specific proteolipide is responsible for all the observed activity. The high concentration of acetal phosphatide in the ether-soluble lower phase suggested that compounds of this type might be the hypothetical non-proteolipide antigen, but this hypothesis was disproved by analytic study of active and inactive materials. The possibility that proteolipide might account for all the antigenic activity was strongly supported by the experimental finding that total lipide and proteolipide progressively lost activity as proteolipide was degraded by adequate processing. The use of an entirely different method for preparing total lipides free of proteolipide (the colloidal iron technique) indicated that this loss of activity did not result from incidental removal of some non-proteolipide antigen. These tentative conclusions are in agreement with those of Tal and Olitsky and provide a satisfactory interpretation of the findings of Goldstein *et al.* The very fact, however, that the suggested proteolipide antigen would amount to no more than 1 per cent of the total chloroform-methanol extractives leaves open the possibility that some unrecognized trace substance may be the antigen.

Skin tests with the various fractions indicated some cross-reactivity between proteolipides A and B and the ether-soluble lower phase fraction and a fair correlation of positive skin reactions with disease. This finding is compatible with the suggestion that the same antigen is present in both of these types of material.

When the disease produced by whole tissue or fractions was evaluated by the use of the proportion of animals developing disease, the day of onset, and the severity of the histologic lesions, it was found that fractions produced milder disease of later onset than intact tissue at all dose levels. The disease-producing activity was not enhanced by increasing the dose; *i.e.*, it appeared to reach an asymptotic maximum below that obtainable with whole fresh tissue. This finding suggests both a quantitative loss of activity and a qualitative change during the initial chloroform-methanol extraction, a procedure which denatures all proteins in the tissue residue. A comparable change appeared to occur in whole white matter stored at $-15°C$. for 15 months and thawed and refrozen several times during this period. The later fractionation steps resulted in no

apparent loss of antigenic activity. A scoring method employing the same type of data to estimate the actual relative antigen contents of different preparations is presented in the Appendix.

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APPENDIX

The Dose-Response Relationship in Allergic Encephalomyelitis in the Rabbit. Estimation of the Encephalitogenic Activity of Bovine White Matter Fractions

In the study of experimental allergic encephalomyelitis (EAE), no method has thus far been put forward which permits quantitative statements regarding the disease response or estimates of the effectiveness of the agent causing disease. In fractionation experiments where one investigates the presence of encephalitogenic antigen in a number of preparations derived from an active source material such as bovine white matter, it becomes imperative to make some statement about the proportion of the original activity present in each fraction. It is also desirable to have a means of comparing groups of animals inoculated under different conditions or receiving particular forms of treatment. Most studies in this field have based their conclusions on the proportion of animals developing disease in groups inoculated at a single dose level, often in the absence of data permitting an estimate of the relation of the given dose to the threshold dose or to the ED_{50}. Such a procedure involves the serious fallacy that at very high doses minor contaminants may impart effectiveness to materials inactive in the gross. Other workers have titrated their inoculation material and estimated the ED_{50} (1).

It is the purpose of this Appendix to ascertain to what extent the measured variables discussed in the preceding paper can be used in setting up a suitable assay technique. The use of graded responses is generally regarded as more informative than the use of quantal responses (disease *versus* no disease), since it makes use of more experimental information (2, 3). When several variables, which basically are determined by the same event, are combined in a single metameter, there results an increased precision of this metameter comparable to that which results from the averaging of replicates in chemical or physical measurement. Thus our aim is to combine suitable measured variables in a metameter which is amenable to conventional statistical manipulations.

It was observed (Table III above) that three different aspects of the disease response varied in a more or less regular manner with the dose of inoculum. To determine whether these show the commonly recognized relationships to the log dose, they were graphed as follows: Histologic severity was plotted directly; the day of onset d, was plotted as $100/d$ and as $-log\ d$ (3); and the figures for the proportion of rabbits developing disease, after smoothing by the calculation of moving averages (4), were plotted as the probit (5). All showed fair linearity, and the lines obtained with different preparations were more or less parallel. However the satisfactory use of such plots of individual variables for the comparison of different preparations would require the use of far greater numbers of animals than were available to us.

We have combined these variables by assigning each animal a graded score based both on the day of onset and the severity of the histological lesion (Table I). Negative animals receive a score of zero. The day of onset is included in the score essentially as $100/d$ since the values $d = 10, 12, 15, 20$, and 30 correspond to equal intervals of $100/d$ (no animals had onsets of disease before 9 days or after 30). This score conforms to the usual statistical requirements of linearity, when plotted against log dose, and roughly equal variability at various dose levels. It has the disadvantage of being asymmetrically distributed at high or low response levels (for a recent detailed explanation of the characteristics of metameters of this type, see Bliss (2)). It weights the variables more or less equally and has the merit of sim-

TABLE I

Graded Scores Assigned to Individual Rabbits with EAE

Histologic severity of EAE*	Day of onset			
	< 12	12–15	16–20	> 20 or unknown
+	4	3	2	1
++	5	4	3	2
+++	6	5	4	3

* Rabbits with questionable histologic lesions or with definite signs of EAE without histologic findings assigned a score of 1. Rabbits without disease scored as 0.
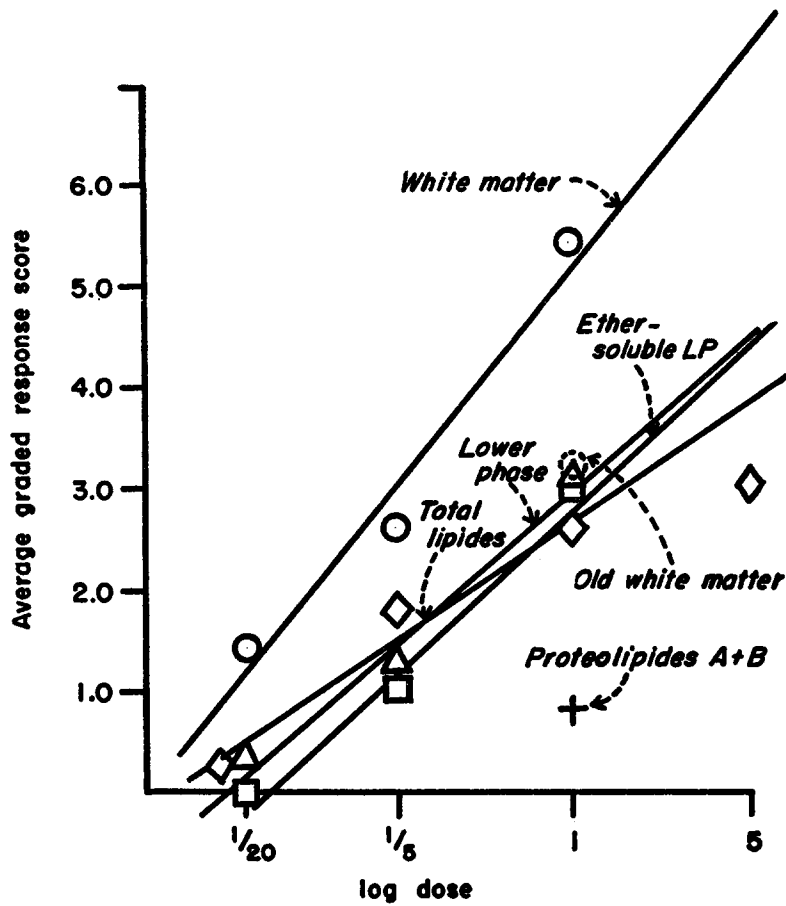

FIG. 1. Regression lines comparing the graded scores of rabbits receiving various antigenically active materials: O, fresh white matter; broken circle, "aged" white matter; ◇, total lipides and proteolipides; △, lower phase; and □, ether soluble lower phase.

plicity of calculation. Values of the score for individual rabbits are plotted against the log dose and a regression line is drawn by the method of least squares. The average score for a group of rabbits injected with the same dose of a given material is a convenient measure of the response of that group of animals.

This scoring technique was applied to the data summarized in Table III above. The average scores are included in the last column of that table. The regression lines calculated from individual values are shown in Fig. 1. For the sake of clarity in this figure, only single points are plotted corresponding to the average values for each dose level of each material. Direct visual examination of the lines brings out clearly the qualitative difference already noted between fresh white matter on the one hand and old white matter and the various fractions on the other, as seen in both the slope and the asymptotic maximum reached by the response. On the basis of our earlier consideration of the effects of fractionation on day of onset and severity as contrasted with incidence of disease, we must withhold judgement whether the difference in position of the lines results from a qualitative alteration of the antigen or represents a real difference in the amount of antigen. The fact that injection of 5 times the stand-

TABLE II

Relative Antigenic Activities of Major Active Fractions Calculated by the Graded Score Method

Preparation	No. of rabbits	M ($\pm S_M$) expressed as per cent of fresh white matter
1. Fresh white matter...........................	21	100
2. Old white matter*...........................	10	25
3. Total lipides and proteolipides‡...............	19	17.0 (7.4–38.9)
4. Lower phase.................................	17	26.7 (13.6–52.5)
5. Ether-soluble lower phase.....................	21	21.4 (11.6–39.7)
6. 3, 4, and 5 combined.........................	57	17.3 (10.2–29.7)
7. Proteolipides A and B*.......................	12	4

* Estimated by inspection from single point.

‡ Omitting dose at 5 times standard level.

ard dose of total lipide extract does not result in an increase of response towards that obtained with fresh white matter suggests strongly that a qualitative difference exists. The single point obtained with old white matter at the standard level falls on the lines obtained for the fractionated material.

These observations can best be summarized by the calculation for each fraction of its relative potency and the standard error of this estimate (Table II). Such calculation is permissible since the slopes of the various regression lines do not differ significantly. It appears from the table that the chloroform-methanol extraction results in an apparent loss of about 80 per cent of the antigenic activity of fresh white matter. The point for old white matter is not statistically distinguishable from the corresponding point obtained with total lipide extract. The lower phase and ether-soluble lower phase have essentially the same activity as the total lipide extract. Proteolipides A and B, on the other hand, appear to have only a small proportion of this activity, approximately one-fifth. The magnitude of S_M is such that estimated differences in antigen content or less than three- or four-fold would not be significant. The value of S_M is basically determined by the variability of the rabbits used in these experiments and could be improved either by using a considerably larger number of animals or by selecting them as randomized groups (2).

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