Table 1. Proportion of cells containing hemoglobins S, C, and F in individuals heterozygous for both HbS and HbC alleles (HbSC disease).

Person	HbF percent	Percentage* of erythrocytes labeled by fluorescent		
		Anti-HbF	Anti-HbS†	Anti-HbC†
J. M.	4.0	18.9	100.6	100.3
R. L.	0.7	4.1	99.1	100.5
J. L.	1.2	5.3	99.7	99.4
S. W.	2.5	16.1	99.4	100.1
D. W.	1.4	4.4	100.3	99.7
P. K.	6.9	35.2	99.6	99.6
M. D.	2.9	12.9	100.9	100.2
W. F.	0.7	2.3	100.5	99.1

*Estimated by counting an average of 5000 (6). †Deviations from 100.0 reflect the expected counting error.

were raised (*4*), the hypothesis can be tested by immunochemical methods for the identification of specific hemoglobins in single erythrocytes. We tested the hypothesis in eight HbS/HbC heterozygotes. In these individuals, variable proportions of red cells contain HbF (F cells) (*5*). Blood smears were reacted with antibody to HbS, antibody to HbC, or antibody to HbF, each conjugated to fluorescein isothiocyanate (FITC) (*4*). Examination of the labeled preparations under white and fluorescent light indicated that all red cells from the eight compound heterozygotes were labeled with FITC-conjugated antibodies to HbS (anti-HbS-FITC) (Fig. 1A); similarly, all red cells were labeled with FITC-conjugated antibodies to HbC (anti-HbC-FITC) (Fig. 1B). Labeling with anti-HbF-FITC (Fig. 1C) disclosed from 2.3 to 35.2 percent HbF-containing red cells (F cells) in the eight subjects (Table 1). Double labeling experiments utilizing anti-HbF-FITC and tetramethyl rhodamine isothiocyanate–conjugated antibodies to HbS or antibodies to HbC demonstrated coexistence of HbS and HbC in the red cells containing HbF. These results thus show that both HbS and HbC are present in the F cells, and that the cells containing fetal hemoglobin derive from nucleated precursors that carry a minimum of three (that is, γ, β^S, β^C) active non-α chain genes. Obviously, at least one γ-chain gene and the β-chain gene that is in *cis* position to it can be synchronously active.

It seems thus that globin gene excision cannot account for the regulation of fetal hemoglobin synthesis and for the patterns of ontogenesis of human hemoglobins; more likely, a situation like that predicted by a "leaky model" (*2*) of hemoglobin regulation prevails during the course of human development; that is, during the transition from, for example, fetal to adult hemoglobin synthesis, the repression of γ-chain synthesis is not necessarily complete in any given maturing cell. Were the repression of γ-chain synthesis to remain incomplete in later life, the "turning on" of γ genes might be a conceivable approach to therapy for the patient with sickle-cell anemia or homozygous β thalassemia; this possibility would be denied were the expression of γ- and β-globin gene activity regulated by gene excision through intracistronic crossing over.

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3. ———, *Ann. N.Y. Acad. Sci.* **241**, 119 (1974). Kabat subjected this prediction to testing using cord blood from HbA/HbS heterozygotes. The methods he used for identification of HbF, HbS, and adult hemoglobin (HbA or HbS) in single cells were based on differences in solubilities of these hemoglobins. The proportions of cells staining for hemoglobin after application of each method were in close agreement with those expected by the mutually exclusive model; however, the lack of sensitivity and specificity of the techniques employed render the results inconclusive.
4. Antibodies against HbF [W. G. Wood, G. Stamatoyannopoulos, G. Lim, P. E. Nute, *Blood* **46**, 671 (1975)], HbS [Th. Papayannopoulou, T. C. McGuire, G. Lim, E. Garzel, P. E. Nute, G. Stamatoyannopoulos, *Br. J. Haematol.* **34**, 25 (1976)], and HbC have been prepared and purified to monospecificity by affinity chromatography. Purification of anti-HbS included extensive absorption against Sepharose HbC, while anti-HbC was extensively absorbed against Sepharose-HbS. Each of these antibodies, when conjugated with FITC and applied to fixed smears of blood, permits the visualization only of cells containing the corresponding antigen. Artificial mixtures consisting of 10 percent SC red cells and 90 percent AS red cells were prepared and labeled with anti-HbC-FITC; only 10 percent of the cells were labeled with the antibodies, indicating the complete absence of cross-reactivity of the antibodies to HbC with HbS. Identical results were obtained when artificial mixtures of 10 percent SC cells and 90 percent AC cells were labeled with anti-HbS–FITC.
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6. To obtain quantitative data, the total number of cells and the number of fluorescent cells were counted in several fields of each of the preparations labeled with anti-HbS-FITC, anti-HbC-FITC, or anti-HbF-FITC. The cells of each microscopic field were first counted under white light to determine the total number of cells per field; next, these cells were counted under fluorescent light to determine the number labeled by each type of anti-hemoglobin antibody. Approximately 5000 cells from each subject were counted, and all contained hemoglobins S and C; in addition, the proportion of the cells indicated in Table 1 contained fetal hemoglobin.
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Sickle Hemoglobin Aggregation: A New Class of Inhibitors

Abstract. *A number of tri- and tetrapeptides have been found to inhibit the aggregation and gelation of deoxygenated sickle cell hemoglobin. These inhibitors have hydrophobic phenylalanine residues at one end and hydrogen bonding lysine or arginine side chains at the other end. The backbone is not very specific. The inhibitors do not modify the oxygen carrying properties of hemoglobin. When the inhibitor and sickle hemoglobin are put inside reconstituted cells, the erythrocytes do not sickle upon deoxygenation. Compounds of this type may develop into useful agents in the therapy of sickle cell anemia.*

The substitution of valine for aspartic acid in the sixth position of the β chain of human hemoglobin is responsible for the aggregation of deoxygenated hemoglobin molecules in the disease sickle cell anemia (*1*). In the deoxygenated state, the hemoglobin molecules form aggregates or gels of elongated microtubular structures which have considerable rigidity within the erythrocyte (*2*). This leads to the sickling of the cells which is ultimately responsible for the pathology of sickle cell anemia. Recent studies have focused on the architecture of the

interaction of sickle hemoglobin (HbS) molecules in these fibers (*3*).

Attempts have been made to develop antisickling agents suitable for clinical use that will either inhibit the gelation of deoxy-HbS or stabilize the normal biconcave configuration of the red cell. For example, carbamylation by cyanate (*4*) alters the oxygen affinity of HbS and thereby prevents gelation. Dimethyl adipimate is a cross-linking reagent (*5*) which alters the oxygen affinity but may also block surface sites on the molecule necessary for aggregation. These are both antisickling agents which combine covalently with the hemoglobin molecule. There are other noncovalent antisickling agents such as the alkylureas whose antigelling effectiveness progressively increases with increased hydrophobicity of the molecule (*6*). Recently, another noncovalent antisickling agent was reported: 3,4-dihydro-2,2-dimethyl-2H-1-benzopyran-6 butyric acid. This compound preserves the normal cell shape (*7*) by becoming incorporated into the membrane, by partial inhibition of intracellular HbS gelation, or by a combination of both of these effects. However, there is still no clinically effective and acceptable method of inhibiting or preventing sickling.

The idea behind our research approach is that it should be possible to synthesize a small molecule which binds noncovalently to HbS with great specificity and competes with the HbS binding sites used in building up the fibrous aggregate.

The important change in sickle hemoglobin is substitution of a nonpolar hydrophobic residue (valine) for a polar residue (glutamic acid). This suggests that hydrophobic interactions are important in stabilizing HbS aggregation. This interpretation is also supported by the negative temperature coefficient for gelation for HbS, indicative of hydrophobic interactions (*8*). Given a small molecule inhibitor with sufficient specificity, we believe this approach is likely to yield an agent that is suitable for the treatment of sickle cell anemia. An effective antisickling agent must have several properties: (i) the molecule must bind sufficiently to HbS and prevent aggregation, yet not interfere with the normal loading and unloading of oxygen; (ii) the molecule must be able to penetrate the erythrocyte cell wall; (iii) when the molecule is inside the erythrocyte, it must prevent or delay sickling; and (iv) the molecule must possess low toxicity. Here we report the development of a new class of molecules which satisfy the first and third conditions.

We have found that certain peptides can act as inhibitors which prevent HbS aggregation. These are bifunctional molecules with a hydrophobic residue at one end, usually phenylalanine, and a hydrogen bonding side chain at the other end, often arginine or lysine. We have examined approximately 30 different peptides, most of which have been synthesized. The results we have obtained with several of these peptides provide some insight into the mode of their action.

Upon deoxygenation near physiological conditions, a solution of HbS rapidly becomes viscous and ultimately gels. We have measured the effect of peptides in retarding HbS gelation by two methods. One involves measurement of the viscosity of the solution of deoxygenated HbS while the other measures the smallest concentration of inhibitor which will prevent gel formation of deoxygenated HbS in test tube gelation experiments (*9*).

Amino acids such as phenylalanine and arginine are inactive in preventing gelation, and so are most dipeptides (*10*). However, the dipeptide phenylalanyl-arginine showed some activity, so we examined related tri- and tetrapeptides. Several of the peptides were succinylated to increase their solubility.

A useful index of the effectiveness of inhibition is the minimal mole ratio of peptide inhibitor to HbS as determined by test tube gelation experiments. Thus, a smaller minimal inhibitor mole ratio (MIMR) indicated a more effective inhibiting compound. Table 1 lists several peptides with their MIMR values measured at a deoxy-HbS concentration of 4.40 m*M* deoxy-HbS solution (14 percent less than the intracellular concentration). Only the peptides containing both the phenylalanine and arginine or lysine residues were effective in preventing gelation of deoxygenated HbS. All the tripeptides have the same MIMR value within experimental error and their antigelation activity far exceeds that of the related dipeptide phenylalanyl-arginine. This suggests that the separation of the hydrophobic group from the hydrogen bonding group may be important: the inhibitor may be interacting with two different parts of the HbS molecule.

The inhibitors in Table 1 have a few unusual features. The tripeptide Arg-

Table 1. Minimal inhibitor mole ratio (MIMR) of peptide to sickle hemoglobin (HbS) necessary to prevent gelation under total deoxygenation (*9*). All MIMR values were determined with 4.4 m*M* HbS. The MIMR values were determined according to (*9*) except for the two values shown in parentheses: the HbS solution was supplemented with 2,3-diphosphoglycerate at a concentration of 4.4 m*M*. Deoxygenation was accomplished at 0°C by continuous stirring of the HbS solution for 2 hours while it was exposed to a flow (100 ml/min) of a water-saturated gas whose composition was 1.8 percent O_2, 5.6 percent CO_2, and 92.6 percent N_2. Under these conditions control samples gelled within 3 minutes at 37°C. Peptides were judged to have no effect if their MIMR values were ≥ 40. The values are means ± standard error [Suc, succinyl; for other abbreviations see (*10, 14*)].

Peptide	MIMR
Suc-L-Phe-L-Phe-L-Arg	16.0 ± 1 (6.2)
Suc-L-Phe-L-Phe-L-Phe-L-Arg	10.0 ± 1 (3.8)
L-Phe-L-Phe-L-Arg	17.5 ± 1
Suc-D-Phe-D-Phe-D-Arg	15.5 ± 1
Suc-L-Arg-L-Phe-L-Phe	16.0 ± 1
L-Arg-L-Phe-L-Phe	15.0 ± 1
L-Lys-L-Phe-L-Phe	15.0 ± 1
Gly-Gly-L-Val	No effect
Gly-Gly-L-Ser	No effect
L-Lys-Gly-Gly	No effect
L-Glu-Gly-L-Phe	No effect
L-Phe-L-Arg	> 26

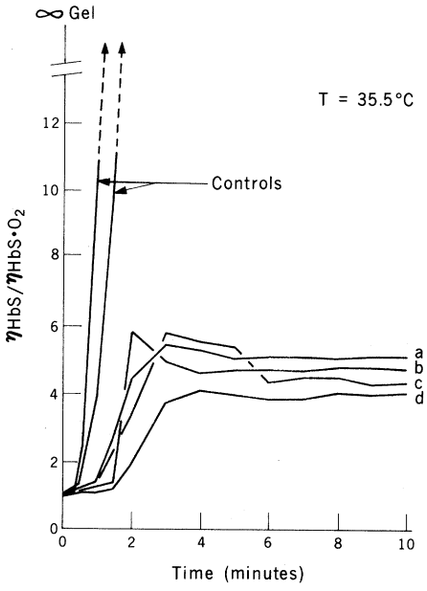

Fig. 1. Time dependent relative viscosity (ηHbS/ηHbS · O_2) in the presence of a ninefold molar excess in the ratio of peptide to HbS for: curve a, Suc-L-Phe-L-Phe-L-Arg; curve b, Suc-D-Phe-D-Phe-D-Arg; curve c, Suc-L-Arg-L-Phe-L-Phe; and a sixfold molar excess for curve d, Suc-L-Phe-L-Phe-L-Phe-L-Arg. A constant viscosity for HbS · O_2 was obtained prior to deoxygenation over a 5-minute time span. For 2 minutes before the rapid anaerobic addition of 15 μl of 10 percent $Na_2S_2O_4$ in 0.05*M* bis-tris-HCl at time $t = 0$, the cone plate chamber was continually flushed with water-saturated nitrogen gas at a flow rate of 75 ml/min. This rate was maintained until the conclusion of the experiment. Viscosities were determined at a constant shear rate of 90 sec^{-1} and a solution volume of 0.40 ml; the HbS concentration was 23.8 g/100 ml. The methemoglobin content was determined to be < 4 percent for the HbS samples used. For the controls, no inhibitor was added.

Phe-Phe has the same inhibitory activity as Phe-Phe-Arg, even though the directionality of the peptide backbone is reversed. Furthermore, the activity of succinylated Phe-Phe-Arg with all the amino acids in the D-configuration has the same activity as that tripeptide when it is synthesized out of the normal L-amino acids. This suggests that perhaps only the side chains of the peptides participate in the inhibitory activity and that the backbone is of secondary importance. The increased activity of the tetrapeptide Phe-Phe-Phe-Arg may indicate that the molecule is interacting with a large hydrophobic region on HbS.

The MIMR values in Table 1 were all obtained under completely deoxygenated conditions except for the values indicated in parentheses, where partial oxygenation was used in an attempt to reproduce conditions close to the physiological conditions in venous blood. In this case, it can be seen that there is almost a threefold decrease in the concentration of peptide needed to inhibit gelation.

Some insight is obtained into the kinetics of the inhibitory process by measuring the viscosity of deoxy-HbS solutions as a function of time. The viscosity of oxy-HbS and deoxy-HbS was determined in a modified Wells-Brookfield cone plate viscosimeter (*11*) and the results are shown in Fig. 1 for several representative peptides listed in Table 1. In the absence of added peptide (control), gelation occurs within less than 2 minutes after deoxygenation. Curves similar to the control are also obtained upon addition of inactive compounds (Table 1). The addition of inhibitory peptides produces a lag time somewhat greater than the controls, followed by a rapid but small increase in the viscosity. Within a few minutes the viscosity remains at a value which is stable with time. However, in the presence of the inhibitor, the viscosity of the deoxy-HbS is four or five times greater than oxy-HbS. Such a viscosity ratio is several orders of magnitude smaller than that predicted by theory (*12*) for fiber formation in vitro, such as that observed with electron microscopy (*3*), which may indicate that such formation is either abolished or greatly attenuated. The effects of several different inhibitors are shown in Fig. 1, but the time-dependent viscosity profile is similar for all of them. Although the tri- and tetrapeptides have different MIMR values (Table 1), the extent of inhibition appears the same. Increases in the concentration of the inhibitor have very little effect on curves a to d in Fig. 1 as long as the threshhold value or MIMR is exceeded. These experiments suggest that the inhibitor is stopping a crucial stage in the assembly of the sickle cell microtubular filament. Not all interactions of deoxy-HbS are stopped, however, since the elevated viscosity after 2 to 4 minutes is probably due to a partial association.

Table 2. Filterability of erythrocyte cells after hypotonic exchange of HbA for HbS in the presence of several antigelling peptides. The measurements were all repeated three times. The conditions are described in (*16*). Values are means ± standard error.

Peptide	Mole ratio of peptide to HbS	Filtration recovery (%)
Suc-L-Phe-L-Phe-L-Arg	6	68 ± 4
Suc-D-Phe-D-Phe-D-Arg	6	65 ± 6
Suc-L-Phe-L-Phe-L-Phe-L-Arg	6	68 ± 7
No peptide	0	11 ± 3
Normal cells with HbA	0	81 ± 4

To measure the oxygen binding properties of HbS in the presence of inhibitors we used an oxygen analyzer (*13*). We found no decrease in the affinity of HbS for oxygen and no change in the cooperativity of the uptake as measured by the Hill coefficient (*14*).

Permeability studies with ^{14}C-labeled tri- and tetrapeptides showed they would not enter the cell in their present form. However, the inhibitor was placed inside erythrocytes through reconstitution in hypotonic exchange experiments (*15*). Erythrocytes were lysed by lowering the ionic strength and were then allowed to reseal at a normal ionic strength. During this process, the erythrocyte takes in hemoglobin. If the solution contains HbS, the final result is an erythrocyte capable of normal sickling upon deoxygenation. The sickling was measured through the use of the filterability assay in a closed system with a small void volume (*16*). Cells were reconstituted with and without inhibitors and their filterability was measured as shown in Table 2. There was a sevenfold difference in the recovery of cells treated with the inhibitor compared to those without inhibitor. The results indicate that cellular flexibility was maintained and no intracellular gelation occurred in the presence of the inhibitor. Without the inhibitor, the reconstituted cells behaved as normal sickle cells in terms of flexibility and were retained in the filter. These experiments demonstrate that when the inhibitor is present inside the erythrocyte, it will effectively prevent HbS aggregation and therefore may abolish the primary cause of sickling.

We have not localized the binding site of these peptides which destabilize HbS fiber formation. Considerable analysis has pointed to a number of specific HbS-HbS interactions which may be responsible for stabilizing the microtubular assembly (*17*). Some of these have strong hydrophobic interactions such as contacts near Phe^{85} or Leu^{88} in the β chain which are possible sites for the interaction with Val^{6} of the β chain of an adjoining molecule (*17*). Most of the potential interaction sites are near bend regions where two α-helical rods in the protein are joined. As a working hypothesis, we suspect that the inhibitors may span across two segments near such a bend region of HbS. This might explain the necessity of a separation of 15 to 20 Å between the terminal hydrophobic and hydrogen bonding side chains of the inhibitors. We have been working with molecular models which suggest plausible interactions between the inhibitor and the residues Leu^{78}, Phe^{85}, and Asp^{79} of the β chain. These are being used to suggest further modification of the inhibitors to yield even greater specificity.

These inhibitors have to be modified in order to facilitate their passage through the erythrocyte cell wall. Once this is accomplished, we believe this will make possible the development of a new class of substances which may prove useful in the development of an effective therapeutic agent to be used in the treatment of sickle cell disease.

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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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18. This research was supported by NIH grant GM 1 P18 HL 15157-01SRC. J.R.V. is a postdoctorate fellow of the M.I.T. Health Sciences Fund. We thank J. Desforges and the Boston Sickle Cell Center for assistance in obtaining blood samples.

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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.