

THE APPLICATION OF TISSUE CULTURE TO THE STUDY OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

II. SERUM FACTORS RESPONSIBLE FOR DEMYELINATION*

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Myelinated cultures of rat and mouse cerebellum undergo a specific and characteristic pattern of demyelination in the presence of sera from animals with experimental allergic encephalomyelitis (EAE) (1, 2). The neuroglia swell; myelin sheaths become disfigured by fusiform swellings and break away as small fragments; and finally, remaining intact sheaths and myelin fragments are reduced to small fat droplets. During this process neuron somas and axons appear little affected (Figs. 1 to 4).

This communication will present evidence for an immunological mechanism responsible for the *in vitro* demyelination.

Methods

The techniques of rat cerebellar tissue culture, induction of EAE in animals, and the preparation of sera have been previously described (1). An additional technique was employed to develop a series of animals with varying incidence of clinical and pathological lesions and varying levels of circulating antibodies. Each of 10 rabbits received 3 intraperitoneal inoculations of bovine cord (250 mg each) over a 3 month period: 5 animals with cord suspension in Freund's adjuvant; and the remaining 5, with cord in saline. Four of the 5 animals inoculated with cord in adjuvant developed hindleg weakness following the third inoculation, which progressed over 2 to 4 weeks to complete paraplegia, urinary and fecal incontinence, and death. At autopsy, 4 months after the first inoculation, characteristic lesions of EAE were found. Two of the remaining animals, *i.e.* without adjuvant, developed hindleg weakness and ataxia following the third inoculation. However, at autopsy, 4 months after the first inoculation and 2 weeks after serum examination and the appearance of symptoms, no microscopic nervous system changes were noted.

Preparation of Serum Fractions.—The globulins were prepared by sodium sulfate or am-

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monium sulfate fractionation of whole sera. Both globulin and albumin fractions were dialyzed against 0.01 M phosphate-buffered saline, pH 7.2, and passed through a Millipore filter. Gamma 2 globulin (comparable to the 7S fraction) was prepared from the active sera of 3 rabbits with known EAE by Dr. S. Kochwa of the Department of Hematology of Mount Sinai Hospital, using a DEAE cellulose column and elution with 0.02 M phosphate buffer (pH 6.3) (3). The remaining serum proteins were eluted with 1 M NaCl. Both fractions were made isotonic and passed through a Millipore filter.

Sera were heated to 56°C for 20 to 30 minutes to inactivate the C'1 and C'2, and diminish the activity of the C'3 and C'4 components of complement (4). Other EAE sera were incubated with 0.15 N NH₄OH at 37°C for 1½ hours and neutralized with 0.15 N HCl to inactivate the C'4 component of complement (4).

Exposure of Sera to Homologous and Heterologous Tissue.—Sera were exposed to homologous and heterologous tissues as indicated in Table I. Rabbit and rat brain suspensions were prepared (75, 37, and 10 mg/cc) and incubated with 1.0 cc of EAE serum for 12 hours at 37°C and centrifuged at 8000 RPM at 4°C for 2 hours. All control tissues, *i.e.* RBC, lung, liver, kidney, were treated in a similar manner. Rabbit and rat RBC were collected in a heparin-rinsed syringe, washed with buffered saline and diluted to a final concentration of 1,000,000 cells per

TABLE I
Exposure of EAE Sera to Homologous and Heterologous Tissue

Tissue	Species	Sera	No. cultures demyelinating
1. RBC	Rabbit, rat	Rabbit	6/6
2. Lung	Rabbit, rat	Rabbit	6/6
3. Liver	Rabbit, rat	Rabbit	3/3
4. Kidney	Rat, guinea pig	Rabbit	12/12
5. Brain	Rabbit, rat, bovine cord	Rabbit	0/12

mm (3). One cc EAE serum or pooled EAE globulin was exposed to 1 cc of cells. Rabbit and rat lung were removed under sterile conditions and homogenized in buffered saline (final concentration 100 mg/cc). Suspensions containing 100 and 50 mg were exposed to 1 cc of EAE serum. Freshly homogenized as well as acetone-dried liver (150 mg/cc former; and 100 mg/cc latter) were exposed to 1 cc of EAE serum. Rat and guinea pig kidneys were homogenized in buffered saline (250 mg/cc) and exposed to 1 cc of EAE serum or globulin. Polylysine (1 mg) (kindly supplied by Dr. Stephen Kornguth) was added to 1 cc of EAE serum, incubated at 37°C for 12 hours, and centrifuged at 7000 g at 4°C for 2 hours.

Application of Sera and Protein Fractions to Cultures.—All normal and EAE sera were tested for sterility and discarded if contaminated. Globulin and albumin fractions were passed through a Millipore filter. All specimens were stored frozen. For testing they were added to aliquots of the usual feeding solution in concentrations ranging from 5 to 40 per cent. Where the sera had been fractionated or exposed to tissue, the specimens were tested with and without normal fresh serum as a source of complement. The various test and control mixtures were used to feed established, randomly distributed, myelinated sister cultures of rat cerebellum. The cultures were subsequently observed for periods of time ranging from 6 hours to more than 1 month. After a period of observation, they were either washed in balanced salt solution and returned to normal nutrient or were fixed for staining.

Fluorescent Antibody Studies.—Following exposure to various sera, cultures were washed

in 0.01 M phosphate-buffered saline for 15 minutes. They were fixed for 30 seconds in 95 per cent alcohol or for 10 minutes in cold acetone. Occasionally they were used unfixed. They were then overlaid with duck anti-rabbit globulin globulin, duck anti-rabbit albumin globulin (supplied by Dr. Konrad Hsu), or goat anti-rabbit globulin globulin (lot 409, Progressive Lab., Baltimore) conjugated with fluorescein isothiocyanate. As a control, demyelinating cultures were initially exposed to unlabeled duck anti-rabbit globulin globulin and subsequently to labeled anti-globulin. All such cultures were examined with a Leitz fluorescence microscope and photographed on Ektachrome or royal X pan film exposed for 3 to 5 minutes.

Frozen Section Incubation.—Normal rabbit and rat spinal cord, brain stem, and cerebellum were quick frozen in isopentane and dry ice, sectioned on a microtome at -20°C at 10 to 20 microns, and thawed at room temperature. The sections were then incubated with normal and known EAE-demyelinating sera in 50 per cent concentrations in normal feeding solution for 12 to 18 hours at 35°C . Sections were then washed in buffered saline, fixed in buffered formalin, and stained with the luxol-fast blue stain.

RESULTS

In addition to the sera of 12 rabbits tested on 366 cultures previously reported (1), the sera of 2 rats, 8 mice, and 15 guinea pigs were tested on approximately 200 myelinated cultures of rat or mouse cerebellum. The 2 rats had been inoculated with rat spinal cord and, following the appearance of symptoms, the sera were removed and applied to myelinated cultures of rat cerebellum. The previously described pattern of demyelination was observed when either homologous or heterologous systems were employed (1). The spinal fluid of 1 rabbit with EAE was also tested and produced the specific pattern of demyelination associated with the EAE sera.

The sera of 12 additional rabbits were tested on 193 cultures (Table II). The sera of all animals with lesions and clinical symptoms of EAE and of 2 animals with clinical but no pathological evidence of the disease, possessed myelinotoxic factors. Four animals without symptoms or lesions produced no demyelinating substances.

Heating the sera to 56°C for 20 to 30 minutes or treating with NH_4OH with subsequent neutralization removed all myelinotoxic activity. In all cases, activity was completely restored by the addition of normal fresh serum as a source of complement.

In the presence of complement, the salt-precipitated globulin fractions of EAE sera demonstrated the myelinotoxic activity of the entire serum, whereas the albumin and alpha globulin fractions were entirely negative. In the absence of complement, the globulin was inactive. The myelinotoxic activity of the DEAE column eluents was entirely in the gamma 2 globulin (7S) fraction, while the remaining fractions were inactive. By dilution with buffered saline, it was determined that *in vitro* demyelination could occur within 24 hours with less than 1 microgram of gamma 2 globulin.

Exposure of EAE sera or globulin to rabbit or rat brain homogenates according to the technique mentioned above completely removed the myelino-

toxic activity. When conditions of exposure were changed, such as reducing the time of exposure to 1 hour and centrifuging at lower speeds, the demyelinating activity was not appreciably lowered. Exposure to homologous or heterologous RBC, liver, lung, and kidney for greater than 6 hours did not remove any of the specific myelinotoxic activity. In fact, on exposure to rat liver it was felt that the onset of demyelination appeared slightly earlier than in unabsorbed active EAE sera.

TABLE II
Correlation of EAE and in Vitro Demyelination

Rabbit	Clinical symptoms	Path. lesions	No. of cultures which demyelinate with serum	
			Preinoculation	Postinoculation
Group I. Rabbits inoculated with bovine cord without adjuvant				
1. 2-79	0	0	0/3	0/6
2. 2-89	+	0	0/3	3/3
2. 2-97	+	0	0/3	6/6
4. 2-98	0	0	Not tested	0/6
5. 7-36	0	0	0/9	0/9
Group II. Rabbits inoculated with bovine cord with adjuvant				
1. 9	+	+	0/12	24/24
2. 11	+	+	0/4	21/21
3. 2-83	+	+	0/6	24/24
4. 2-87	+	+	0/6	6/6
5. 2-93	0	0	0/3	0/6
6. 2-94	+	+	0/9	15/15
7. 2-95	+	+	0/3	6/6

Addition of polylysine to EAE sera produced an immediate precipitate. However, the myelinotoxic activity in the supernatant was completely preserved. One mg of polylysine in itself produced no morphological alteration of a normal healthy myelinated culture. Thus, *in vitro* there appears to be no affinity of the demyelinating factor for a non-specific basic protein, nor does the protein initiate or facilitate demyelination *in vitro* as it has been demonstrated to do *in vivo* (5).

Whereas heparin alone had no effect on cultures, it interfered with the demyelinating activity of a known positive sera. An experiment was constructed to determine whether the effect of heparin depended on binding to the myelin sheath (perhaps to a postulated basic protein, reference 6) or whether the effect was mediated through extracellular factors; *viz.*, complement. Three

cultures were exposed to 100 units of heparin. Another three cultures were exposed to 100 units of heparin plus 10 per cent EAE serum. At the end of 72 hours, the myelin sheaths in all six cultures appeared unchanged. At this time, the cultures were washed. Those previously exposed to heparin alone were now exposed to EAE serum alone. The three cultures previously exposed to heparin plus EAE serum were now exposed to normal fresh serum. All six cultures demyelinated within 4 to 6 hours. These results are consistent with an action of heparin on complement and suggest that EAE globulin rather than heparin had attached to the myelin sheath.

Normal as well as EAE serum had no effect upon unfixed frozen sections of rabbit and rat CNS. Myelin as well as adjacent glia appeared unchanged as determined by the luxol-fast blue stain. There was no evident swelling, fragmentation, or myelinolysis in the section; whereas such changes were striking with the same sera applied in similar concentration to myelinated cultures of rat cerebellum.

Fluorescent Microscopy.—Approximately 135 cultures were exposed to various sera and then treated with anti-rabbit globulin or anti-rabbit albumin conjugated with fluorescein isothiocyanate. The pattern observed has had the following consistent features:

1. After short term exposure to EAE serum and prior to demyelination, cultures stain positively for globulin on the myelin sheath as well as on the surface of some neuroglial cells (Fig. 5).
2. As fusiform swellings and fragmentation occur during the process of demyelination, the fluorescent antiglobulin appears on these ballooned membranes as well as diffusely throughout the cytoplasm of many clusters of neuroglial cells (Figs. 6 to 9).
3. Cultures which were unaffected after treatment with heat-inactivated EAE rabbit sera also stained positively for globulin on glial surface membranes. Myelin sheath staining was present but was far less intense unless a source of complement had been added.
4. Cultures unaffected by treatment with normal rabbit sera or normal feeding solution demonstrated a pattern of antiglobulin staining which was punctate and sparse. Often no observable staining was present (Fig. 10).
5. All cultures exposed to EAE or normal serum responded to staining for albumin with a similar punctate distribution.

In demyelinated cultures and cultures exposed to heat-inactivated serum, myelin sheath, and neuroglial membrane localization of fluorescent antiglobin could be blocked by prior treatment of the culture with unlabeled duck and anti-rabbit globulin. The punctate activity, however, could not be completely blocked. This may indicate that a portion of this response is due to pinocytosis by incompletely fixed cells or to inadequate washing of the fluorescent-stained cultures. In either case, it represents a non-specific response.

The consistent and specific localization of globulin on the myelin sheath and glial cell membrane during the process of demyelination represents the most conspicuous and characteristic feature of the fluorescent antibody study.

DISCUSSION

Although several patterns of glial, neuronal, and myelin sheath alterations have resulted from the action of different agents on cultured rat and mouse cerebellum (22), the response to serum from animals with EAE remains unique and follows a consistent and predictable morphological pattern. The data from the present experiments suggest an antigen-antibody reaction as the mechanism responsible for this *in vitro* demyelination:

1. The system is complement dependent.
2. In the presence of complement, the serum globulins or, more specifically, their gamma 2 globulin fractions, possess the same activity as the whole serum. The remaining fractions are inactive.
3. Exposure to homologous or heterologous brain removes the total activity, whereas homologous or heterologous non-nervous tissue does not.
4. Fluorescent antibody studies localize the EAE globulin on the myelin sheath and glial cell membranes during the process of demyelination.

No gross morphological alteration occurs in frozen sections of adult rabbit and rat spinal cord incubated with EAE serum although the EAE globulins are localized on the myelin sheaths (8, 9). It appears, therefore, that the EAE effect is not simply due to the action of a myelinotoxic serum enzyme. It would seem, rather, that demyelination of the cultured tissues results from a complex interaction involving antibody, complement and the living myelin sheaths and neuroglia. Whether or not the initial event of *in vitro* demyelination is an antigen-antibody reaction affecting the myelin sheath or the glial cell membrane directly cannot be determined from the fluorescent antibody experiments. Preliminary electron microscopic observations suggest that morphological alterations in neuroglial cytoplasmic constituents may precede those of the myelin sheath (7, 10). Biochemical changes of the myelin sheath might be present, however, and be undetected by the techniques employed.

That gamma 2 globulins and complement in the sera of animals with EAE will demyelinate cultured tissue does not necessarily signify a similar role *in vivo*. Recent theories have emphasized "delayed sensitivity" and cell-bound immune factors as opposed to circulating antibodies as explanations for the etiology of EAE. Support for the "delayed sensitivity" theory has come from three main sources: (a) the reported correlation between skin testing and the incidence and severity of the disease (11); (b) the lack of correlation of incidence and severity of disease with circulating antibodies as measured by complement fixation titers (12); and (c) the passive transfer with cells but not serum (13).

The reported correlation of skin test results with the presence and severity

of allergic encephalomyelitis is obscured by the absence of a purified brain or cord extract which contains the complete biological activity of intact brain tissue (11). As a result a trace antigenic component unrelated to the production of encephalomyelitis could be responsible for skin test activity (14, 15).

A serum which demyelinate tissue culture may have no detectable complement-fixing antibody *versus* brain tissue; and conversely a serum with complement-fixing antibody may have no *in vitro* demyelinating antibody (16, 17). It is thus clear that no correlation exists between complement fixation tests for antibody and the myelinated tissue culture assay for antibody. The two tests may in fact be measuring different antibodies or different amounts of the same antibody. Less than one microgram of EAE gamma 2 globulin is required for *in vitro* demyelination, and this amount may not be detectable by the complement fixation technique. One would then not be surprised that complement-fixing antibody titers do not parallel the severity or even the presence of EAE if the test is not sensitive enough to detect the presence of myelinolytic antibody. Therefore a failure to correlate tests of circulating antibody with the presence and severity of the disease would not exclude circulating antibody in the pathogenesis of experimental allergic encephalomyelitis.

The ability to transfer EAE with cells *in vivo* (13) or *in vitro* (23) certainly represents an important demonstration of the immunological character of the process but does not further define it because of the concomitant transfer of antibody-producing capacity. In fact, one of the tests of viability of the transferred cells within the recipient is the increasing levels of antibody produced (13). The question must then still be raised as to whether antibody can be completely excluded in explaining development of brain lesions in the recipient. Although labeled lymphocytes will localize in the brain at sites of perivascular inflammation (18), the specificity of such localization is still under active investigation. The nucleic acid and antibody constituents of the inoculated cells may act directly to produce the specific defect of EAE, or the macromolecular components may function indirectly as specific transforming factors or inducers of the host lymphoid system. In any event the evidence at hand is inadequate to accept or reject the mediation of antibodies in the ultimate inflammatory and demyelinating lesions *in vivo*.

The failure to transfer EAE *in vivo* with large amounts of serum is a serious objection to the etiological significance of circulating demyelinating antibodies, but need not invalidate an antibody mechanism. Small amounts of a highly specific antibody may not achieve sufficient concentration at the myelin sheath and glial membrane both because of serum dilution and more significantly because of an intact "blood-brain" barrier which prevents direct access to the antigenic site. Myelinated cultures, on the other hand, provide both sensitivity and direct access to the myelin sheath and neuroglia. Alteration of blood-brain permeability does in fact occur in EAE (19), and may represent the means

by which a significant amount of specific antibody or antibody-producing cells gain access to the tissue. This is supported by the demonstration that electrolytic brain lesions (20) and cyanide encephalopathy (21), both of which alter blood-brain permeability, localize EAE lesions in the affected areas. The pathology of experimental allergic encephalomyelitis would then be determined not by a single factor but by several possibly independent factors: one of which may be directed against the vascular endothelium, and others against the nerve tissues.

The sera of all animals with EAE as well as of two animals with symptoms but no demonstrable histological alteration possessed demyelinating antibody. The latter two cases are of extreme interest because they indicate that neurological dysfunction may be associated with minimal or no light microscopic pathology despite biochemical or immunochemical alterations. On the other hand, these data may also indicate that an antibody which results in demyelination *in vitro* may be only one component of a series of factors responsible for the *in vivo* pathology.

The significance of a demyelinating antibody *in vivo* is at present unknown. Yet, because of its specific and consistent biological activity *in vitro*, its possible role must be considered in any explanation of demyelination in animal and man. For demyelination *in vivo* the specific antibody may have to be produced in the brain by infiltrating lymphocytes to be present in sufficient concentration at the myelin sheath and glial membrane. An initial induction of antibody at the lymph follicles by the inoculated antigen might result in low levels of serum antibody rendered ineffectual *in vivo* by dilution and subsequent inability to cross the blood-brain barrier in sufficient concentration. It may be undetectable by techniques such as complement fixation, yet may be detectable by its specific *in vitro* demyelinating activity. Subsequently, an anamnestic response induced in sensitized lymphocytes by antigenic stimulation within the nervous system may exhibit biological activity because of direct access of the antibody to the antigenic site within the neural tissue. Such a theory is in accord with known experimental data and appears testable with available techniques.

SUMMARY

1. In the presence of a source of complement, the gamma 2 globulin fraction of rabbit EAE serum results in complete demyelination of myelinated cultures of rat cerebellum. Exposure of the serum to homologous or heterologous brain specifically removes the myelinotoxic activity whereas exposure to non-nervous tissue does not. Polylysine has no effect upon the cultures or upon the demyelinating potency of EAE, whereas heparin inhibits activity presumably through an effect on complement.

2. With the fluorescent antibody technique, the EAE globulins are specifically localized to the myelin sheaths and glial cell membranes during

the process of demyelination. As demyelination proceeds, the globulins become localized within the neuroglia in a homogeneous manner which contrasts sharply with the punctate pattern observed in control experiments.

3. The "delayed sensitivity" and classical antibody interpretations of experimental allergic encephalomyelitis are discussed. It is suggested that several factors may be responsible for the pathogenesis of EAE, one of which may well be a myelinotoxic antibody.

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EXPLANATION OF PLATES

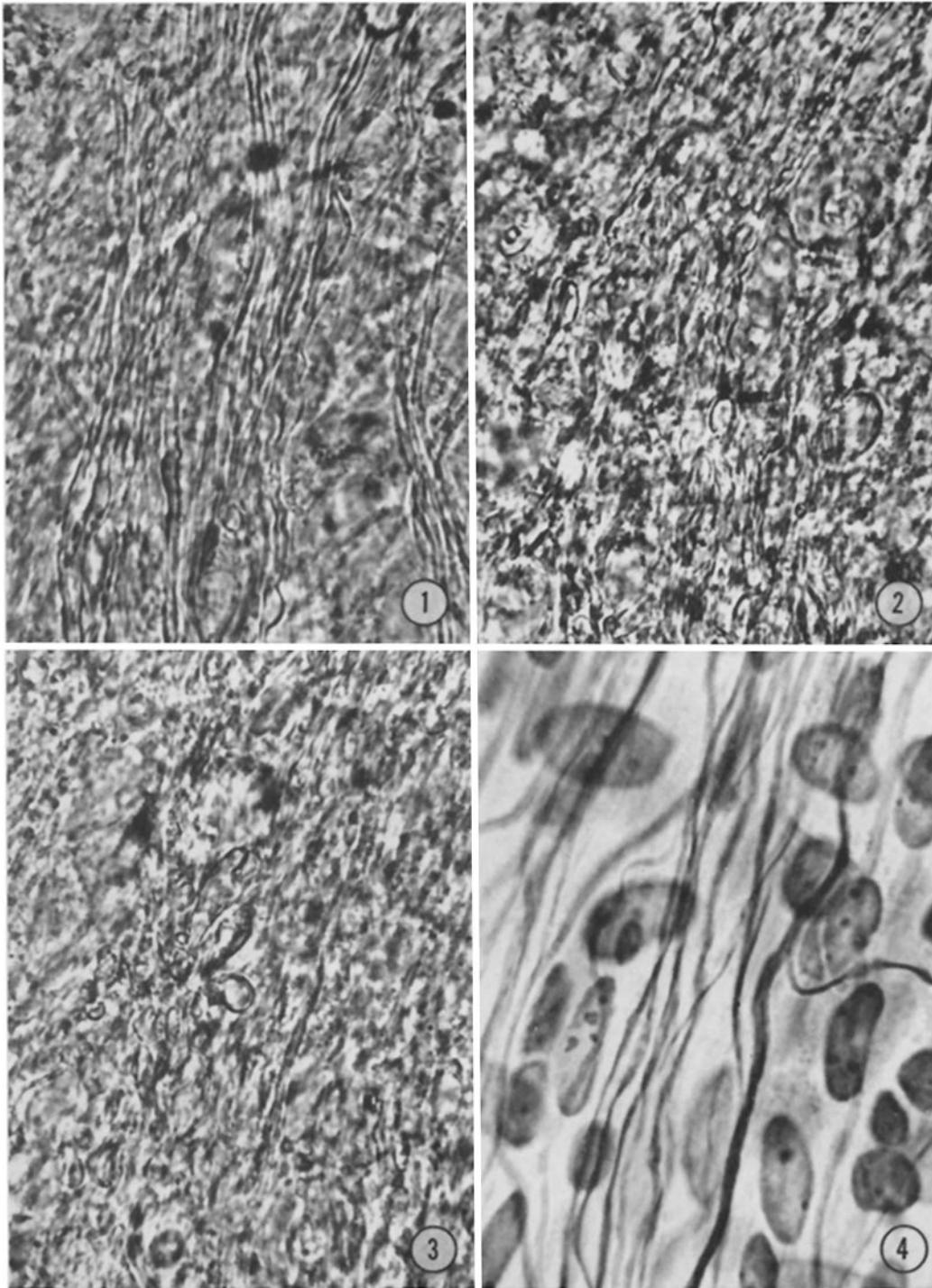
PLATE 24

FIG. 1. Rat cerebellum. 27 days *in vitro*. Appearance of myelinated axons prior to exposure to a 25 per cent concentration of rabbit 14 serum. $\times 450$.

FIG. 2. Same field, 3 hours after exposure to EAE serum. Note the fragmentation of the myelinated axons and the numerous irregularities and slight ballooning of the sheaths. $\times 450$.

FIG. 3. Same field, 5 hours after exposure to EAE serum. By this time there has been complete disruption of the myelinated axons which gave an appearance of linear strands in Fig. 1. The ballooning and fragments of Fig. 2 have disappeared to a large extent. $\times 450$.

FIG. 4. Same field. Bodian silver impregnation of the culture fixed after 5 hours' exposure, indicating the intact axons and soma. $\times 450$.



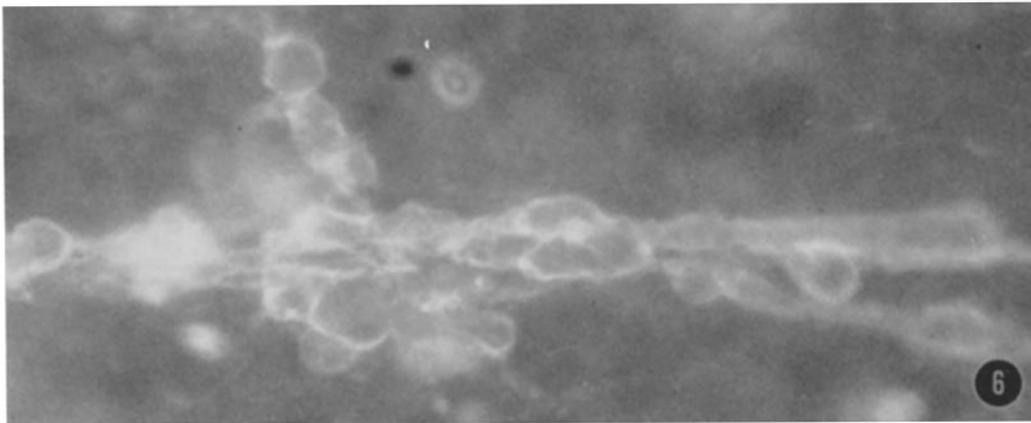
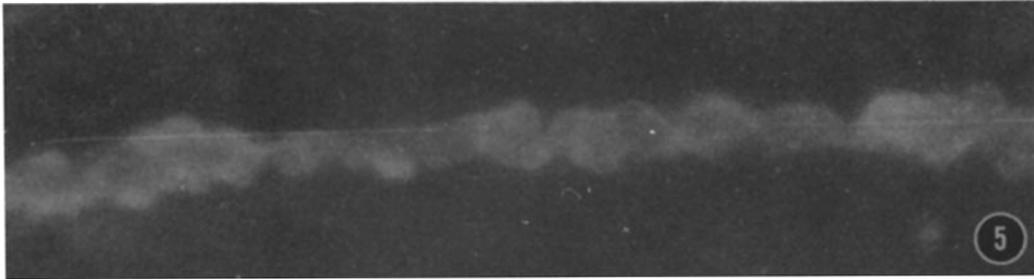
(Appel and Bornstein: Allergic encephalomyelitis. II)

PLATE 25

FIG. 5. Rat cerebellar tissue culture, 2 hours after exposure to rabbit EAE serum (10 per cent). Exposed to duck anti-rabbit globulin labeled with fluorescein isothiocyanate. At this time there was no visible evidence of demyelination. The fluorescent labeling, however, indicates irregularities in the myelin sheath. $\times 1000$.

FIG. 6. Rat cerebellar tissue culture, 3 hours after exposure to 20 per cent rabbit EAE serum, and during a period of visible demyelination. Exposed to duck anti-rabbit globulin conjugated with fluorescein isothiocyanate. Note marked ballooning of myelin sheaths and contiguous neuroglia. $\times 800$.

FIG. 7. Rat cerebellar tissue culture, 3 hours after exposure to 10 per cent rabbit EAE serum, during process of demyelination. Exposed to fluorescent duck anti-rabbit globulin. There is scalloping of myelin sheaths as well as discontinuities in each of the sheaths depicted. $\times 500$.



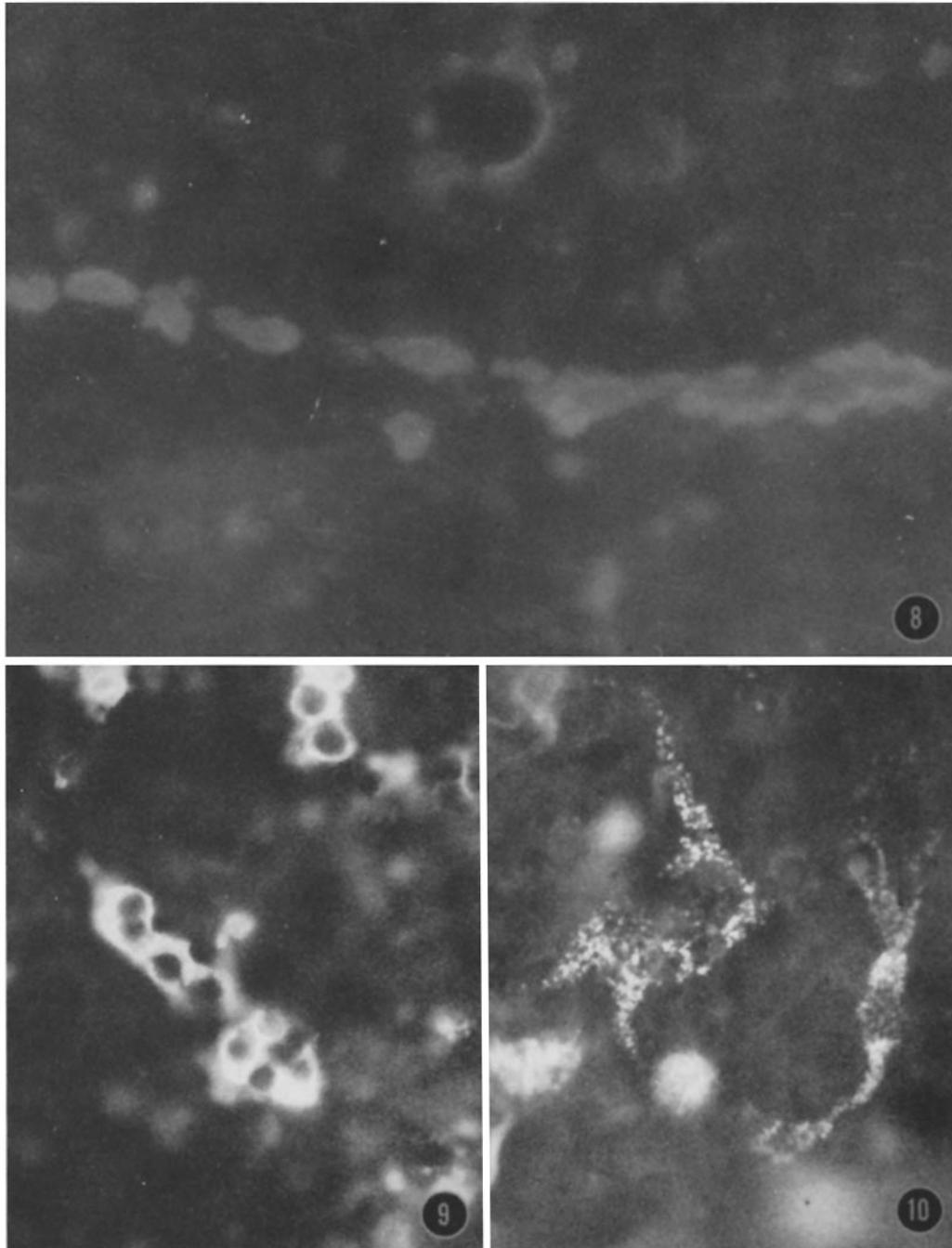
(Appel and Bornstein: Allergic encephalomyelitis. II)

PLATE 26

FIG. 8. Enlargement of a section of Fig. 7 emphasizing the scalloping effect and discontinuity in the affinity for fluorescent-labeled globulin. $\times 1000$.

FIG. 9. Rat cerebellar tissue culture, 5 hours after exposure to rabbit EAE serum (10 per cent). Exposed to fluorescent-labeled duck anti-rabbit globulin. Note the homogeneous fluorescence throughout the cytoplasm of clusters of neuroglial cells. $\times 450$.

FIG. 10. Rat cerebellar tissue culture, 5 hours after exposure to 25 per cent normal rabbit serum. Exposed to duck anti-rabbit globulin labeled with fluorescein isothiocyanate. There is no affinity of the fluorescent-labeled globulin for myelin sheaths or neuroglial membranes. Instead the fluorescence is present in a punctate distribution throughout neuroglial cells, most especially the microglial cells. $\times 450$.



(Appel and Bornstein: Allergic encephalomyelitis. II)