9. The HbS was prepared from heterozygous AS blood by DEAE-cellulose chromatography according to the method of R. W. Briehl and S. Ewert [*J. Mol. Biol.* **80**, 445 (1973)]. The HbS was concentrated by Amicon ultrafiltration and dialyzed under N_2 against 0.05*M* bis-tris-HCl, 0.1*M* NaCl, *p*H 7.3. The methemoglobin S content was less than 4 percent. Test tube gelation of deoxy-HbS was conducted by a modified method of R. M. Bookchin, R. L. Nagel, and H. M. Ranney [*J. Biol. Chem.* **242**, 248 (1967)]. To 0.1 ml of an HbS-peptide solution at 0°C under N_2, 5 μl of 10 percent $Na_2S_2O_4$ in 0.05*M* bis-tris-HCl (*p*H 7.2) were added anaerobically. The deoxygenated solution was incubated at 37°C for 30 minutes, followed by transfer to 0°C for 10 minutes and then returned to 37°C for 30 minutes. The MIMR values were based on lack of gelation of HbS during both incubations at 37°C. In the absence of added peptide, controls gelled within 2 minutes at 37°C.
10. The following dipeptides of the L-configuration did not exhibit any inhibitory activity: Arg-Arg, Lys-Ser, Lys-Ala, Gly-Arg, Gly-Gly, Arg-Lys, Lys-Lys, Gly-Asp, Lys-Asp, Arg-Val, and Glu-Lys. (The abbreviations are, respectively, arginine, lysine, serine, alanine, glycine, aspartic acid, valine, and glutamine.) Peptides were prepared from commercially available intermediates by means of the *N*-hydroxysuccinimide ester method of G. W. Anderson, J. E. Zimmerman, and F. M. Callahan [*J. Am. Chem. Soc.* **86**, 1839 (1964)].
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14. The p_{50} (partial pressure of oxygen at which hemoglobin is 50 percent saturated) in millimeters of mercury was determined for HbS solutions in the presence and absence of peptide inhibitors. The HbS concentration was 0.51 m*M* and the solution was in 0.137*M* phosphate buffer (*p*H 7.4), 0.1*M* NaCl, 0.51 m*M* 2,3-diphosphoglycerate. A peptide to HbS mole ratio of 4 was used. The following values (where Suc is succinyl and Phe is phenylalanine) were found: Suc-(L-Phe)$_3$-L-Arg, p_{50} = 26.5, Hill coefficient 2.98; Suc-(L-Phe)$_2$-L-Arg, p_{50} = 23.5, Hill coefficient 2.30; Suc-(D-Phe)$_2$-D-Arg, p_{50} = 24.5, Hill coefficient 2.42; no peptide added, p_{50} = 23.9, Hill coefficient 2.78.
15. The hypotonic exchange of erythrocyte HbA in normal cells for HbS + peptide inhibitor was carried out by a slight modification of the method described by T. R. Whitaker, G. P. Sartiano, L. J. Hamelly, Jr., W. L. Scott, and R. H. Glew [*J. Lab. Clin. Med.* **84**, 879 (1974)]. Peptides labeled with ^{14}C are used in order to determine the amount of the total compound incorporated in the reconstituted erythrocytes (details will be published elsewhere). The final HbS concentration in exchanged cells was 28 ± 2 g/100 ml, as determined spectrophotometrically by the conversion of hemoglobin to cyanmethemoglobin with Drabkin's reagent, a molar extinction coefficient of 1.1×10^4 at 540 nm being used. The amount of HbA present was less than 5 g/100 ml as determined by polyacrylamide gel electrophoresis.
16. Filterability of the resealed erythrocytes prepared by hypotonic exchange loading was determined by initially diluting a 25-μl portion (hematocrit = 90) of the erythrocytes with 2.5 ml of a buffered isotonic solution (0.85 percent NaCl, 10 m*M* glucose, 1 m*M* sodium phosphate, *p*H 7.2). The cell suspension was added to the upper chamber of a small void volume filter assembly. Deoxygenation of the red cell suspension was carried out by water aspiration for 10 minutes at 37°C. The suspension was then filtered through a 3.0-μm Nuclepore filter with a negative pressure differential of 1.5 cm-Hg established by a nitrogen gas atmosphere. The filtered suspension was collected and the percent by recovery weight of cells was measured as well as the cellular hemoglobin concentration.
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Experimental Allergic Encephalomyelitis in Lewis Rats: Chemical Synthesis of Disease-Inducing Determinant

Abstract. *Two amino acid sequences from the same regions of guinea pig and bovine myelin basic protein which induce experimental allergic encephalomyelitis in Lewis rats were synthesized. The sequences of these two regions may be defined by residues 69 to 84 of the bovine basic protein. The encephalitogenic sequence from guinea pig basic protein (peptide S49), H-Gly-Ser-Leu-Pro-Gln-Lys-Ala-Gln-Arg-Pro-Gln-Asp-Glu-Asn-OH, is a much more potent encephalitogen than that of H-Gly-Ser-Leu-Pro-Gln-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn-OH (peptide S8) found in the bovine protein. The primary structures of the two determinants are similar; however, a Gly-His deletion from the guinea pig sequence is noted. Study of the encephalitogenicity of peptide S49, peptide S8, and the parent proteins suggests that the difference in the encephalitogenic potency of the parent proteins in Lewis rats is due to a natural modification in the primary structure of their respective encephalitogenic determinants.*

The myelin basic protein (BP) isolated from the central nervous system (CNS) of a number of species was shown to induce experimental allergic encephalomyelitis (EAE) (*1*), an autoimmune demyelinating disease of the CNS similar in many respects to multiple sclerosis in man (*2*). The BP from guinea pig CNS tissue is a much more potent encephalitogen in Lewis rats than the BP isolated from CNS tissues of bovine, chicken, turtle, and frog (*3, 4*).

The complete amino acid sequences of BP from bovine and human myelin were elucidated (*5, 6*). Physical, chemical, and immunological properties of the BP from human and bovine myelin are similar (*5, 6, 7*) despite 11 amino acid substitutions at various positions along the polypeptide chain and the presence of His-Gly (*8*) sequence in the human BP between residues 10 and 11 (*6*). The residue numbering system reported for the sequence of bovine BP (*5*) is used throughout this report. Of particular relevance to this study is the amino acid sequence of two specific regions from bovine and guinea pig BP, which induces EAE in Lewis rats (*4, 9*). The two regions may be defined by residues 44 to 89 of the bovine sequence. Comparison between the amino acid sequence of the bovine BP and the corresponding region of the guinea pig protein reveals a serine substitution for alanine and proline, at residues 75 and 80, and the deletion of the Gly-His, residues 77 and 78, from the guinea pig sequence (*10*). We now describe the chemical synthesis of two peptides and study their respective encephalitogenicity in Lewis rats. The results show that the difference in the encephalitogenic potency between the two synthetic sequences and the parent proteins may be related to alterations in the amino acid sequence of the corresponding regions of the polypeptide chains.

The EAE assays were carried out in 350- to 400-g Lewis rats (Microbiological Associates). Each male Lewis rat was challenged in the left hind foot pad with 0.1 ml of emulsion containing the desired antigen concentration and 0.1 mg of heat-killed *Mycobacterium butyricum* (Difco). Control rats were similarly challenged with 0.1 ml of emulsion without antigen. Synthetic peptides were prepared from BOC-glycine-resin ester (Schwarz/Mann) by an adaptation of the Merrifield solid phase method (*11*). Details of the synthesis including methods of coupling, cleavage, and peptide purification have been described (*12*). High-voltage electrophoresis and paper chromatography of each purified peptide showed a single spot with ninhydrin stain. Amino acid analysis of each peptide (110°C for 24 hours in 6*N* HCl) gave values close to whole integers of expected amino acids. The synthesis of these peptides was uneventful. The coupling reaction at several amino acid positions along the chain showed the expected residues, indicating complete synthesis. Recovery of synthetic peptides ranged between 40 and 60 percent of the theoretical yield.

Animals challenged with bovine BP, guinea pig BP, or with synthetic peptides developed clinical signs of EAE between day 10 and 22 (Table 1). The development of hind leg paralysis (HLP) and incontinence in challenged rats followed weight loss, lethargy, and weakness of the tail and limb. The development of front limb weakness was observed only in rats with severe HLP. Although these clinical signs did not severely impair the ability of the individually housed animals to reach for food and water, diseased rats continued to lose body weight even after they recovered from HLP. Nevertheless, the majority of these animals recovered from disease.

Table 1. Encephalitogenicity of bovine, guinea pig myelin basic proteins, and synthetic peptides in Lewis rats. Saline solutions of the antigens were emulsified with complete Freund's adjuvant and 0.1 ml of emulsion containing the desired antigen concentration in addition to 0.1 mg of *M. butyricum*. The amino acid sequence of the bovine BP which contains the sequence of peptide S8 has been reported (*6*). Also, the sequence of a region of the guinea pig protein containing the sequence of peptide S49 has been reported (*10*). The residue numbering system of the bovine BP (*6*) is used and deleted residues are designated by —. The number of animals with hind leg paralysis (HLP) are listed separately from those developing weakness of tail and limb without HLP. The total clinical incidence is the summation of those with HLP and those with weakness.

Challenging antigen	Dose (μg)	Day of onset		Weakness of tail and limb	HLP incidence	Total clinical incidence	Mortality
		Weakness	HLP				
Bovine BP	50	10–16	11	5/6	1/6	6/6	2/6
Guinea pig BP	50	10	10	–	8/8	8/8	1/8
Peptide S8 (69–84)	100	18–22	21–26	3/6	3/6	6/6	1/6
	50	20–22	21–26	2/6	4/6	6/6	0/6
Peptide S49	25	11	13–15	–	6/6	6/6	2/6
	10	11	11–16	2/6	4/6	6/6	3/6
	2.5	11	12–25	3/6	3/6	6/6	4/6
M. butyricum	100			0/6	0/6	0/6	0/6

The encephalitogenic potency of synthetic peptides S8 and S49 is demonstrated in Table 1. There was no difference in the total clinical score between the proteins and synthetic peptides. Regardless of the species from which the proteins or the sequences of the peptides originated, 100 percent of the animals developed signs of disease at the doses tested; however, the day of onset and the severity of the clinical signs of disease varied with the origin and the dose of the respective antigens. A single challenging dose of 50 μg of the guinea pig BP induced HLP and incontinence in eight of eight rats compared with one of six rats which developed HLP in the same dose of the bovine BP. The day of onset of clinical signs of EAE in rats challenged with bovine BP ranged between day 10 and day 16. In contrast, all animals challenged with guinea pig BP developed tail and limb weakness, including HLP on day 10 after challenge.

The development of EAE in rats challenged with peptides S49 and S8 followed a similar course observed for BP-challenged rats. The onset of clinical signs of EAE in rats challenged with peptide S49 occurred on day 11 with a range of 11 to 16 days for the development of HLP. In addition, one of the rats challenged with 2.5 μg of peptide S49 developed HLP on day 25. The number of animals developing HLP increased with increasing doses of peptide S49. At the highest concentration tested (25 μg) peptide S49 induced HLP in six of six rats. Doses of 10 and 2.5 μg induced HLP in four and three out of six rats per group, respectively. The appearance of clinical signs of EAE was delayed and was less severe in rats challenged with peptide S8. At 100 and 50 μg, the onset of clinical signs including HLP ranged between 18 and 26 days. On the basis of the incidence of HLP, peptide S8 was less potent than peptide S49. Challenging doses of 50 and 100 μg of peptide S8 were necessary to induce HLP in the same number of animals observed with 2.5- and 10-μg doses of peptide S49. The development of HLP in animals challenged with peptide S8 was not accompanied by incontinence and did not lead to a high mortality observed for lower doses of peptide S49.

The encephalitogenic activity of the myelin BP and its ability to induce delayed type hypersensitivity in experimental animals have been related to the primary structure of specific peptide regions of the polypeptide chain (*12*). Deletion or substitution of specific residues from the linear sequences of these regions destroyed the encephalitogenic activity of the corresponding peptides (*13, 14*). In contrast, deletion of Gly-His, residues 77 and 78, from the bovine peptide S8 gave rise to the guinea pig sequence, peptide S49. Such deletion did not destroy or reduce the encephalitogenic potency of the resulting sequence but enhanced it.

The EAE response of the Lewis rat varied with the species from which the BP was derived. These findings confirm previously published results (*4, 9*) and further show that the difference in the encephalitogenic potency between the two proteins is not related to the alanine substituted for serine (residue 75) or for proline (residue 80) but to deletion of the Gly-His from the bovine sequence. This conclusion is supported by the data presented and defines the encephalitogenic sequence from both proteins responsible for EAE in Lewis rats. The difference in the encephalitogenic potency between the bovine and guinea pig proteins is demonstrated by a similar difference in the potency of peptides S8 and S49, respectively. Comparison of the sequences of the two peptides revealed several interesting features in common with the respective parent molecules. Both peptides are encephalitogenic and contain a Gln-Lys at position 73 and 74, previously shown to be essential not only for the encephalitogenic activity of the H-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-OH sequence in guinea pigs (*12, 13, 15*) and H-Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-OH sequence in rabbits (*16*), but also for the induction of delayed type skin hypersensitivity in both species (*12*). The sequence of peptide S8, H-Gly-Ser-Leu-Pro-Gln-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn-OH, contains a Gln-Lys at position 5 and 6 from the N-terminal end. Deletion of Gly-His from the peptide S8 brought the Gln-Lys into proximity to the Gln-Arg sequence and gave rise to a more potent encephalitogen found in peptide S49, H-Gly-Ser-Leu-Pro-Gln-Lys-Ala-Gln-Arg-Pro-Gln-Asp-Glu-Asn-OH.

In a study of synthetic polypeptides, Gill and Doty (*17*) proposed that the immunologic reactive sites may consist of short amino acid sequences found in the nonhelical regions of the polypeptide chain; others concluded that the size of the combining site of poly-L-lysine with antibody could be represented by a penta- to hexapeptide range (*18*). In this regard, the encephalitogenic determinant for the Lewis rat might be defined by a five- to six-residue sequence around the Gln-Lys or the Gln-Arg. Deletion of the Gly-His which separated the Gln-Arg gave rise to a Gln-Lys-Ala-Gln-Arg sequence and enhanced cellular recognition of the resulting antigen. Another glycine and histidine residue deletion was reported for the bovine BP when the sequence of the latter was compared to that of the human BP. Although the relevance and significance of this deletion from the bovine BP and its insertion between residue 10 and 11 of the human BP to the encephalitogenic potency and species susceptibility of the respective protein is not readily recognized, it is important to recognize that the insertion of His-Gly in the human BP did not alter the position or the proximity of Gln-Lys and Gln-Arg found in the NH_2-terminal region of the human BP, NH_2-Ac-Ala-Ser-Ala-Gln-Lys-Arg-Pro-Ser-Gln-Arg-His-Gly-Ser-Lys-Tyr-. In the human BP sequence, the Ala-Ser-Ala-Gln-Lys se-

quence has been shown to induce delayed type hypersensitivity (*12*) unaccompanied by clinical or histological signs of EAE; however, the encephalitogenic potency and species susceptibility to this region of the myelin BP need further definition.

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Prelytic Damage of Red Cells in Filtrates from Peroxidizing Microsomes

Abstract. *When liver microsomes are incubated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), their constituent lipids undergo peroxidative degeneration. If erythrocytes are present in such a peroxidizing system, they hemolyze. Filtrates obtained by ultrafiltration of peroxidizing microsomal systems were found to have the capacity to produce prelytic damage in red cells. Filtrates obtained from microsomes that had not undergone peroxidative lipid decomposition were inert. The toxic activity in the active filtrates was not due to continuing oxidation of NADPH nor to continuing liver microsomal lipid peroxidation. Neither the chemical identity of the toxic product or products in active filtrates nor the mechanisms involved in the erythrocyte damage are known at this time.*

Peroxidation of lipids in biological membranes is an abnormal and destructive phenomenon. It has been implicated in a variety of pathophysiological conditions that often, although not always, arise from toxigenic sources (*1–10*). If one assumes that lipid peroxidation is a critical vector in a particular condition, the question remains as to the exact mechanisms whereby the pathological involvement of the structure and function of the cell as a whole can result from a process that, at least initially, occurs at a circumscribed locus. In other words, if lipid peroxidation is induced at one location within a cell, for example, the cytochrome P450 locus of the endoplasmic reticulum, what are the critical events which eventually result in destructive manifestations at other locations? One possibility is that toxic metabolites arising from a particular site have the capacity of inducing pathological effects elsewhere in the cell. We use erythrocytes as a biological device to give evidence that such toxic metabolites of lipid peroxidation most likely exist.

When liver microsomes are incubated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), without any exogenous electron accepting substrate, lipid peroxidation occurs (*11*). It has been reported that when erythrocytes are added to a peroxidizing microsomal mixture they hemolyze (*12*). Given the generally deleterious effects with which lipid peroxidation is associated, it is possible that the agent responsible for the red cell lysis is a product of this microsomal peroxidation. Supportive of this idea would be the recent finding that erythrocytes incubated in the presence of linoleic hydroperoxide undergo hemolysis (*13*). However, when this phenomenon was previously investigated, the conclusion was reached that microsomal lipid peroxidation was in no way responsible for the erythrocytic hemolysis (*14*). Rather, it was concluded that a free radical species (most likely hydroxyl radical) emerged from the microsomal electron transport system and directly induced the hemolysis. On reinvestigating this phenomenon we have obtained evidence that microsomal lipid peroxidation plays a more critical role in the hemolysis than previous investigators suspected.

Among the experiments that suggest that microsomal lipid peroxidation may be the decisive event expressed ultimately in the erythrocytic damage are the following. When malonic dialdehyde (MDA) production and hemolysis are monitored in the same incubation mixture, MDA appears well before the red cells lyse (Fig. 1). Since the appearance of MDA is used to infer that microsomal lipid peroxidation has occurred, this particular sequence of events is consistent with the idea that some product arising from the peroxidation process in the microsomal membrane is responsible for the subsequently occurring erythrocytic damage. MDA is most certainly not the hemolytic agent since, when it is added to erythrocytes at concentrations one hundred times in excess of what is observed in Fig. 1, no hemolysis can be detected.

Furthermore, no hemolysis occurs when red cells are incubated in the presence of microsomes, NADPH, and the terminal substrate aminopyrine. Active electron transport occurs in this system as evidenced by production of formaldehyde; however, lipid peroxidation does not occur in presence of the substrate. Thus, mixed function oxidase activity per se is insufficient to induce erythrocytic damage; rather, peroxidation of microsomal lipids seems to be the requisite precondition for subsequently occurring red cell hemolysis.

These experiments led us to carry out much more critical experiments. These experiments have demonstrated that filtrates derived from peroxidizing microsomes retain the capacity to produce prelytic damage in red cells. This phase of our work was facilitated by use of microsomes recovered from calcium-rich media (*15*). These aggregated calcium