Experimental "allergic" encephalomyelitis (EAE), produced by the injection of brain-containing vaccines, has been studied for many decades (1, 2). In the earliest studies no adjuvants were used, and in the human counterpart which still occurs, rabies post-vaccinal encephalomyelitis, no known adjuvant is involved. In later experiments, even with adjuvants such as heterologous serum, repeated injections over many months resulted in disease which was quite irregular in incidence and severity (1, 2). The histologic changes with characteristic demyelination were best demonstrated in the central nervous system of monkeys by Rivers et al. (3, 4), but the long incubation period made further refinements in the definition of etiology or pathogenesis impractical.

The development by Freund et al. (5–7) of adjuvants capable of regularly inducing delayed sensitivity to a variety of antigens renewed interest in EAE, since it could be readily produced in a high percentage of experimental animals within a few weeks after only one or a few injections (8–16). The effect of these adjuvants, killed mycobacteria in water-in-oil emulsion, is further potentiated by the use of the intracutaneous route (17–20) and, in some species, by a "priming" injection of killed Hemophilus pertussis (19, 20).

Opinions have differed, however, concerning the relative importance of these adjuvants; indeed, some reports have indicated that even the brain need not be included in the encephalitogenic emulsion (21–24). Bell and Paterson (25, 26) have recently reported that adjuvants are not necessary in the rat, whereas...
Lee and Schneider (27) have demonstrated in mice that the relative amounts of brain and adjuvant are of critical importance. Our own results have indicated that both the neural and the adjuvant components are essential for the production of EAE in the guinea pig and that their relative proportions exert profound quantitative effects. Furthermore, although briefly described earlier (28, 29), the fact that relatively large amounts of adjuvant may mask the encephalitogenic potential of only moderate amounts of neural tissue has not been widely recognized in the past and may be of fundamental importance in reconciling some of the discrepancies in the literature.

![Figure 1](image-url)

**Fig. 1.** Severity of EAE produced by various combinations of lyophilized homologous brain and *M. butyricum*. The mean disease index (maximally 9) for each group of five or more guinea pigs is plotted at the coordinates of the weights of each component contained in the 0.1 ml of water-in-oil emulsion injected intracutaneously into each animal over the sternum.

**Methods**

Male guinea pigs of about 500 gm were obtained from two sources: in the experiments performed in Bethesda the closed but not inbred NIH stock of mixed colors was used, whereas in the experiments performed in Houston and Seattle white but not inbred animals were obtained from a single source (May Rabbitry, San Antonio). The animals were maintained under daily observation in the laboratory for 1 or 2 weeks before being challenged with an intracutaneous injection over the sternum of 0.1 ml of a vaccine containing brain in water-in-oil emulsion with killed mycobacteria. The vaccine contained one part water, one part aquaphor, and two parts light mineral oil (bayol F) with a variable concentration of heat-killed, dry *Mycobacterium butyricum* or *tuberculosis* and variable concentrations of guinea pig brain or spinal cord. All weights are expressed as dry weights of mycobacteria, dry weights of lyophilized tissue, or equivalent dry weights of fresh spinal cord (based on a water content of 70 per cent) contained in 0.1 ml of the final vaccine. The vaccines were homogenized, heated to about 60°C, and emulsified by repeated aspiration and ejection through a syringe. Groups of at least five animals received the same vaccine. The animals were observed daily.
for evidence of paralysis or other signs of EAE (30) and killed under nembutal anesthesia by perfusion with 10 per cent formalin when moribund or at the end of the experiment (generally 30 days after challenge). The brain and spinal cord were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Comparison of the signs of EAE in vivo and post mortem allowed the determination of a numerical index of severity of EAE (maximally 10) for each animal and an average for each group (30).

The day of onset of EAE was defined as the day of onset of definite paralysis or as the preceding day if questionable signs had been previously noted. The median day of onset for each group (i.e., the day on which 50 per cent or more of the animals became sick) was not only the easiest to calculate but was also considered the most reliable since it was least influenced by the more delayed cases, which are generally milder and more difficult to define precisely.

![Graph](image)

**Fig. 2.** Severity of EAE (mean disease index) produced by various combinations of lyophilized homologous brain and *M. tuberculosis*.

**RESULTS**

During the past 7 years most of our studies have been concerned with the bio-assay of encephalitogenic fractions of bovine spinal cord and guinea pig brain (31–34). In order to establish reference points for determining the relative activity of the fractions, similar bio-assays have been made on various whole tissues and adjuvants and the results summarized in Figs. 1 and 2. These figures are essentially three-dimensional graphs and indicate, by the numbers plotted at the coordinates of particular combinations of lyophilized guinea pig brain with *M. butyricum* (Fig. 1) or *tuberculosis* (Fig. 2), the average severity of EAE which resulted from intracutaneous injection of a vaccine containing that combination into a group of five or more guinea pigs. (For simplicity, only single digit numbers are used, 9 rather than 10 thus becoming the maximum). The dashed lines separate those combinations which produce EAE
from those which do not. The threshold for brain has not been sharply defined in Fig. 1 (0.03 to 0.1 mg), but in Fig. 2 is seen to be 0.03 to 0.05 mg. The thresholds for the two mycobacteria have also not been sharply defined, but as little as 0.02 mg \textit{M. butyricum} (Fig. 1) can be effective whereas the threshold for \textit{M. tuberculosis} (Fig. 2) is probably closer to 0.1 mg. For both mycobacteria, however, large amounts no longer function effectively as adjuvant with small or moderate amounts of neural tissue.

Fig. 3 summarizes the results with \textit{M. tuberculosis} and fresh guinea pig spinal cord. The dashed line indicates the combinations of spinal cord and adjuvant which will produce half-maximal disease and the solid line the combinations which will produce maximal EAE. The threshold for the spinal cord is just below 0.06 mg, but about ten times as much is required for maximal EAE. As little as 0.01 mg \textit{M. tuberculosis} can serve as adjuvant, but at least five times as much is required for maximal disease. As in the previous experiments large amounts of adjuvant mask the encephalitogenic potential of moderate amounts of spinal cord. Only with large amounts of neural tissue does this masking effect disappear.

A horizontal section through Fig. 3 at the level of 2.5 mg spinal cord is given in Fig. 4, which demonstrates the range of variation in the severity of EAE with variations in the amount of \textit{M. tuberculosis}, the spinal cord concentration being kept constant at 2.5 mg. As might be expected, the greatest variation occurs in the region of the 50 per cent effective dose (ED$_{50}$) of \textit{M. tuberculosis}, which is about 0.01 mg.
Fig. 5 is to be compared with Fig. 4 and gives the variation in median day of onset of paralysis after injection of the challenge vaccine rather than the variation in disease index in these same experiments. With the combination used most frequently (2.5 mg spinal cord and 1.0 mg \textit{M. tuberculosis}) the median day of onset of EAE in groups of five or more guinea pigs was 11 to 13 days in 46 of 56 experiments involving 405 guinea pigs. In only one experiment was the median day of onset delayed beyond 16 days after challenge. Since all
405 animals had histologic evidence of EAE, this vaccine can be considered to contain a 100 per cent effective dose (ED_{100}); indeed, only 25 guinea pigs failed to become paralyzed by the 17th day and only seven remained clinically well until the 30th day. As a result, in 98 per cent of the experiments half or more of the guinea pigs had become paralyzed by the 16th day, and in 77 per cent of the experiments all of the animals were paralyzed by the 19th day (Fig. 6).

Within Figs. 3 to 6 are included results obtained with three different preparations of pooled lots of three strains of *M. tuberculosis* (C, DT, and PN), obtained from three different sources and suspended in oil for varying periods of time up to 3 months or more. Within the limited variations summarized above no significant differences were found in the effectiveness of these different preparations.

**DISCUSSION**

From the results presented in this paper and in the accompanying paper of Lee and Schneider (27), it is apparent that the mouse and guinea pig require

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1 We wish to thank Dr. A. W. Tallman, Supt., Bacteriological Production Department, Lederle Laboratories, Pearl River, New York, and Dr. J. V. Iron, Director, Texas State Department of Health, Austin, for generously providing us with two preparations of steam-killed organisms. Smaller amounts of the same organisms, obtained from the Animal Inspection and Quarantine Division, United States Department of Agriculture, were also prepared under similar conditions in the Department of Microbiology, Baylor University College of Medicine.
mycobacterial adjuvants for the production of EAE, whereas the rat may not
(25, 26). Furthermore, in the mouse (27) and guinea pig, a quantitative adju- 
vant effect can be demonstrated, and definite thresholds can be defined for both the neural and mycobacterial components of the encephalitogenic vaccine.

From the general shape of the boundaries defined in Fig. 3, it would appear 
that there is a minimum amount of encephalitogenic tissue (~0.05 mg) for 
which a rather sharply defined amount of mycobacteria (~0.1 mg) is essential 
for the production of EAE. Smaller or larger amounts of mycobacteria do not 
bring out the encephalitogenic potential of this threshold amount of spinal 
cord. As the amount of encephalitogen is increased, however, the range of 
mycobacterial content which can function as adjuvant broadens considerably 
(0.01 mg to 4 mg). In the mouse (27), on the other hand, there appears to be a 
linear ratio between the amounts of encephalitogen and adjuvant which is 
critical for the production of EAE. Although the two species behave similarly 
with large amounts of adjuvant, they behave differently with small amounts 
of adjuvant. The significance of this discrepancy is not known.

With the volume of vaccine constant at 0.1 ml, it becomes technically im-
possible to increase the amounts of either neural tissue or adjuvant beyond 
those shown in Figs. 1 to 3, so that the upper boundaries, if any, cannot be 
determined. If it were possible to extrapolate from the data in Fig. 3, one would expect that with very large amounts of neural tissue EAE production in the guinea pig might become independent of the presence or amount of mycobacteria, which could then no longer be considered as an adjuvant. If this were correct, one might have an adequate frame of reference for including those cases of EAE produced in man or animals by brain-containing vaccines without adjuvants, especially since such cases generally occur infrequently and following large doses of neural tissue. An alternative speculation might be that the lipids or other non-encephalitogenic components of brain may function as an adjuvant when present in sufficiently large amounts. Conversely, one would not expect the pure encephalitogenic protein to have such adjuvant properties, and hence it should not be encephalitogenic in the absence of adjuvants no matter how much is used.

Such speculations at first suggest a frame of reference for understanding why 
rats may develop EAE without adjuvants: perhaps rats are more susceptible to EAE. The fact that rats require over 30 times (18) as much neural tissue to become paralyzed indicates, however, that rats cannot be considered more susceptible than guinea pigs, and some other explanation must be sought. Levine (35) has recently observed differences in adjuvant effectiveness in different strains of rats: some require mycobacteria and some do not. In either, however, adjuvants increase the incidence of EAE. He has attributed these differences to specific genetic differences, and such an hypothesis gains further support from data with the mouse, only a single strain of which appears to be susceptible to EAE (20). Although perhaps an adequate explanation for differ-
ences between strains, it does not seem quite satisfactory for differences between species.

In view of previous arguments regarding the importance of the day of onset of EAE (36) it may be of interest to record here our belief that it is indeed a useful index of the intensity of EAE, especially useful when one wishes to perform a long series of experiments, each dependent on the results of the previous ones. Within 2 to 3 weeks one can know the results with a high degree of accuracy and reproducibility, without waiting for the final results of histologic and statistical analysis (30). As with most short cuts, however, one must expect somewhat greater variability, such as is shown in comparing Figs. 4 and 5.

In all three species (rat, mouse, and guinea pig) the data are quite consistent in requiring central nervous tissue or specific extracts thereof for the production of EAE. In the guinea pig in particular we cannot give any confirmation for the reports that EAE can be produced without such specific encephalitogens (22–24). One can only suggest that such reports are due to the contamination of the experimental animals with some organism, such as *encephalitozoon cuniculi* (37).

The present experiments provide a base line for the comparison of various adjuvants or encephalitogens. Such comparison may be made in terms of the amount which can produce half-maximal disease, i.e., the 50 per cent effective dose or ED₅₀ (30), but in actual practice the degree of variation in the results of the bio-assay is so great at this dose level that comparisons accurate to within a factor of 5 are very difficult to obtain. Even within this range of error, however, it is of interest that if Freund’s “complete adjuvant” is used as directed (7), 0.1 ml contains only 0.025 mg *M. butyricum*, which is just about the threshold amount shown in Fig. 1 and is almost certainly suboptimal. In another paper (38) Freund and Lipton used ten times this amount of *M. tuberculosis* (0.25 mg), which according to Fig. 3 is within the optimal range.

When one is titrating the adjuvant, one must not only keep the neural component constant, but must also be careful to titrate the adjuvant over a wide enough range to avoid false negative results due to the use of too large an amount of adjuvant. Such a precaution was not used and may invalidate some of the negative results of White and Marshall (39) and Katsh (40) with various types and extracts of mycobacteria. This masking effect may be minimized, if not completely avoided, by using large amounts of encephalitogen, but such amounts are truly massive (2.5 mg spinal cord).

The mechanism of this masking effect of large amounts of mycobacterial adjuvant, demonstrated in both mouse (27) and guinea pig, is not known. Whether the physical state of the various emulsions is truly comparable cannot be stated, but the masking effect does not seem to be due to grossly visible differences in the degree of ulceration and sloughing out of the inoculum. It may be due to toxic factors (41) in the mycobacteria which modify or kill cells
more or less non-specifically, or it may be due to specific competition of various antigens of the mycobacteria for the immunogenic mechanism at the expense of the relatively smaller amounts of neural antigens. Support for this last possibility is given by the fact that the masking occurs with the relatively non-toxic *M. butyricum* as well as with the relatively toxic *M. tuberculosis*. It becomes somewhat difficult, however, to reconcile this masking effect as a competition of antigens with Burnet's (42) theory that sensitized clones arise as spontaneous mutants which are selected out either for proliferation with relatively small doses of an antigen or for destruction by relatively large doses of antigen: one would have to postulate that a significant number of clones spontaneously arise specifically sensitive to antigens of both mycobacteria and brain. From this point of view the phenomenon of competition of antigens fits best with the theory that antigenic determinants actively induce sensitization of particular clones rather than passively select out those which arise spontaneously.

**SUMMARY**

Quantitative relationships between the amounts of encephalitogenic neural tissue and mycobacterial adjuvant are presented for the guinea pig, for comparison with similar results obtained in the mouse and presented in an accompanying paper by Lee and Schneider (27). Definite threshold amounts of both neural encephalitogenic and mycobacterial adjuvant can be defined. With the proper amounts of each component, 100 per cent of guinea pigs can be made to develop EAE, 97 per cent dying of it, and over 50 per cent becoming paralyzed by the 12th day after challenge. With moderate amounts of encephalitogen the severity and incidence of EAE can be very great, but this encephalitogenic potential can be masked if large amounts of mycobacteria are employed. The mechanism of this masking effect by excess adjuvant is not known, but speculation centers upon the possibility of competition of antigens of the mycobacteria at the expense of those of the encephalitogen.

**BIBLIOGRAPHY**


