some crabs in the experimental tank developed poor coordination on day 4. By day 5 all experimental crabs were uncoordinated. Instead of scurrying away when threatened by a hand, as usual with the control crabs, they moved a few centimeters, lost coordination, and rolled over once or twice before regaining equilibrium. This was repeated several times as the crab moved 10 or 15 cm. Such awkward and sluggish behavior is unusual and would almost certainly affect survival under natural conditions.

On day 11, the crabs were killed and the muscles of the large claw were analyzed. Controls showed no change in residue concentration (0.240 ppm) or metabolite distribution. The mean of the DDT residues found in the crabs that had fed on the contaminated detritus was 0.885 ppm (34 percent DDT, 58 percent DDE, 8 percent DDD), representing a threefold increase during the 10-day experiment. Wilcoxson's two-sample test showed that the probability was 95 percent that this difference was not due to chance. The poor coordination coincided with the increase in residues within the experimental group, a factor that may explain the disappearance of fiddler crabs from the Carmans River marsh more than a decade ago.

Organic detritus particles with their associated bacteria and other microorganisms in marsh sediments appear to be a reservoir of DDT residues in the environment, small particles sometimes containing residues thousands of times greater than the concentration occurring in water. Fiddler crabs assimilate and concentrate these DDT residues in their muscle tissues from the organic detritus, a process that probably also occurs among other marsh inhabitants.

W. E. ODUM
Institute of Marine Sciences, University of Miami, Miami, Florida 33149
G. M. WOODWELL
Biology Department, Brookhaven National Laboratory, Upton, New York 11973
C. F. WURSTER
Department of Biological Sciences, State University of New York, Stony Brook 11790

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6. The DDT residues include DDT and its decay products (metabolites) DDE and DDD: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DDD, also known as TDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane.
7. Plant detritus consumers include: amphipods, isopods, harpacticoid copepods, various filter- and deposit-feeding bivalves, annelid worms, caridean and penaeid shrimp, fiddler crabs, and fish such as the mullets (*Mugil* spp.).
8. Generally less than 5 percent of these plant materials is consumed on the stalk by herbivores, leaving 95 percent to the detritus food chain [A. E. Smalley, *Ecology* **41**, 785 (1960); E. J. Heald, personal communication].
9. Use of DDT by the Suffolk County Mosquito Control Commission was halted in 1966 by court injunction and later by agreement of the commission when local scientists demonstrated that the accumulation of DDT residues was deleterious to diverse natural resources of the area (*2*).
10. Inorganic particles were separated by washing each size class in a beaker; the organic debris, being lighter, was decanted. The technique could not be used with particles smaller than about 50 μ, but examination showed that more than 95 percent of these fine particles were organic matter.
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12. Supported in part by Brookhaven National Laboratory under the auspices of the AEC, and in part by a NASA predoctoral fellowship through the University of Miami to W.E.O. Contribution No. 965 from the Institute of Marine Sciences of the University of Miami.

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Encephalitogenic Protein: Structure

Abstract. *Amino acid sequences of encephalitogenic proteins from bovine cord and rabbit brain are reported. The bovine protein contains 45 residues. The rabbit protein is identical except for two isopolar substitutions, a dipeptide and amino acid deletion. Analysis of this protein and a 140-residue myelin basic protein indicates that the smaller protein is a portion of the larger encephalitogen. The larger myelin protein contains at least two encephalitogenic sites.*

Experimental allergic encephalomyelitis (EAE) was initially produced in animals by injection of whole central nervous system tissue (*1*). It has now been shown that the encephalitogenic activity of whole tissue resides in a basic protein (or proteins) of myelin (*2*).

We have reported the isolation and partial amino acid sequence of proteins of small molecular weight (4700) from bovine and rabbit central nervous system tissues that are encephalitogenic in rabibts (*3, 4*). The full sequences of these proteins have now been ascertained, and their relation to the basic protein of myelin has been established.

Isolation of the proteins used in these structural studies has been described (*3*). Bovine spinal cord or rabbit brain was defatted in a mixture of acetone and ether and extracted in sodium citrate buffer, *p*H 4.3; the extract was then chromatographed on carboxymethyl-cellulose and Sephadex G-50.

Twenty-four-hour tryptic and chymotryptic digests of the protein were separated by two-dimensional chromatography and electrophoresis. The papers were stained with 0.01 percent trinitrobenzenesulfonic acid (TNBS) in butanol, developed in pyridine vapor, and viewed against a black light (*5*). As soon as the spots became visible they were eluted from the paper in 0.01*N* HCl. Excess TNBS was removed by chromatography on Sephadex G-10. The following procedures were carried out on each peptide: hydrolysis in 6*N* HCl at 110°C for 24 and 96 hours; amino-terminal analysis by the fluorodinitrobenzene method; analysis of amino acid sequence by the subtractive Edman degradation; and timed digestion with leucine aminopeptidase and carboxypeptidases A and B. All amino acid analyses were carried out on the Beckman-Spinco model 120C analyzer used in an accelerated system (*6*).

The results are shown in Fig. 1. All seven of the primary tryptic peptides except T_2 and T_6 were sequenced in a straightforward manner. The sequence of peptide T_2, Gly-Ala-Pro-Lys, was assumed to be correct since leucine aminopeptidase should not have removed glycine if the order were Gly-Pro-Ala-Lys. Peptide T_6 proved resistant to attack by both carboxypeptidase and the Edman degradation, and digestion with leucine aminopeptidase was limited. This problem was resolved by treatment of the peptide with 0.03*N*

HCl for 12 hours at 110°C. After separation of this dilute acid digest on Sephadex G-25 and paper electrophoresis at *p*H 6.5, two peptides were isolated—peptides 24 to 27 and 28 to 35. Removal of the NH_2-terminal asparagine, residue 28, of the second peptide by more prolonged hydrolysis (24 hours) in dilute acid was required for sequence analysis, since this residue had been converted during hydrolysis to aspartic acid (Fig. 1).

Glutamine and asparagine residues were proven by analysis of complete enzyme hydrolyzates of the appropriate peptides on the amino acid analyzer. Residue 28 was assumed to be asparagine by virtue of the net charge on peptide T_6, which was determined from its migration in electrophoresis at *p*H 3.6 and *p*H 6.5. Other peptides also migrated in electrophoresis as predicted.

Reaction of the whole protein with fluorodinitrobenzene is followed by liberation of the entire phenylalanine content of the protein as dinitrophenylphenylalanine (DNP-Phe) (*3*). This serves to establish T_1 as the NH_2-terminal peptide. Peptide T_8 was considered to be the COOH-terminal peptide since it contained no lysine or arginine; and histidine, tyrosine, and threonine were released in that order from the whole protein by carboxypeptidases A and B. The overlap peptide T_3 was present to some degree even after prolonged tryptic digestion, whereas T_9 disappeared during the first few hours. Peptide C_1 was isolated from a chymotryptic digest. Treatment of the whole protein with TNBS to block hydrolysis at the lysine residues gave the following tryptic peptide: Gly-Ala-Pro-Lys(trinitrophenyl)-Arg. The latter established the link between T_2 and T_4, and, thereby, suggested the order of the remaining peptides, T_3 and T_6. This order was confirmed by the following: dilute acid hydrolyzates of the protein were chromatographed on Sephadex G-50, and the major peaks were subjected to paper electrophoresis at *p*H 6.5; peptides 16 to 45 and 22 to 45 were isolated from a 1½-hour hydrolyzate, and peptide 28 to 41 was isolated from a 12-hour hydrolyzate.

Analysis of the protein prepared from rabbit brain by these same techniques revealed that glycine-2 and alanine-29 had both been replaced by serine, and the dipeptide glycine-16–histidine-17 and glutamine-30 had been deleted.

The relation of this protein to the higher-molecular-weight encephalitogen isolated by Kies *et al.* (*7*) has been explored. The preparative method of Kies and Martenson (*8*) was followed: fresh bovine cord was defatted in 2:1 chloroform-methanol and the dried residue was extracted once in distilled water and then in dilute HCl at *p*H 3.0; the acid extract was dialyzed and lyophilized. Further purification was achieved by chromatography on Sephadex G-150. Total amino acid analysis is shown in the first column of Table 1. A 24-hour tryptic digest of the protein was separated by two-dimensional paper chromatography and electrophoresis. Time for full-color development was allowed after staining with TNBS, and 20 major spots were identified. These were eluted and hydrolyzed for 48 hours in 6*N* HCl for amino acid analysis. The results are shown in Table 2. Peptides T_a, T_b, T_e, and T_h correspond exactly to the amino acid composition of peptides T_3, T_4, T_5, and T_2, respectively, of our low-molecular-weight protein. These peptides also appeared in identical positions on paper. In order to obtain a value of around 400 nmole per residue for peptides T_b and T_e, the residue values of T_a must be divided between the two, since T_b and T_e are derived from T_a. Peptide T_i of Table 2 appears to be either an incompletely digested core or a mixture of several peptides. In either event it contains the proper amounts and ratios of the appropriate amino acids to constitute peptides T_6 and T_9 of our protein except for low amounts of histidine and valine. We are aware, however, that in our protein the Val-Val-His sequence is resistant to acid hydrolysis and requires 96 hours or more to liberate the individual amino acids. Peptide T_i also contains amino acids other than those in peptides T_6 and T_9. Peptide T_q corresponds to T_1, the NH_2-terminal peptide of our protein, except for an additional phenylalanine residue; that is, Phe-Phe-Gly-Ser-Asp-Arg. This peptide moved further in chromatography than did T_1, as expected.

These data indicate that our protein is a portion of the large encephalitogen. The split which has occurred between the Phe-Phe linkage of T_q is at a site consistent with the suggestion of Einstein and others that a brain acid protease is still active after whole tissue is defatted with an acetone-ether mixture (*9*). This enzyme is presumably inactivated during extraction with the chloroform-methanol mixture.

The number of times that each amino acid occurred and the total number of residues (last column of Table 2) compare favorably with the corresponding figures for the whole protein (column 1, Table 1), in view of the many factors tending to reduce recovery of certain peptides and amino acids. For example, the extent of elution from paper varies for different peptides and low yields of the NH_2-terminal residues and of lysine would be expected since full-color development of the peptide maps with TNBS was permitted. In addition, acid hydrolysis was carried out for only 48 hours, and this could have led to low values for certain amino acids.

Amino acid compositions of other encephalitogens of higher molecular weight (*9–12*) are compared in Table 1 to the Kies and Martenson preparation. The analysis of Kornguth (*10*) reported as moles percent has been converted to percentage of residues, 140 residues being considered the total. In the analysis of Einstein and Chou (*9*) the total number of residues has been adjusted from 100 to 135 and, in that of Eng *et al.* (*11*), from 82 to 141. There is surprisingly good overall agreement. Some of the variations can be explained on the basis of the procedure for acid hydrolysis of the protein. As already discussed, the Val-Val-His sequence in our protein is quite resistant to acid hydrolysis.

Also included in Table 1 are the amino acid compositions of two other active fragments of the larger encephalitogen. Einstein and Chou obtained their fragment by digestion with a purified acid protease from brain (*9*). The NH_2-terminal residue was phenylalanine, but no sequence data are available. Eylar and Hashim (*12*) have isolated a peptic peptide which is active and have sequenced 16 out of the total 26 residues as follows: Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Ser-Pro-Phe-Gly-Lys followed by Lys, 2 Arg, Asp, Ser, 3 Gly, Ala, Tyr in some sequence. The sequenced portion, except for the NH_2-terminal Ser-Arg, constitutes a tryptic peptide which in composition corresponds closely to a tryptic peptide of the Kies and Martenson preparation (T_r, Table 2). Each contains a tryptophan residue, presumably the only one in the molecule. Therefore, the fragment obtained by Einstein and Chou, which also contains a tryptophan, probably includes the peptide of Eylar and Hashim, and although our fragment could be included it fits the other half of the Einstein-

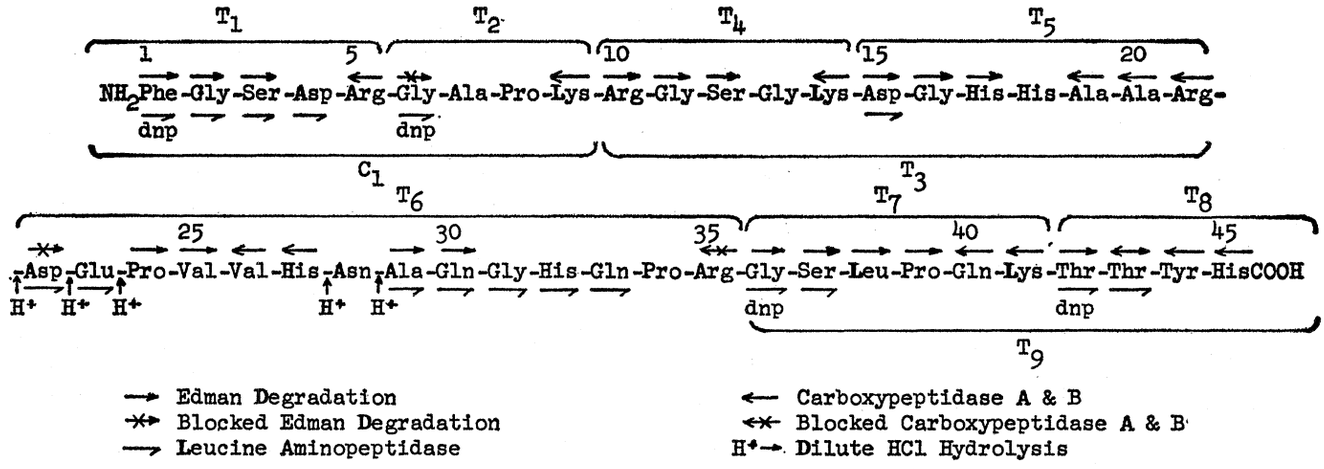

Fig. 1. Structure of an encephalitogenic basic protein from bovine spinal cord; T_n and C_1 refer to tryptic and chymotryptic peptides; *dnp* signifies the position of the dinitrophenyl group when these peptides are treated with fluorodinitrobenzene; NH_2Phe is the NH_2-terminal residue; HisCOOH is the COOH-terminal residue, and the protein is a continuous polypeptide chain of 45 residues.

Table 1. Amino acid composition of encephalitogenic protein and fragments.

Amino acid residue	Basic protein (No. of residues)					Active fragments—bovine (No. of residues)		
	Bovine			Human (Eng *et al.*)	Swine (Kornguth & Tomasi)			
	(Kies & Martenson)	(Einstein & Chou)	(Eylar & Hashim)			(Einstein & Chou)	(Eylar & Hashim)	(Kibler & Shapira)
Lys	12	10	13	12	11	6	2	3
His	8	8	9	10	8	4	0	5
Arg	14	14	16	15	14	6	3	4
Asp	10	9	9	10	9	4	1	4
Thr	6	6	6	7	6	3	0	2
Ser	13	13	13	13	12	6	4	3
Glu	10	9	9	8	9	5	2	4
Pro	11	10	9	10	11	6	1	4
Gly	21	20	20	20	21	12	7	7
Ala	12	11	11	10	13	5	2	4
Val	3	2	4	3	3	1	0	2
Met	2	2	2	2	2	0	0	0
Ile	3	2	3	3	3	1	0	0
Leu	8	8	8	7	8	3	0	1
Tyr	3	3	3	3	4	2	1	1
Phe	7	7	6	7	7	3	2	1
Trp	1	1	1	1	1	1	1	0
Total	144	135*	142	141*	140*	68*	26	45

* Total residues adjusted to values comparable to other preparations. See text.

Table 2. Amino acid analysis of tryptic peptides from the low-molecular-weight encephalitogen. The tryptic peptides are given at the head of each column. The corresponding tryptic peptides from the low-molecular-weight protein (Fig. 1) are indicated at the foot of the appropriate columns. Results are expressed as nanomoles of amino acid and, for the common peptides, as molar ratios relative to lysine or arginine (figures in parentheses). Tryptophan was identified with Ehrlich's reagent. The total number of residues was calculated on the basis of 400 nmole for one residue; the total number is 127.

Amino acid	Content of tryptic peptide (nmole)																			Total residues
	T_a	T_b	T_c	T_d	T_e	T_f	T_g	T_h	T_i	T_j	T_k	T_l	T_m	T_n	T_o	T_p	T_q	T_r	T_s	T_t	
Lys	157(1)	177(1)	336				341	423(1)	668	680	400		415	287				255			10
His	324(2)		332	200	296(2)				874	450			210								7
Arg	268(2)	156(1)		280	150(1)	594	488		450	130		315	280		343	900	321(1)	185	115	399	13
Asp	175(1)				124(1)				770	556	543	251				350	352(1)	100	150	782	10
Thr									809	649						425				395	6
Ser	181(1)	185(1)	316			248	318		604	767	536	314				360	355(1)	295	450	424	13
Glu						200			1420	952								545			8
Pro						200		455(1)	1130	1304		230	400			479		225	237		12
Gly	529(3)	370(2)			158(1)		192	326(1)	832	1079			638		605	280	364(1)	1100	376	829	19
Ala	350(2)		410		288(2)			464(1)	450	502	499							280			8
Val									200							180					1
Met									112										122		1
Ile														144		167				345	2
Leu									650	414			770		270	390				699	8
Tyr									368		342							155			2
Phe									566				400	233			545(2)	376	163		6
Trp																		+			1
	T_3	T_4			T_5			T_2	T_6 and T_0 plus								T_1				

Chou protein just as well. More work such as tryptic peptide composition and sequence analysis of the Einstein-Chou fragment and the larger protein is necessary to identify the relative order of these three active fragments in the larger encephalitogen. In any event, there is no overlap between our protein and the Eylar-Hashim peptide, thus indicating the presence of at least two separate encephalitogenic sites. Together these two fragments represent approximately half of the larger protein.

Since our active fragment has been tested only in rabbits, and the other two only in guinea pigs, it will be important to determine activities in other species. In rabbits, the critical amount of our protein for encephalitogenic activity seems to be 50 μg. Injections of 100 or 200 μg produced experimental allergic encephalomyelitis (EAE) in over 90 percent of injected rabbits with the average day of onset being around 14 days (*3*). In most experiments, 50 μg exhibited the same degree of activity, while in other experiments the incidence was lower and the day of onset was prolonged. Below 50 μg activity fell off; that is, 25 μg produced EAE in three-fourths of the rabbits with an average onset of 17 days, and 15 μg produced this disease in five-eighths of the rabbits with an average onset of 25 days. A tryptic digest of our protein was inactive but peptide 16 to 45, obtained by dilute acid hydrolysis, produced EAE in two out of two rabbits on days 14 and 21, respectively, in a dose of approximately 25 μg.

The amino acid sequence of the encephalitogenic protein from bovine cord and rabbit brain reported here is also of importance since we are dealing with a membrane protein. One might predict that such proteins must have unique properties that result primarily from their amino acid sequence. It is interesting to note (Fig. 1) that portions of our protein can be fitted to a repeating sequence of three apolar amino acids followed by two polar ones (residues 1 to 15 and 37 to 44 in the bovine preparation, and residues through the NH_2-terminal aspartic acid of peptide T_6 and 37 to 44 in the rabbit preparation). In a discussion of interaction of membrane protein and lipid, Wallach (*13*) "conjectured that the distribution of polar amino acids is such that their side chains lie at the membrane surfaces, or cluster round the central axis of each subunit, or both, possibly producing aqueous channels which penetrate the membrane." This requires that the protein segments have a polar face opposite the apolar one. Such an arrangement could be made with a repeating sequence of polar and apolar residues.

The foregoing evidence suggests that only one basic protein exists in bovine myelin and that this protein contains more than one encephalitogenic site, as demonstrated by sequence analysis on encephalitogenic fragments which have been shown to be part of the larger protein. It is conceivable that other portions of the protein are also active.

ROBERT F. KIBLER, RAYMOND SHAPIRA
SARAH MCKNEALLY, JULIA JENKINS
PAMELA SELDEN, FRANK CHOU
Department of Medicine (Neurology)
and *Department of Biochemistry,*
Emory University,
Atlanta, Georgia 30322

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Plasma Saluretic Activity: Its Nature and Relation to Oxytocin Analogs

Abstract. *Plasma natriuretic activity was evoked in cows and dogs by infusion of saline with or without dextran. Deproteinized samples were fractionated on both Sephadex and Bio-Gel columns; the activity was separated, the approximate molecular weight being in the region of 1000. Incubation with chymotrypsin destroyed the activity, suggesting that it might be a polypeptide. A similar activity in blood resulted from intracarotid injection of either oxytocin or either of two synthetic analogs. Possibly the latter are saluretic by virtue of a releasing action on some intracranial structure for another natriuretic peptide.*

Knowledge of the chemical nature of "natriuretic hormone" has thus far been acquired from an active factor in the plasma of dogs, cats, and cows sampled after a variety of stimuli (infusion of saline with and without colloid, carotid-artery occlusion) as well as from uremic man (*1–3*). This evidence suggests that the molecule concerned is not precipitated with plasma proteins, is heat-stable after protein is removed, is polypeptide in nature, contains at least one basic amino acid and at least one free amino group, and has a molecular weight of less than 20,000 (*2*) or less than 2000 (*3*). Until the exact chemical identity has been established, there is no way of determining whether the various experimental results deal with the same molecular entity. The cow is an adequate experimental animal for the production of saluretic responses to such "volume" stimuli as the infusion of isosmotic, isooncotic dextran, accompanied by the appearance of natriuretic activity in plasma (*4*). There is the particular advantage that fairly large blood samples can be withdrawn.

Six cows were investigated by described methods (*4*). A cardiac catheter was introduced through the external jugular vein into the innominate vein in order to collect blood mixed with the