report occurred among lambs not affected with cyclopia.

Thin layer chromatography of crystalline preparation from benzene extracts(3) of *V. californicum* roots was done on alumina. The mixed crystalline material was obtained by evaporation of nearly all the benzene from the extract *in vacuo* whereupon crystallization occurred. Benzene methanol (6:1 v,v) was used as developer and bromphenol blue-acetone (20 mg/100 ml) followed by 0.5 M phosphate-citrate buffer pH 3.95 as spray reagents to visualize the spots on the thin layer plates. The plates were photographed immediately after spraying, prior to fading.

Results and discussion. The production of the veratramine-induced type malformations by the plant *V. californicum* is described in Table I and Fig. 1-3. The results were in every way similar to those obtained with pure veratramine including the marked improvement or recovery of afflicted animals within a few days to weeks after birth(1).

These results suggested the presence of veratramine in the plant. We had previously isolated veratrosine, the glucoside of veratramine, from this plant(3) further suggesting the likelihood of the presence of the latter in at least precursor concentrations. We sought, therefore, to identify veratramine in mixed crystalline preparations from benzene extracts of the plant roots by thin layer chromatography. Fig. 4 shows 3 such benzene extracts chromatographed with authentic veratramine. All 3 possess spots of identical R*f* with veratramine. It thus appears probable that the veratramine-induced type malformations caused by *C. californicum* root or top may be due to the presence of that alkaloid in the plant.

Summary. Congenital malformations of the type produced by maternal ingestion of the alkaloid veratramine occurred from maternal ingestion of the plant *Veratrum californicum.* A preliminary identification of veratramine in benzene extracts from the plant by thin layer chromatography was achieved.

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Altered Distribution of Lesions After Repeated Passive Transfers of Allergic Encephalomyelitis.* (32475)

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Several investigators have studied the protection against active induction of experimental allergic encephalomyelitis (EAE) afforded by a variety of treatments (reviewed in (1)). It is likely, though not proven, that such treatments act by inhibition of the process of immunization against the neural tissue antigen-adjuvant mixture. There are few studies on the influence of one attack of EAE upon a subsequent attack(1,2). Such studies must take into account effects of the first attack on subsequent active immunization as well as effects on the target organ, the central nervous system itself. The production of EAE by passive transfer of living immunized lymphoid cells(1,3) simplifies this problem. The recipient animal can be subjected to repeated attacks of EAE induced by waves of actively immunized, encephalitogenic cells, each wave derived from fresh and immunologically naive donors. In this way, effects of previous attacks on active

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immunization are eliminated. With this method, we have exposed aspects of lesion distribution, protection and enhancement that are probably determined at the level of the nervous system.

Methods. Passive transfer of EAE was accomplished by transfusion of living, immunized lymph node cells. Donors and recipients were inbred, isohistogenic, 8-10 week old male Lewis rats, obtained from Microbiological Associates Inc., and maintained on Purina Laboratory Chow and tap water. Donors were immunized with guinea pig spinal cord tissue emulsified in Freund's complete adjuvant and injected into the right hind foot pad; immediately thereafter concentrated pertussis vaccine was injected in the dorsum of the same foot. Seven or 8 days later, as soon as some donors developed signs of EAE, lymph nodes draining the sites of inoculation were removed from all donors and processed rapidly into a cell suspension. The suspension was washed twice and injected in a volume of 1 or 2 ml through a 25 gauge needle into the dorsal penile vein of anesthetized recipients. The donor:recipient ratio was 1:1. The 3 experiments reported here required 9 separate transfers, in which cell counts varied from 1.2 to 3.3 $\times$ 10^8 cells per donor. For details of immunization and transfer, see(4).

Recipients were checked daily for signs of EAE: weakness or paralysis of tail and extremities. Seven days after the last transfer (10 days for half of each group in the third experiment), they were killed by exsanguination under ether anesthesia. Tissues were fixed in Bouin's solution. Coronal slices of most of forebrain and all of hindbrain, and longitudinal segments of entire cord were embedded in paraffin, sectioned and stained by hematoxylin-eosin. Slides were randomized and scored for EAE without knowledge of origin. Cord, cerebellum, medulla and forebrain were scored separately and independently of each other to avoid bias. Perivascular inflammation was graded as follows: 1+, up to 4 or 5 lesions in the entire area under study; 2+, several scattered groups of lesions; 3+, many groups of lesions; 4+, lesions in nearly every low-power field.

For electron microscopic examination, rats were anesthetized by an intraperitoneal injection of nembutal. The trachea was cannulated and attached to an automatic respirator. The chest was opened and the central nervous system was fixed by perfusion through the aorta with 5% glutaraldehyde in phosphate buffer. Specimens of spinal cord, cerebellar and cerebral white matter were dissected, post-fixed in Dalton's chrome osmium fixative, infiltrated and embedded in epon(5). Sections were cut with diamond knives, on LKB Ultratome I or Sorvall Porter-Blum MT1 ultramicrotomes and examined with an RCA EMU-3F electron microscope after staining with 2% uranyl acetate in absolute methanol. Stains for myelin (Luxol fast blue) and glia fibers (Holzer) were performed on remaining neural tissue from these animals and several other specimens.

Experimental design (Table I). In the

TABLE I. Effects of Repeated Passive Transfers of EAE.

	Clinical EAE, incidence*				Histologic EAE, score†	
Exp.	Transfer A	Transfer B	Transfer C	Transfer D	Cord	Cerebellum
1	8/8	3/7	—	—	3.1	3.2
	No transfer	8/8	—	—	3.0	1.0
2	6/6	5/6	1/6	—	2.2	2.7
	No transfer	6/6	2/3	—	2.8	3.5
	" "	No transfer	6/6	—	3.3	.8
3	10/13	12/12	7/12	0/12	1.4	1.7
	No transfer	10/10	4/7	0/7	2.6	1.7
	" "	No transfer	8/8	0/7	2.5	2.1
	" "	" "	No transfer	11/11	3.1	.4
	Non-EAE	Non-EAE	Non-EAE	7/8	2.7	.4

* Numerators represent number of rats with weakness or paralysis of limbs or tail. In every case signs had disappeared by the time of the next transfer. Denominators represent total No. of rats. Decreases in denominators in later transfers were caused by deaths.

† Avg. severity of lesions, graded individually from 0 to 4.

first experiment one group of rats was given 2 successive transfers while another group received only the second. In the second experiment, one group was given 3 successive transfers, while other groups received only the last two or last one transfer. In the third experiment, one group was given 4 successive transfers, while other groups received only the last three, two or one transfer. Thus, each experiment recapitulated its predecessor and added an additional stage. At the beginning of each experiment, all rats were selected from a single shipment even though some did not receive a transfer for several weeks. The interval between transfers was 2 weeks. In every instance, clinical signs of EAE from the preceding attack had subsided by the time of the next transfer. This made it possible to detect clinical effects of each successive transfer. Histologic effects could be evaluated of course, only after the last transfers.

Controls. It was important to determine whether the inflammatory lesions observed after multiple transfers were caused only by the last transfer or represented a summation of effects of several transfers. For this purpose, 7, 5 and 10 control animals were included in Exp. 1, 2 and 3, respectively, (not recorded in Table I). They received all the transfers *except* the last. They were sacrificed at the same time as the experimental groups. Histologic examination revealed residual changes (demyelination, gliosis) but there was nearly complete resolution of perivascular inflammation in the brain and cord. Therefore, the histologic scores following repeated transfers represented EAE acquired from the last transfer only, and not a cumulation of lesions from successive earlier transfers.

The third experiment included an additional control group whose first 3 transfers consisted of non-encephalitogenic cells. These cells were derived from lymph nodes of donors stimulated with Freund's adjuvant and pertussis vaccine but without neural antigen. When this group received the fourth transfer (encephalitogenic cells), they developed as much EAE as animals that were left untreated during the first three transfers (Table I). Therefore, changes in incidence and distribution of lesions after multiple attacks of EAE were not caused by any non-specific effects of repeated cell transfers.

Results. Almost every animal developed signs of EAE after its first exposure to encephalitogenic cells (80 of the 84 rats represented in Table I). Second transfers caused clinical signs in only 26 of 42 rats. Clinical signs were much less frequent and less severe following third and fourth transfers (8 of 25 rats and zero of 12 rats, respectively). The signs in these 8 rats were invariably mild, restricted to the tail, and unattended by the limb paralysis observed in these same animals during earlier attacks. In the last experiment, sacrifice of half of each group of rats was delayed 3 days but there was no instance of late onset of disease. Therefore, previous attacks of EAE had induced amelioration and not merely delay in appearance of clinical signs.

Despite clear evidence in each experiment of clinical amelioration following the last of successive transfers, histologic examination revealed a different picture (Table I). In fact, inflammatory lesions of EAE were abundant in the spinal cords of most animals, including many that had exhibited no clinical signs after the last transfer. The 3 groups that had received 2 transfers and one of the groups that had received 3 transfers revealed just as many spinal cord lesions as the groups that had only one attack. A slight decrease in histologic score was found in one group that had received 3 transfers. Definite inhibition of histologic lesions in cord was found only in the group that received 4 transfers.

Even more dramatic controversion of the clinical impression was afforded by examination of the cerebellum. *Every* group of rats that had received more than one transfer had evidence of *enhancement* of disease in the cerebellum (Table I). Cerebellar lesions were relatively mild and infrequent after one transfer. In contrast, the cerebellum was as severely affected as the spinal cord in rats that received more than one transfer.

It is important to emphasize that the increase of cerebellar lesions could not have been a mere cumulation of lesions from suc-

cessive attacks because, in each experiment, the control groups in which the last transfer was omitted had almost complete resolution of perivascular inflammation. However, poorly demarcated zones of partial demyelination and fibrillary gliosis were present in both spinal cord and cerebellum of these animals. In the last experiment, three rats of this control group were examined by electron microscopy. Although these rats had gone through 3 separate attacks of EAE with clinical signs on each occasion, this examination also revealed no evidence of residual inflammation. The presence of gliosis was confirmed, but it was not localized around vessels, nor was there evidence of intrinsic vascular abnormality.

The forebrain had a minimal increase in EAE lesions after multiple transfers in each experiment, but the lesions in this area were so few in number that no significance could be ascribed to these results. No consistent change in the lesions in medulla oblongata was found.

Discussion. Clinical evaluation of the effect of multiple passive transfers was unreliable. The apparent amelioration of clinical signs was contraverted by clear histologic evidence of enhancement of EAE in the cerebellum. This lack of correlation is probably due to the difficulty of detecting neurologic signs of cerebellar dysfunction in rats. Weakness, hypotonia and paralysis, the usual evidences of EAE, probably reflect lesions in spinal cord and brain stem. Even here, however, correlation was poor as many rats without clinical signs had innumerable lesions in cord and medulla. Amelioration or absence of clinical signs of EAE in the presence of abundant histologic lesions has been observed in the past following second attacks of EAE(2), and after protective treatments with unmyelinated central nervous system tissue and adjuvants (2), purified brain fractions(6), or stress(7).

Nevertheless, one cannot disregard the clinical observations, especially because histologic confirmation of inhibition of EAE was obtained in the spinal cord after four consecutive transfers. The failure to detect slight inhibition in the cord after 2 or 3 transfers may represent an inadequacy of histologic scoring: the lesions were too numerous to count, and simple scoring might be too crude to detect small differences in the presence of severe disease. Furthermore, discrepancies between clinical and histologic evaluations have not been the rule in investigations of first attacks of EAE, either in our hands or in other laboratories. There is no doubt that rats recover function after a first attack of EAE before their cord lesions have disappeared; it seems possible that functional recovery involves the utilization of accessory neural pathways and that this adaptive response persists and modifies the clinical effects of subsequent attacks of EAE.

It remains to explain the histologic effects of multiple passive transfers, namely, decreased involvement of spinal cord after the fourth attack and increased involvement of cerebellum after 2, 3 or 4 attacks. For purpose of discussion, we assume that these two phenomena are related. If they are related, it is possible to rule out most systemic factors, such as antibodies with either inhibitory† or facilitatory effects, altered nutritional state, or non-specific stress. Systemic factors are not likely to have opposite effects on spinal cord and cerebellum. It is more logical to suppose that EAE lesions in the cord have a local blocking effect, which increases with each attack until most available sites are rendered immune. Circulating encephalitogenic cells, blocked from access to the cord, are available to cause cerebellar lesions. It may be presumed that the cerebellum itself is subject in its turn to such a blocking effect, but our data have not established this; perhaps more than 4 transfers would be needed. Local blockade in the cord might be related to the gliosis demonstrated by light and electron microscopy. However, the lack of perivascular localization makes the significance of this change un-

† The Lewis rats used in the present study are poor producers of complement fixing antibodies for which a protective role has been hypothesized, and are relatively susceptible to repeated active sensitization with neural tissue and adjuvants(1). The effects of repeated passive transfers might be different in Wistar rats that produce large amounts of protective antibodies and are relatively insusceptible to repeated active sensitization. Unfortunately, Wistar rats are not isohistogenic and therefore not easily used in passive transfer experiments.

certain. Loss of myelin was too slight to cause significant decrease of the encephalitogenic antigen which is presumed to be responsible for the influx of specifically immunized cells. In view of the absence of ultrastructural lesions in vessel walls, the uncertain significance of gliosis, and the minor degree of myelin loss, one may consider local release of a blocking antibody from the infiltrating lymphoid cells within the lesions. Local blockade in the nervous system should be considered in the interpretation of experiments done with active sensitization as well as passive transfer of EAE. If EAE is related to multiple sclerosis, these same considerations might apply to the formation and growth of plaques.

An opposite sequence of events might be conjectured. Minimal attack on the cerebellum in the first attack might be sufficient to break the blood-brain barrier(8), facilitate localization of EAE in cerebellum in subsequent attacks(9) and reduce the number of cells available to attack the cord. However, any mechanism of this type should apply with even more force to the spinal cord where EAE lesions and disruption of blood-brain barrier are greater from the outset, and this vitiates the logic of the argument.

Either view presupposes that the total number of encephalitogenic cells is fixed, and any increase of cells in one area of the nervous system must imply a decrease in some other area. In EAE produced by active sensitization, the evidence is quite the opposite(10): diversion of encephalitogenic cells into the forebrain did not act to spare the spinal cord, probably due to continuous immunization of lymphoid cells by the antigen-adjuvant depot. In the passive transfer system, the absence of a depot and the limited mitotic potential of lymphoid cells removed from their source of stimulation, make the presupposition seem reasonable. We have made two unpublished attempts to prove this concept by causing some of the passively transferred cells to localize preferentially in cyanide lesions in the forebrain(9,4). This maneuver did cause delay and amelioration of clinical signs and decrease of histologic lesions in spinal cord and cerebellum of recipients. However, this effect could not be confirmed in adrenalectomized recipients. Therefore, sparing of cord and cerebellum was largely or entirely an adrenal-mediated stress phenomenon(7) rather than the result of deviation of encephalitogenic cells into the forebrain. The concept of a fixed total number of encephalitogenic cells still seems reasonable but remains to be proved.

Summary. Repeated attacks of experimental allergic encephalomyelitis (EAE) were induced in rats by 2, 3 or 4 consecutive biweekly passive transfers of living, encephalitogenic, lymphoid cells. The incidence and severity of clinical signs of EAE decreased progressively during successive attacks. A decrease in spinal cord lesions was found histologically only after the fourth transfer. Unexpectedly, the cerebellum revealed a marked increase of EAE lesions after the second, third or fourth transfers. This redistribution of lesions may be due to local blockade at the sites of original attack in the spinal cord.

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