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The Isolation and Characterization of Soluble Brain Proteins

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THE ISOLATION AND CHARACTERIZATION OF SOLUBLE BRAIN PROTEINS

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PREFACE

Although electrophysiological experiments have resulted in a detailed description of the role of inorganic ions and of certain small transmitter molecules in the propogation of the nerve action potential, our present knowledge of the role of macromolecules in excitability and in psychological memory is very incomplete. The experiments I shall describe in this thesis were carried out in the belief that in order to determine the role of proteins in nerve function, it would be necessary to identify and isolate particular molecules which might have an activity other than the necessary supportive activities that proteins are known to have in all types of cells.

After presenting the background and rationale for the experimental approaches used, I shall describe certain characteristics of the soluble proteins of rat brain. I shall then report the purification and some chemical properties of a nerve specific antigen. Finally, I will discuss studies on *in vivo* biosynthesis of soluble brain proteins.

In planning and carrying out these experiments, I am indebted to my research advisor, Dr. Gerald M. Edelman, for his critical guidance and constant encouragement. I am also grateful for the generous help and advice received from other members of his laboratory, particularly Dr. William O. McClure and Mr. William Einar Gall. The skillful technical assistance of Mrs. Helvi Hjelt in the preparation of the animals was invaluable. I should also like to acknowledge the contribution of Dr. Frederick A. Dodge in planning and evaluating the experimental approaches. Finally, Dr. Stephan Chorover of the Massachusetts Institute of Technology gave me instruction in the preparation of animals for spreading depression, and provided valuable discussion of these experiments.

ABSTRACT

The experiments described in this thesis were undertaken in order to define and purify proteins that might have a specific function in nerve activity. The studies were limited to rat brain proteins soluble in dilute aqueous buffers at neutral pH. Fractionation procedures for these proteins, and two means of identifying individual proteins of interest in the fractions were developed.

The soluble proteins were divided into two classes according to their solubility in mildly acidic conditions: the pH 5 soluble and pH 5 insoluble fractions. Two different large anatomical areas of the brain, referred to as "cortex" and "brainstem," contained the same amount of pH 5 soluble proteins but differed considerably in the pH 5 insoluble protein content. Using analytical disk electrophoresis on polyacrylamide gels as a tool for examining individual components of the pH 5 soluble fraction, the following comparisons were made: Brain extracts from a single animal species gave a characteristic pattern, which differed qualitatively from extracts of other tissues and from serum. Extracts of brains from different animal species all differed from each other, but different strains of rats were identical. Finally, the patterns obtained with different regions of the central nervous system of a given animal were identical, but peripheral nerve differed.

One means of characterizing the soluble brain proteins was by studying their in vivo biosynthesis during variations in electrical activity. For this purpose an experimental system was employed which utilized the phenomena of unilateral spreading cortical depression in conscious animals. It was found that after administration of tritiated leucine the specific activity of "cortical" proteins was about 16% lower in the depressed hemisphere than in the control hemisphere of the same animal, while the specific activity of "brainstem" proteins remained the same on both sides. The pH 5 soluble and insoluble fractions were affected to the same extent. Further subfractionation of the pH 5 soluble proteins failed to show individual proteins whose biosynthesis was altered to a greater extent than the average over all soluble proteins. The significance of these results

is discussed with respect to the use of biosynthetic criteria for identifying proteins of importance in nerve tissue.

The second means of characterizing individual brain proteins was the investigation of their tissue specificity, using immunological methods. For this purpose antisera to rat brain extracts were prepared. About twelve antigenic components could be demonstrated by immunoelectrophoresis; most were present in extracts of other tissues as well. The various antigens, most of which were in the pH 5 soluble fraction, could be separated according to their electrophoretic mobility by zone electrophoresis on starch. Preparation of antisera to the six subfractions of the soluble proteins obtained in this manner made possible the identification and purification of a protein, designated antigen α, which could not be demonstrated in other tissues.

Antigen α was found to be an acidic protein, consisting of a single type of subunit having a molecular weight of about 39,000. Evidence was obtained which indicated that the molecule exists as a dimer stabilized through disulfide bonds. Higher order aggregates were also observed. The protein is found in both the central and peripheral nervous system of the rat, and immunological evidence for the existence of related proteins in other animals was also obtained. The properties of antigen α were compared to those of certain other brain proteins, especially the "S-100" protein, which is the only other highly purified soluble protein found only in the central and peripheral nervous system.

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I. INTRODUCTION

I. INTRODUCTION

This thesis is concerned with the attempt to define and purify proteins that might be specifically involved in the activity of the nervous system. The experimental approach used was to develop fractionation procedures for soluble nerve tissue proteins, and concurrently to apply specific screening methods to identify those fractions of potential significance. The underlying hypothesis is that although many proteins in nerve tissue play the same structural and metabolic roles that they have in other tissues, there may be some proteins that are specifically involved in those properties specific to nerve.

It is helpful first to examine the evidence for such a hypothesis, and then to review briefly the experimental approaches that have been used to identify proteins in the nervous system. For the convenience of the reader, the experimental schemes, and the proteins identified through them, are summarized in Table I. They can be divided into two categories, which will be discussed separately.

First, there are several known or proposed specific functions which are likely to be carried out by a single protein. Included in this category are various enzymes and trophic and pathologic factors. In all cases, the activity of the protein can be stated in sufficiently precise terms to design an *in vitro* assay necessary to purify the protein.

To date, it has not been possible to describe completely the functions of the nervous system in molecular terms on the basis of the known actions of particular molecules alone. Therefore, a second type of experimental approach has been used. This approach has centered on the search for proteins which may have some function that has not yet been discovered. The need of a functional assay for the purification of such a molecule has been circumvented by the use of some other criterion. While the precise nature of these alternative criteria vary, most of them have at their basis an implication that such a protein should be present only in neural tissue.

TABLE I

Examples of Experimental Approaches for Identifying Proteins of Particular Importance in Nerve Function

Approach	Examples of Proteins
Purification and characterization of enzymes	Enzymes of transmitter metabolism Acetylcholinesterase (Nachmansohn, 1959) Choline acetylase Tyrosine hydroxylase (Udenfriend, 1966) L-Glutamic decarboxylase (Susz, et al., 1966) $Na^+ + K^+$-ATPase (Skou, 1957) Proteases (Marks and Lajtha, 1963)
Purification of transmitter or drug binding proteins	None
Purification of a "gross" biological activity	
Trophic activity	Nerve growth factor (Levi-Montalcini, 1965)
Pathologic activity	Encephalitogen (Kies, 1965)
Purification of proteins from specific nerve constituents	
Myelin	Proteolipids (Folch-Pi, 1963) Basic proteins (Wolman, 1962)
Nerve endings	None
Development of Fractionation Schemes	
Purification of fractions absent from non-neural tissue	"S-100" (Moore, 1965)
PURIFICATION OF NERVE SPECIFIC ANTIGENS	ANTIGEN α
PURIFICATION OF PROTEINS WHOSE SYNTHESIS IS PREFERENTIALLY AFFECTED BY ALTERATIONS IN NEURAL ACTIVITY	NONE

Because of the considerable number of different methods, and the variable degrees of success which they have met, no attempt will be made to cover all the approaches completely. Instead, emphasis will be placed on studies which are related to the approaches used in the present experiments. These involved the development of two precise criteria which did not imply a specific biological activity, for screening fractions of soluble nerve proteins. One criterion was immunological tissue specificity. The second was the relative degree of alteration in rate of *in vivo* biosynthesis induced by changes in neural activity.

A. Evidence for a Specific Role of Proteins in Nerve Function

Although the role of proteins in neural function has been the subject of considerable speculation (see, for example, Schmitt and Davison, 1965), very little concrete evidence exists as to how they may be involved. Even less information is available about which specific proteins are particularly important.

In order to examine the available evidence, it is helpful to discuss the properties of nerve tissue at two levels. There is first the ability of nerve to respond to changes in its immediate environment by rapid specific and reversible changes in the permeability of the cell membrane to cations. This property is shared by all types of neural tissue, can be demonstrated *in vivo* and *in vitro*, and is exhibited by organized tissue, single cells, and parts of cells (isolated axons) alike. Furthermore, it is likely to be a phenomenon restricted to the cell membrane, since a giant axon, after replacement of its cytoplasm with suitable inorganic salt solutions, maintains its excitability (Baker *et al.*, 1961). At another level, there is the ability of the nervous system as a whole to respond to changes in the environment of the organism by memory and learning. At this level, the nature of the changes in neural tissue are much less clear, but they can be generalized as involving alterations in the interactions of nerve cells which are relatively stable over long periods of time. Suitable *in vitro* analogues for these phenomena have not yet been developed.

Some suggestive evidence that proteins may be specifically involved at both levels has been obtained.

1. Possible Role of Proteins in Excitability A few experiments have been reported which give indirect evidence that proteins are involved in some way in the permeability changes accompanying the action potential. For example, proteolytic enzymes applied intracellularly to giant axons have been shown to block excitability (Rojas and Luxoro, 1963; Rojas, 1965; Rojas and Atwater, 1967; Tasaki and Takenaka, 1964). Alterations in the action potential could usually be demonstrated before any deterioration of the resting potential had occurred. Other experiments imply a possible role of protèins because of the effects on the action potential of chemical modifications of the side groups of amino acid residues. Huneeus-Cox et al. (1966) investigated the effects of a number of sulfhydryl reagents on the electrical properties of the squid giant axon and were able to produce some alterations in excitability. All of these experiments are difficult to interpret because of the lack of specificity for particular proteins of the reagents employed.

A few experiments have been reported in which reagents of much greater specificity (antibodies) were shown to affect excitability. These experiments are of great interest because they immediately suggest a means of examining the role of individual proteins in nerve function.

The first report that antisera to nerve tissue affected excitability was that of Mihailovic and Jankovic (1961), who prepared antisera against saline extracts of rat caudate nucleus. Injection of the purified γ-globulin into the lateral ventricle of rats resulted in electrical disturbances of the caudate nucleus, but not in several other regions of the brain. Although the regional specificity of the effect is still controversial, several other reports of similarly produced disturbances have since appeared. Mihailovic et al. (1965) reported that γ-globulin from rabbits immunized with an extract of lobster nerve, when externally applied to lobster nerve, resulted in a fall and eventual block of the action potential. Immune γ-globulin against squid axoplasm, when applied internally to squid axons, produced conduction block in these nerves (Huneeus and Fernandez, 1967). Antisera against isolated nerve endings

were shown to cause disturbances in the electrical activity of the visual cortex of the cat, when topically applied (De Robertis et al., 1966). The antisera, however, were not specific for the nerve ending fraction.

Gamma globulin from rabbits with experimental encephalomyelitis produced alterations in electrical activity of several regions of rabbit brains in vivo, when administered into the lateral ventricle (Jankovic et al., 1966) and also when applied to rabbit cerebral cortex in tissue culture (Bornstein and Crain, 1965).

All of these experiments are still quite preliminary. The antisera were all complex, and were shown to react with more than one component in the antigen preparation. Moreover, the nature of the antigens was not investigated. The results are, however, sufficiently interesting to encourage a more complete investigation of nerve tissue antigens.

2. Possible Role of Proteins in Memory and Learning The molecular events responsible for psychological memory, which is a long-lasting modification of the integrated action of organized nerve tissue, are far less well established. Although it is reasonable to assume that these changes are the result of the electrical events occurring at the membrane, and that they in turn modify the excitability of the membrane, the chemical nature and cellular location of these "changes" is as yet unknown. Evidence implicating proteins has been very indirect. A number of in vivo and in vitro experiments have been published reporting changes as a result of electrical activity in such parameters as incorporation of amino acids into protein, protein configuration, and activity of various enzymes. These studies have been thoroughly reviewed by Lajtha (1964b). In general, it has been difficult to relate the reported changes directly to any physiological neural function.

Some more recent experiments have utilized drugs to affect changes both in protein synthesis and in memory. Inhibitors of protein synthesis, such as puromycin and acetoxycycloheximide, have been reported to block the storage of memory in mice (Flexner et al., 1964; Barondes and Cohen, 1967a, b), and in goldfish (Agranoff et al., 1966). Drugs stimulating the synthesis of ribonucleic acid (RNA) and protein have been used in attempts to facilitate learning and retention, but conflicting results

have been obtained. Some of the compounds employed were "TCAP" (1,1, 3-tricyano, 2-amino, 1-propene) (Brush et al., 1966; Daniels, 1967) and Magnesium Pemoline (Glasky and Simon, 1966; Plotnikoff, 1966a, b; Morris et al., 1967; Goldberg and Ciofalo, 1967). Other reports, still highly controversial, have attempted to show the involvement of macromolecules (RNA or protein or both) in the acquisition and storage of memory, by injecting brain extracts of trained animals into naive animals. These experiments have recently been evaluated and reviewed by Booth (1967) and a general review of the effects of drugs on learning and memory has also appeared (McGaugh and Petrinovich, 1965).

All of these studies point out the need for examining individual proteins, rather than total proteins, before definite correlations between nerve function and the role of proteins can be made. Therefore, some individual proteins will be discussed in the following pages.

B. Nerve Proteins with Known Functions

Proteins that immediately come to mind as being of particular significance in neural function at both levels discussed above are enzymes involved in the synthesis and metabolism of transmitters. Innumerable ways of examining these enzymes and the regulation of their activity in vivo have, of course, been developed, and a considerable amount of information has been accumulated. Also, very specific means of inhibiting the action of individual enzymes are available. Histochemical techniques, especially, have been widely used to examine enzymes in the nervous system. Very few enzymes, however, have been purified to an extent that would permit chemical characterization.

The best established transmitter is acetylcholine, and the enzyme that catalyzes its hydrolysis, acetylcholinesterase, has been extensively studied (Nachmansohn, 1959; Wilson, 1954). Much of the information about the active site of the enzyme, and its distribution in the nervous system has come from experiments done in situ or on partially purified preparations, but recently acetylcholinesterase has been obtained in highly purified form, and some of its physicochemical characteristics examined (Leuzinger and Baker, 1967; Leuzinger et al., 1968; Grafius and Miller, 1967). The enzyme catalyzing the synthesis of acetylcholine from acetyl

coenzyme A and choline, choline acetylase, has not been extensively purified and studied.

Enzymes involved in the synthesis and degradation of other transmitters have not been so completely examined, but a few have been purified to some degree. For example, tyrosine hydroxylase, catalyzing the apparently rate limiting step in the synthesis of norepinephrine from tyrosine, has been studied and partially purified by Udenfriend (Udenfriend, 1966; Nagatsu et al., 1964). L-Glutamic decarboxylase, responsible for the formation of the transmitter γ-aminobutyric acid from glutamate has recently been highly purified (Susz et al., 1966).

The transmittor "receptors," presumably located at the post synaptic membrane, have been the subject of considerable pharmacological investigations, but the precise chemical nature of the receptor has not been established for any transmitter, nor has any protein been isolated that has been shown to interact with or bind a transmitter.

Because of the strong dependence of nerve excitability on the intra- and extra-cellular ionic environment, considerable attention has been focused on the energy-dependent "active transport" of cations. An enzyme strongly implicated in this phenomenon, a sodium- and potassium-activated ATPase, was first demonstrated in crab nerve by Skou (1957), and in red blood cells by Post (Post et al., 1960). Since then, this enzyme has been demonstrated in a number of tissues (see review by Judah and Ahmed, 1964); this is not surprising in view of the fact that active cation transport is a common property of all cells. The enzyme is found in close association with cell membranes, and with subcellular organelles as well. Although the ATPase has not yet been extensively purified, considerable effort is currently being directed towards the solubilization and purification from several sources (Cooper and McIlwain, 1967; Medzihradsky et al., 1967; Schoner et al., 1967; Brown et al., 1967). This should lead to a comparison of the properties of the enzyme in nerve tissue and other tissues. There is some evidence that ATPase is a lipoprotein (Judah and Ahmed, 1964) and/or a phosphoprotein (Bader et al., 1967).

In any consideration of the role of proteins in neural functions, proteolytic enzymes would be of great interest. Very little, however, is known about such enzymes in nerve, although they have recently been studied

by Marks and Lajtha (1963, 1965). Two groups of proteases in brain, active at neutral and acidic pH, were described and separated from each other by various fractionation techniques. At least one of the proteases active at acid pH was obtained in highly purified form. Peptidases have also received some attention, and partial purification of brain amino peptidases have been reported (Brecher and Sobel, 1967).

A few other proteins have been purified which are of considerable interest because of their gross functional activity. One of these is the "nerve growth factor" (NGF), originally discovered by Levi-Montalcini (1965), and purified from both mouse submaxillary gland and snake venom by Cohen (1960). Despite the unusual source of NGF in the parotid gland, it exhibits a highly specific trophic action on sympathetic ganglia. It is necessary for the embryonic development of the sympathetic nervous system, as shown by the inhibitory action of antisera to NGF on differentiation of sympathetic ganglia of chick embryos in tissue culture, and mouse embryos *in vivo*. NGF is not necessary for the maintenance of sympathetic ganglia in mature animals (Levi-Montalcini, 1965). Although the exact molecular form of active NGF is still being investigated, there appears to be single basic protein subunit having a molecular weight of about 20,000 (Angeletti *et al.*, 1967; Varon *et al.*, 1967a, b).

Another protein purified from the nervous system is the so-called "encephalitogen." Nothing is known about the normal function of this molecule, and it has been investigated solely because of its pathologic activity in the production of the disease known as experimental allergic encephalomyelitis. A number of groups have purified encephalitogenic proteins or peptides from brain and spinal cord (Einstein *et al.*, 1962; Kies, 1965; Carnegie and Lumsdem, 1967; Kibler and Shapira, 1968), and while the different extraction and purification procedures employed make it difficult to estimate the number of different proteins having encephalitogenic activity, a comprehensive study has indicated that a basic peptide having a molecular weight of about 4,000 to 5,000 is common to all active preparations (Lumsden *et al.*, 1966). Immunofluorescent studies have shown that the encephalilogen(s) is confined to myelin (Ranch and Raffell, 1964), and although purified only from central nervous tissue, they can be detected in small amounts in peripheral nerve as well.

C. Methods for Identifying Additional Proteins

A number of nerve tissue proteins have been selected for purification and characterization because of some property other than a known or postulated function. Indeed, in all cases their activity is still unknown.

1. Myelin Components Folch-Pi has pioneered in the chemical fractionation of the constituents of myelin, a structure unique to the nervous system, and has defined and extensively examined the class of proteins "proteolipids," making up about 25% of white matter proteins (Folch and Lees, 1951; Folch-Pi, 1963; Matsumoto et al., 1964). Several different protein constituents have been reported, and at least partially purified (Wolman, 1962; Lowden et al., 1966). Chemical investigations of myelin have been undertaken largely because they may shed light on the structural components of cell membranes in general. In this context it is worth noting that myelin consists primarily of electrically inexcitable glial cell membranes, and only a small proportion of electrically excitable axonal membrane. A recent report, however (Wolfgram, 1967), shows that although the amino acid composition of total proteolipids from a variety of non-neural tissues is identical, central nervous system proteolipids, though similar, are not identical. This result suggests that some of the protein constituents of myelin are peculiar to that structure, and therefore may be of particular significance in neural function.

Another morphological structure unique to the nervous system is the synapse. Development of cell fractionation techniques has made possible the isolation of nerve endings and parts of nerve endings such as synaptic membranes and synatpic vesicles (De Robertis et al., 1962; Whittaker, 1966). Although proteins specific to these structures have not yet been identified, some work directed towards this aim is in progress. De Robertis et al. (1966) prepared antisera to isolated nerve endings, but could not identify any components specific to that fraction. Cotman and Mahler (1967) have begun to examine insoluble proteins from synaptic vesicles and synaptosomes and to compare them to proteins from myelin and mitochondrial membranes.

2. Development of Fractionation Schemes There have been several attempts at fractionation of total brain proteins, on both analytical and preparative scales, with the overall aim of identifying specific subfractions that might be of particular importance in neural function. Early preparative experiments employed purely chemical methods of fractionation of total protein, such as fractional precipitation and extraction with salts, heat, acid, alkali, and other solvents (McGregor, 1916; Dingman et al., 1959). With the development of electrophoretic techniques capable of high degrees of resolution of soluble proteins, recent analytical work has centered on the use of these methods. Robertson (1957) used electrophoresis on paper to classify soluble brain proteins into various fractions according to their electrophoretic mobility, and defined the fractions by reference to serum proteins. This type of classification has been used by most workers. Karcher et al. (1959) used microelectrophoresis in agar gel, and compared proteins of cerebrospinal fluid and brain from normal and diseased humans, and other animals. Starch gel electrophoresis was used by Bailey and Heald (1961a, b) and by Bondy and Perry (1963). Electrophoresis on analytical polyacrylamide gels was first used for brain proteins by Vos and van der Helm (1964). Concurrently, attempts were made to maximize the percent of total protein that could be obtained in solution (Le Baron and Folch, 1959; Brunngraber and Aguilar, 1962). Separation of soluble nerve tissue proteins by chromatography has also been reported. Bondy and Perry (1963), Bogoch et al. (1964), Moore and McGregor (1965), and MacPherson and Liakopoulou (1966) all used ion-exchange chromatography on DEAE cellulose for the preparation of protein fractions. Fractionation by chromatography on calcium hydroxyapatite was carried out by Brunngraber and Occomy (1965).

Although a number of these studies have pointed out qualitative or quantitative differences in various protein fractions, when comparisons were made between neural and non-neural tissues, different regions of nerve tissue, brains from normal individuals and those with neurological diseases, only one study has so far resulted in the identification and purification of a specific protein. Moore (Moore and McGregor, 1965; Moore, 1965), using a combination of DEAE-cellulose chromatography and starch gel electrophoresis, identified an acidic protein in brain extracts which was not present in liver.

3. Nerve Specific Antigens The existence of organ specific antigens in nervous tissue has been recognized for some time. Early work (Brandt et al., 1926) indicated that lipid components of the central nervous system were responsible for the production of brain specific antisera, and considerable interest in the lipid determinants of myelin has been maintained up to the present (Rapport et al., 1964). The possible existence of protein antigens in nerve was also noted early (Schwab, 1936). Since then, a number of studies have been published.

Van Alten and LaVelle (1966) were able to prepare precipitating antibodies to homogenates of whole myelinated and unmyelinated hamster brain, and demonstrated some brain specific components. The chemical identity of the antigens was not investigated. Friedman and Wenger (1965) prepared complement-fixing antibody to whole chicken brain homogenates. They detected a brain specific antigen presumed to be protein in nature because of its presence in the chloroform-methanol insoluble fraction, but did not further characterize it.

Some information about the nature of specific antigens has been obtained by immunoelectrophoretic analysis of antisera and various antigenic fractions. Warecka and Bauer (1967) reported a brain specific antigen with an electrophoretic mobility in the α_2-range in aqueous extracts of human brain. They presented some evidence indicating that this antigen is a glycoprotein containing neuraminic acid. Kosinski and Grabar (1967) reported eleven antigenic components in rat brain extracts, including as many as five specific to brain. A more extensive fractionation of brain protein antigens was carried out by MacPherson and Liakopoulou (1966) and also by Bogoch and coworkers (Rajam and Bogoch, 1966; Rajam et al., 1966). The former group prepared antisera to aqueous extracts of rat brain, and identified twelve different antigenic components by immunoelectrophoresis. The proteins were then separated on DEAE-cellulose into seven fractions. The presence of two brain-specific antigens was demonstrated; one of these was specific to rat brain, but the other was not species specific. The DEAE fractions containing these antigens also contained several components not specific to brain and no attempt at further purification was reported. Bogoch and coworkers (Rajam and Bogoch, 1966; Rajam et al., 1966).have developed extensive fractionation

schemes for water soluble proteins from human brain, and have reported the existence of three brain specific antigens among basic protein fractions, but to date, none of these has been extensively purified or characterized.

Antisera to squid axoplasm proteins were prepared by Huneeus-Cox (1964). The six antigenic components demonstrated in immunodiffusion were not present in squid blood, but no other tissues were studied. A qualitative separation of squid axoplasm proteins by polyacrylamide gel electrophoresis showed at least fourteen discrete components.

The only water soluble protein specific to nerve tissue which has been extensively purified is an acidic protein, named the "S-100" protein because of its solubility in 100% saturated ammonium sulfate at neutral pH. It was originally identified by Moore (Moore and McGregor, 1965; Moore, 1965) because of its rapid mobility in starch gel electrophoresis. No protein with a corresponding mobility could be detected in a similarly prepared extract of liver. The "S-100" protein was found to be not antigenic in its native state. By treating purified "S-100" with methylated bovine serum albumin, however, both complement-fixing and precipitating antibodies could be prepared, and the antisera thus produced reacted with the native protein (Levine and Moore, 1965). It could then be shown that "S-100" was indeed specific to nerve tissue, and not species specific. It was subsequently shown that this acidic protein was present primarily in glial cell cytoplasm, although possibly present in neuronal nuclei as well (Hyden and McEwen, 1966).

The potential significance of nerve specific antigens is indicated by experiments described earlier in which antisera to nerve were reported to affect neural activity. In view of the demonstration by several workers that antisera against nerve tissue do contain some nerve specific components in addition to several components not specific to nerve, it is tempting to postulate that the effects produced by the complex antisera employed in physiological experiments were due to the presence of nerve-specific antibodies. This in turn would suggest that the corresponding antigen had a specific role in excitability. It appeared to the author that an extension of these studies was necessary in order to examine this hypothesis critically. It would be necessary to obtain

physiological effects with a univalent antiserum, containing antibodies to a single antigen. In order to prepare a specific antiserum, it would be necessary to purify the antigen. It would also be necessary to show that antisera not containing that antibody component, however complex, did not have any effects on excitability. A chemical characterization of the antigen would also be necessary to suggest the precise way in which the molecule might be involved in excitability. One of the purposes of the experiments described in this thesis, therefore, was to purify and characterize a nerve-specific antigen, so that an investigation of the precise role of a specific molecule in neural activity could be attempted.

4. Protein Fractions Preferentially Affected by Alterations in Nerve Activity There have been few attempts to identify particular proteins of the nervous system by examining the effects on the in vivo synthesis of individual proteins of alterations in nervous activity. It has been reported that agents affecting the electrical activity of brain tissue also affect the incorporation of amino acids into total brain proteins. For example, Gaitonde and Richter (1956) reported a decreased incorporation of (^{35}S) methionine into rat brain proteins during ether and pentobarbitone anesthesia, insulin hypoglycemia, and electrical stimulation. The magnitude of the decrease relative to control animals was between 19 and 34 percent. One study has examined individual protein fractions under similar conditions. Dignman et al. (1959) measured (^{14}C) proline incorporation into seven fractions of total rat brain proteins after electroconvulsive shock treatment, but reported no differences between control and experimental groups in any of their fractions.

The use of such means of altering nervous activity as those mentioned above have several disadvantages. They can be expected to affect tissues other than nerve, thus making it difficult to distinguish direct effects on brain from indirect effects mediated through the blood supply. Further, a separate group of animals must be used as controls. The latter disadvantage leads to two additional variables. The extent of individual variation in "normal" rate of amino acid incorporation into protein, and statistically significant variation in dose of labeled amino acid administered can limit the magnitude of the change in incorporation due

to a given experimental situation. Also control and experimental animals may respond variably to the stress produced by the injection of isotope and by other handling during the incorporation period. In fact, a recent series of experiments has indicated that some if not all of the observed effects of more physiological alterations in nerve activity, such as motor exercise and visual training, on incorporation of radioactive amino acids into brain proteins can be attributed to differences in general stress (Altman and Das, 1966; Das and Altman, 1966; Atlman et al., 1966).

On the other hand, the use of drugs and of electroconvulsive shock has the advantage of affecting a sufficiently large amount of nerve tissue to make an examination of individual fractions of brain proteins feasible.

An examination of various means of altering neural activity in order to limit the disadvantages mentioned above led to the choice of unilateral spreading cortical depression (SCD) for the present experiments. As will be shown below, SCD provides a suitable experimental design for an investigation of alterations of amino acid incorporation into individual fractions.

The phenomenon of spreading depression was originally described by Leao (1944), and has been thoroughly reviewed by Marshall (1959) and by Ochs (1962). The most important aspects of spreading depression are: 1) it consists of a wave of decreased spontaneous electrical activity which spreads across the entire cortex from a single point of stimulation. This wave can be elicited by mechanical or electrical stimulation, and also by the application of potassium chloride solutions to the cortex (Leao, 1944). Of these means, potassium chloride has been widely used and is generally the most reliable (Marshall, 1959). 2) Spreading depression, when initiated in the neocortex, spreads throughout it, but not to the hippocampus (Weiss and Fifkova, 1960). Most pertinent, is the fact that it does not spread to the opposite hemisphere (Bures, 1959; Russel and Ochs, 1963). Although low concentrations (0.1 - 0.2%) of KCl will elicit spreading depression, higher concentrations (10-25%) are necessary to depress electroencephalographic activity for periods as long as 3 to 4 hours (Bures and Buresova, 1956; Ochs, 1962).

Although spreading depression was originally investigated in anesthetized animals, it could also be demonstrated in conscious animals by means of chronically implanted electrodes. In recent years, such chronic preparations have been used extensively for the production of KCl induced spreading depression.

Unilateral spreading depression can thus be seen to have several advantages over other experimental designs for a study of the effects on protein synthesis of alterations in electrical activity. It involves a sufficiently large amount of nervous tissue to permit fractionation of proteins, while leaving unaffected an equally large amount of tissue, thus making possible the use of the same animal as its own control. The disadvantages of procedures such as electroconvulsive shock mentioned above are thereby avoided.

Only one report has appeared describing the effect of spreading depression on the in vivo amino acid incorporation into brain proteins. Ruscak (1964) reported a lower specific activity of the total proteins in the depressed hemisphere relative to the control hemisphere in anesthetized rats after injection of (^{35}S) methionine. He did not, however, use a chronic preparation, nor did he look at any subfractions of the total proteins. An extension of this experimental design therefore seemed to be well suited for a comparison of the biosynthesis of individual protein fractions during altered neural activity. Part of this thesis will be concerned with experiments investigating the in vivo incorporation of amino acids into fractions of brain proteins during unilateral spreading depression in conscious rats.

II. EXPERIMENTAL

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A. Purpose of the Experiments

The functions of the nervous system have become increasingly well described in terms of the actions of specific chemicals, elements and compounds. Despite the considerable information presently available about the role of inorganic cations and chemical transmitters in the propagation of the action potential, a complete description of the events occurring at the excitable membrane in terms of specific molecules is thus far not possible. Even less is known about the chemical changes accompanying long-term memory. It is generally thought that macromolecules, such as proteins and nucleic acids, have specific roles in these neural activities. With the exception of a few enzymes, however, no proteins have been identified, which have an activity that could extend the molecular description of nerve function.

The experiments described in this thesis were undertaken to identify specific proteins that might be of importance in those physiological activities unique to nerve tissue. This was done by developing fractionation procedures for the soluble proteins of rat brain, and concurrently applying two methods for screening the fractions for individual proteins of sufficient potential interest to merit their complete purification and characterization. One method was to examine the *in vivo* incorporation of amino acids into soluble proteins with the aim of identifying individual fractions whose synthesis was preferentially affected by alterations in neural activity. In order to alter the electrical activity of the brain in a carefully controlled way, the experimental design consisted of the use of unilateral spreading cortical depression in conscious animals. The other method was to identify nerve-specific antigens, using immunological techniques, on the hypothesis that any protein present only in nerve tissue would be a good candidate for a specific role in nerve activity.

B. Materials and Methods

1. Animals Except as indicated, adult, male, Sprague-Dawley rats were used in all experiments. They weighed generally between 250 and 350 gm, and were purchased mostly from the Blue Spruce Farms (Altamont, N. Y.). In some experiments other strains of rats were used. Long Evans Hooded were obtained from The Research Animal Corporation, Pittsburgh, Pa. The following lines were obtained from Dr. David Krech, Department of Psychology at the University of California at Berkeley, where they were developed (see Bennett et al., 1966): HK, HG, LK, LG, S_1 and S_3. These lines will be described in section C. Balb C mice were supplied by the Jackson Laboratories, Bar Harbor, Me.; guinea pigs were selected at random from The Rockefeller University Animal House, and female, white, New Zealand rabbits, weighing two kilograms were purchased from Carver's Rabbitry, Somerville, N. Y.

Beef brains were obtained from Max Insel Cohen Co., Newark, N. J. Samples of human brain were obtained from Harvard University Medical School through the courtesy of Dr. William Sweet of the Department of Neurosurgery.

2. Reagents and Buffers All inorganic salts were reagent grade. The following reagents used for polyacrylamide gels were obtained from Eastman Organic Chemicals, Rochester, New York: Acrylamide, N,N'-Methylenebisacrylamide, N,N,N',N'-Tetramethylethylenediamine. Riboflavin, U. S. P. was obtained from Merck and Co., Rahway, N. J. Tris(hydroxymethyl)aminomethane ("Trizma Base") was purchased from Sigma Chemical Co., St. Louis, Mo.

All phosphate buffers were made up from the sodium and potassium salts. Ionic strength was calculated in molar units. Barbital buffer, pH 8.6, ionic strength 0.05, was prepared by adding solid barbituric acid to a 0.05 M solution of NaOH. For Tris buffers, a solution of the desired molar concentration of "Trizma Base" was prepared, and the pH adjusted with HCl. Na acetate, pH 5.0, ionic strength 0.1, and pH 4.7, ionic strength 1.0 were prepared by adjusting with acetic acid the pH of 0.1 M and 1.0 M NaOH respectively.

3. Removal of Brain Tissue Animals were fasted for tweleve hours and anesthetized with sodium pentobarbital (Nembutal or Diabutal). To obtain brains free of blood, a cardiac perfusion with 0.9% NaCl (saline) was performed. The heart was exposed and the right atrium opened. An 18-gauge needle attached to the saline reservoir was inserted into the left ventricle. The height of the reservoir was about 50 inches above the animal, to approximate the blood pressure, and flow was continued until the perfusate was colorless. The brain was then quickly exposed and removed, rinsed in saline, blotted, and frozen in a mixture of dry ice and acetone. When whole brains were used, a transverse section was made through the medulla at the level of the foramen magnum, and another transverse section made through the olfactory bulbs at the anterior tip of the cerebrum. When the brain was subdivided, the following additional sections were made in situ: a transverse section through the posterior colliculus to separate forebrain and hindbrain, and a sagittal section through the longitudinal fissure, to separate left and right hemispheres. In some experiments, the hemispheres were further divided roughly into "cortex" and "brainstem." This was done after freezing the separated left and right hemispheres, by prying away the brainstem along a crack which consistently developed during the freezing procedure and extended anteriorly and ventrally from the choroid fissure into the recesses of the third ventricle. This led to the operational definition of the "brainstem" as thalamus, hypothalamus and midbrain, and "cortex" as the remaining tissue. Thus the portion designated "cortex" contained the entire telencephalon, including the basal ganglia.

4. Preparation of Rat Brain Extracts (RBE) The frozen tissue was weighed and homogenized in a Tenbroeck Tissue Grinder in either 2 ml or 10 ml per gram of Na-K phosphate buffer, pH 7.4, ionic strength 0.1 at 0-4°C. The homogenates were centrifuged for one hour at 50,000 x g in the No. 40 rotor of the Spinco Model L centrifuge. The clear supernate containing the soluble protein was retained; the sediment was discarded. The supernate is hereafter referred to as RBE (rat brain extract).

5. Determination of Protein Concentration Protein concentration of solutions was determined with the phenol reagent according to the method of Lowry et al. (1951) or by absorbance at 280 mμ. Bovine serum

albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was used as a standard. To determine the protein content of trichloracetic acid (TCA) precipitates, the method of Lowry et al. was slightly modified. TCA precipitates were dissolved in 97-100% formic acid. An aliquot of the formic acid solution containing an appropriate amount of protein was placed in a test tube, and the formic acid was evaporated by drying in an oven at 80-90°C. The residue then dissolved readily in 1 N NaOH, and the protein determination was completed in the usual way by omitting NaOH from the reagents. Bovine serum albumin was treated in the same manner, and the standard curve agreed well with that obtained by the conventional method.

6. Preparation of Antisera Rabbits were used for the preparation of all antisera. An emulsion was prepared of 0.5 to 1.0 ml antigen solution with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Subcutaneous injections of the emulsion were made at approximately three-week intervals for up to nine months. The rabbits were bled either from an ear vein or by cardiac puncture. The sera were stored either frozen or at 0-4°C after addition of Merthiolate (Lilly) to a final concentration of 0.01% (wt/vol). When used in agar gel diffusion, the sera were first dialyzed against phosphate buffer, pH 7.4, ionic strength 0.05. Rabbit antiserum to "S-100" protein was the gift of Dr. Lawrence Levine of Brandeis University.

7. Analysis of Antisera and Antigenic Activity Rabbit sera were tested for precipitating antibody against the antigen solution using the qualitative microcapillary precipitin method given by Kabat and Mayer (1961). Serum from unimmunized animals, and solvent without antigen were used as controls. For a more sensitive test, double diffusion in agar on glass microscope slides was performed (Scheidegger, 1955). One percent agar (Special Noble Agar, Difco Laboratories, Detroit, Mich.) was prepared in either Na-K phosphate buffer, pH 7.4, ionic strength 0.05, or in pH 8.6 barbital buffer, ionic strength 0.05. Merthiolate (Eli Lilly and Co., Indianapolis, Ind.) was added (0.01% wt/vol) to prevent bacterial growth.

For analysis of mutliple antigenic components, immunoelectrophoresis was carried out in agar prepared in the same way as for double diffusion, usually with the pH 7.4 buffer system. Electrophoresis was at a potential

gradient of 6 volts/cm for 90 minutes. Precipitin arcs developed generally within 12-24 hours, but slides were observed for several days to detect weakly reactive components.

8. Disc Electrophoresis on Polyacrylamide Gels Standard analytical gels, 7% acrylamide monomer concentration, pH 8.3 Tris-glycine buffer system, were prepared according to the procedure of Davis (1964) in glass tubes 6 mm I.D. Usually no sample gel was prepared; instead the sample was made to about 20% in sucrose and layered directly over the spacer (large pore) gel. Electrophoresis was carried out at a constant current of 1 to 3 milliamps (mA) per tube for about 3 hours. After electrophoresis gels were stained either in Amido Black 10B according to Davis (1964) or in Coomassie Blue using the method of Chrambach et al. (1967).

For some purposes larger gels were prepared in tubes of 12.5 mm I.D. The gel concentration and buffer system was the same as for the analytical gels. Electrophoresis was done at 6 to 7 mA per tube for 6 hours and the gels were usually cut into cross sectional slices, 2.5 mm thick, with a gel slicer designed for that purpose. A small-pore gel 10 cm long thus yielded 40 slices. The material in each slice was then eluted by soaking in 1-2 ml of buffer, or the slices were prepared for the determination of radioactivity using the method of Boyd and Mitchell (1966).

9. Zone Electrophoresis on Starch This was carried out according to Kunkel (1954) except that pH 7.4 phosphate buffer was used rather than pH 8.6 barbital. The size of the starch block was 15" x 18" x 1/4" to 1/2". Up to 800 mg of protein in a volume of 15 ml was applied. Electrophoresis was carried out for 16 to 24 hours at a potential gradient of 20 volts per inch. After electrophoresis, the starch was cut into 1/2" strips and eluted with phosphate buffer. For smaller amounts of material (25 mg in approximately 1.5 ml), a starch block of 2" x 18" x 1/4" was used.

10. Starch Gel Electrophoresis in Urea Vertical starch gel electrophoresis was carried out according to Smithies (1959). The starch was made 8 M in urea, and the formate buffer system of Edelman and Poulik (1961) was used. Hydrolyzed starch was obtained from Connaught Medical Research Laboratories, Toronto, Canada. Electrophoresis was carried out at a

potential gradient of 6 volts/cm for 16 hours. The gel was stained in 0.2% Amido Black 10B.

11. Column Chromatography on DEAE-Cellulose Diethylaminoethyl-cellulose ("Cellex D," Bio Rad Laboratories, Richmond, Calif.) was prepared according to Peterson and Sober (1962), and equilibrated with Na-K phosphate buffer, pH 7.2, $\Gamma/2 = 0.01$. A column 1.0 cm in diameter was packed to a height of 50 cm. The sample was applied in the same buffer and a linear ionic strength gradient, from 0.11 to 0.51 was used for elution. NaCl was added to the starting buffer to obtain the initial and final ionic strengths. Two hundred ml of the 0.51 ionic strength buffer was run into a mixing chamber initially containing 200 ml of the 0.11 ionic strength solution.

12. Gel Filtration Sephadex G-100 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.05 M Tris, 0.85% NaCl, pH 7.8-8.0. Column dimensions were 1.0 cm x 110 cm and the sample volume was usually 1 ml.

13. Analytical Ultracentrifugation All experiments were performed with a Spinco Model E Ultracentrifuge. Sedimentation velocity experiments on antigen α were carried out with phase plate schlieren optics. The sedimentation coefficient, s, was calculated according to the definition of Svedberg and Peterson (1940):

$$s = \frac{dx/dt}{\omega^2 x}$$

where ω is the angular velocity, x is the distance from the center of rotation, and dx/dt is the velocity of sedimentation. The s values were corrected to water at 20°C, and are thus expressed as $s_{20,\omega}$. In view of the polydispersity, no attempt was made to calculate $s^{o}_{20,\omega}$.

Apparent molecular weight of antigen α was determined according to the high speed equilibrium method of Yphantis (1964), for dilute solutions, using interference optics.

$$M_{app} = \frac{2RT}{\omega^2(1-\bar{V}\rho)} \cdot \frac{d(\ln c)}{d(x)^2}$$

Where R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{V}$ is the partial specific volume of the solute, ρ is the

density of the solvent, c is the solute concentration, and x is the distance from the center of rotation. $\bar{V}$ was taken as 0.73 as calculated from the amino acid composition of antigen α. Kielley and Harrington (1960) have shown for myosin and ribonculease that $\bar{V}$ in water and in 5 M guanidine-HCl differ by only 1%, and this difference was ignored in the present calculations.

14. Determination of Amino Terminal Residue The dansyl end group technique of Cunningham et al. (1968) was used.

15. Determination of Amino Acid Composition This was performed according to the method of Spackman, Stein, and Moore (1958), using the automatic amino acid analyzer (Beckman Model 120C) equipped with an integrator (Infotronix Model CRS-11AB). Triplicate samples were hydrolyzed with 6 N HCl for 20, 44, and 68 hours. The values for threonine and serine were obtained by extrapolation to zero time. Those for valine, isoleucine and leucine were obtained by averaging the results obtained from the 44 and 68 hour hydrolysates. For all other amino acids except half-cystine the three hydrolysates agreed well, and were averaged. Half-cystine was determined as cysteic acid after oxidation with performic acid according to Moore (1963). Tryptophan content was not determinted.

16. Carbohydrate Determination Total hexose was measured with the anthrone reagent (Mokrash, 1954) according to the procedure of Muller-Eberhard and Kunkel (1956). A solution consisting of 9 parts each of mannose and galactose and 2 parts of fucose was used as a standard.

17. Two-Dimensional High Voltage Electrophoresis of Tryptic Hydrolysates (Peptide Mapping) The procedure of Schwartz and Edelman (1963) was used. After overnight digestion with trypsin in 0.05 M ammonium bicarbonate, the tryptic peptides were applied to Whatman 3MM filter paper. Electrophoresis in the first dimension was carried out in pyridine acetate buffer, pH 4.7 (pyridine: acetic acid: water, 25: 25: 950) for one hour at a potential gradient of 50 volts/cm. Electrophoresis in the second dimension was performed in formate-acetate buffer, pH 2.0 (acetic acid: 88% formic acid: water, 87: 25: 888) for 3.5 hours at a gradient of 20 volts/cm. The paper was then dried in a stream of warm air and stained with ninhydrin.

18. Assay for Acid Protease Activity The procedure described by Marks and Lajtha (1965) was followed. The 30 minute incubation at 37°C was carried out at pH 3.8 in Na-Acetate buffer. Two milligrams of hemoglobin (Hemoglobin substrate powder, Worthington Biochemical Corp., Freehold, N. J.) per 1.0 ml reaction mixture were used as substrate. For control tubes, hemoglobin was omitted. The reaction was stopped by addition of 0.5 ml 18% TCA, and the amount of α-amino group in 1.0 ml of TCA supernate measured with ninhydrin, using leucine as a standard.

19. In Vivo Incorporation of Amino Acids Animals were fasted for about 12 hours before incorporation experiments. Leucine-4, 5-H^3 (Tracerlab, Waltham, Mass.), specific activity 5C/mM, was injected intraperitoneally in a dose of 3.5μc per gram body weight, at a concentration of 1 mc/ml in 0.15 M Tris buffer, pH 7.5. During the incorporation period the animals were kept in individual cages. Three to five minutes before the scheduled end of the incorporation period, generally from 15 minutes to three hours, the animals were given Na Pentobarbital, perfused, and their brains removed as described above in section 3. In most experiments the brain was subdivided into cortex and brainstem. Hind brain was discarded.

20. Measurement of Radioactivity Specific activity of RBE proteins was determined in the following way. RBE was prepared using 10 ml phosphate buffer (pH = 7.4, $\Gamma/2$ = 0.1) per gram tissue. An aliquot of the extract was added to 0.5 volume of 30% TCA. The precipitate was separated by centrifugation and washed in 10% TCA by resuspension and centrifugation. The precipitate was dissolved in 97-100% formic acid and 0.2 ml of the formic acid solution was added to 20 ml of scintillation fluid consisting of 50 gm naphthalene, 500 ml dioxane, 70 ml Liquifluor (Pilot Chemicals Inc., Watertown, Mass.) brought to 1 liter with toluene. The samples were counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.) equipped with an external standard, and corrected for quenching due to formic acid. Self absorption or quenching due to protein was found to be negligible. Protein concentration was determined either in the original RBE or in the formic acid solution. Specific activity (CPM/mg) was calculated from the protein concentration (mg/ml) and the CPM (in protein) per ml.

Acid soluble radioactivity was determined by adding an aliquot of the TCA supernate directly to the scintillation fluid. The efficiency of counting was 25% in unquenched samples.

21. Preparation of Rats with Cranial Cannulas Under Na Pentobarbital (Diabutal, Diamond Laboratories, Des Moines, Iowa), anesthesia, two trephine holes, 3 mm in diameter, were drilled into the skull just anterior to the lambdoid suture and on either side of the sagittal suture, leaving the dura intact. Nylon cannulas, having an internal diameter of 2 mm were fitted into the openings and held in place with denture acrylic (Cold Cure, Hygenic Dental Manufacturing Co., Akron, Ohio). Two stainless steel screws, generally placed just anterior to the bregmatic suture, served to hold the acrylic to the skull. The cannulas were filled with sterile 0.9% NaCl (saline) and fitted with plugs made from polyethylene tubing. The detailed construction of this assembly is shown in Figure 1.

C. Preliminary Examination of Soluble Brain Proteins

1. Soluble Protein Content of Rat Brain Whole rat brains which were free of blood were homogenized in 10 ml phosphate buffer (pH 7.4; $\Gamma/2 = 0.1$). Total brain protein was determined by drying an aliquot of the homogenate at 80-90°C and treating the residue in the same way as TCA precipitates (described in Methods). A value of 140 mg protein per gram wet weight of brain was obtained. Total soluble protein was defined as that protein in RBE which was detectable by the method of Lowry et al. (1951). Forty-five mg per gram wet weight, or about 30% of the total protein was included in RBE.

2. Qualitative Comparison of Soluble Proteins in Acrylamide Gels This comparison was made in order to see if there were any consistent similarities or differences in the soluble protein components of brains from different animals or species or in different regions of the nervous system, which might lead to the identification of a component of particular interest.

a. Different tissues Extracts of several rat tissues were prepared in the same way as described in "Methods" for brain. Aliquots of each were subjected to electrophoresis on polyacrylamide gels. Two examples of the resulting stained gels are shown in Figure 2. Each tissue gave

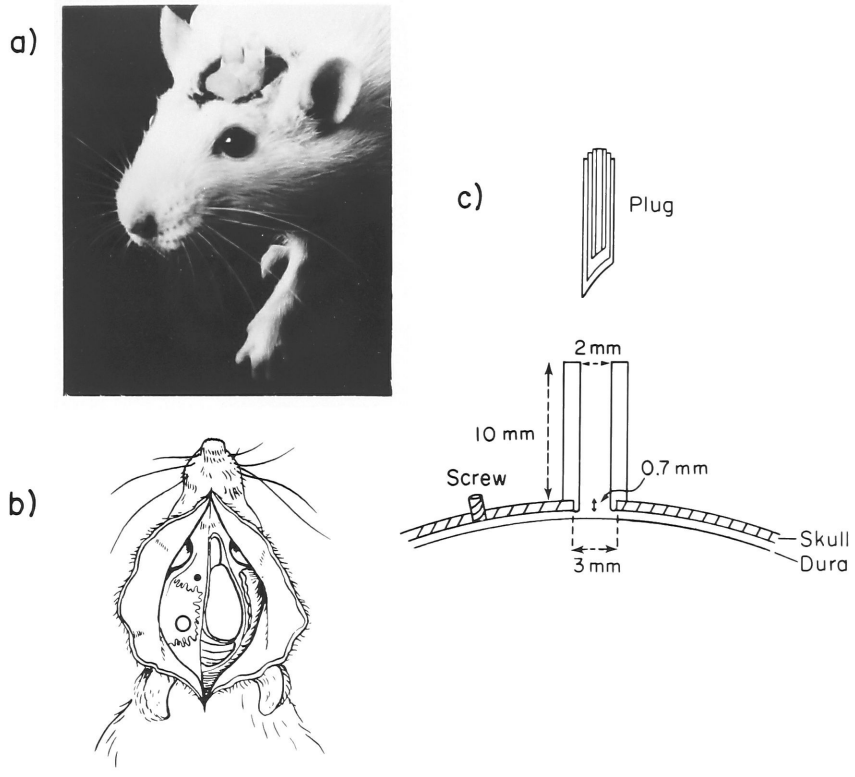

Figure 1. a) Rat with bilateral cannulas. b) Position of trephine hole (O) and screw (●) with respect to cranial sutures. Skull has been cut away on right side to show corresponding position on surface of the brain. c) Sagittal section through a cannula. The plugs were made from polyethylene tubing (size PE 50, 100, and 200) cut so that the plugs did not contact the dura.

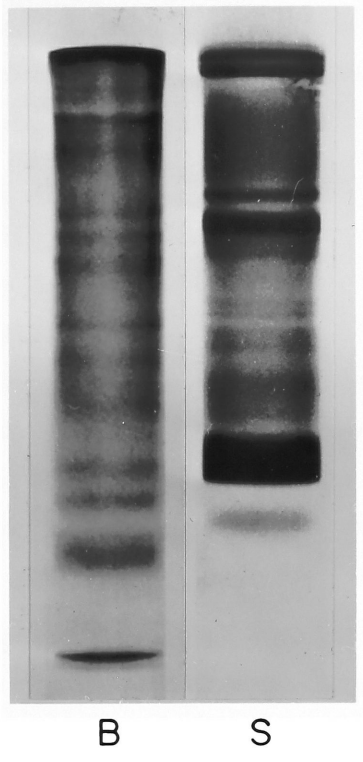

Figure 2. Analytical acrylamide gels comparing rat brain extract (B) and rat serum (S). The origin is at the top. Migration was towards the anode, at the bottom. The lowest band in B corresponds to the buffer front and dye marker. This band is not visible in S, but was at the same position in both gels.

rise to its own characteristic pattern, which differed considerably from all other tissues. This result gave support to the hypothesis that there may be soluble proteins in nerve tissue that are not present in other tissues. At least 15 discrete bands could be discerned in brain extract, superimposed on a background of unresolved protein; no one component predominated. A considerable amount of protein did not enter the gel, resulting in an intensely stained sample gel, or in an intense band at the top of the spacer gel, when the sample gel was omitted.

b. Different animal species The patterns obtained with brain extracts from several different animal species are shown in Figure 3. Each species gave rise to its own characteristic pattern, but there were several bands shared by all. The appearance of a band in the same position with different extracts does not, however, necessarily mean that it represents the same protein, especially in a complex mixture of many different proteins, such as the brain extract. Thus additional information is necessary before assigning any particular significance to the proteins having the same electrophoretic mobility from brains of different animals.

c. Different strains Extracts of brains from several different rat strains were compared in the same way. The strains examined were Sprague-Dawley, Long Evans Hooded, and some lines developed at Berkeley. The latter included strains selected for high acetylcholine (HK + HG), low acetylcholine (LK + LG), Tryon maze bright (S_1), which are high in both acetylcholine and acetylcholinesterase, and Tryon maze dull (S_3), which are low in both acetylcholine and acetylcholinesterase. Both males and females were included. Representative gels are shown in Figure 4. Brain extracts of all strains resulted in identical gel patterns. Thus, the differences in the behavior and brain chemistry of the strains (see Bennett et al., 1966) are not reflected in qualitative or gross quantitative differences in soluble brain protein composition.

d. Different regions of nervous system Different areas of the rat central and peripheral nervous system were also compared. Extracts of cortex, brainstem (defined in Methods section), cerebellum spinal cord, and sciatic nerve were prepared. The acrylamide gel patterns are shown in Figure 5. All areas of the central nervous system resulted in identical patterns.

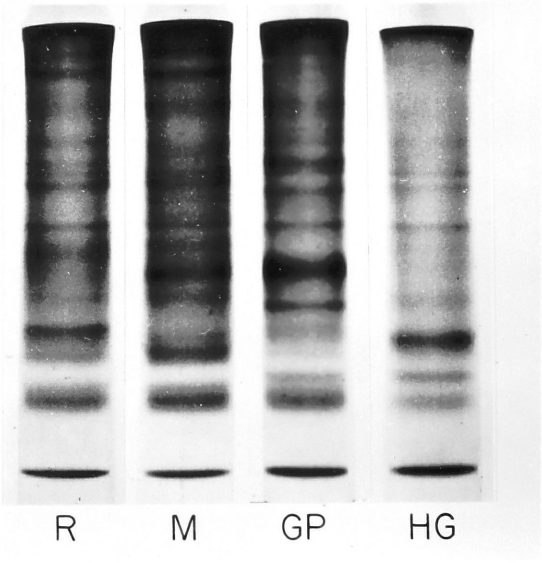

Figure 3. Analytical acrylamide gels of brain extracts from rat (R), mouse (M), guinea pig (GP) and human gray matter (HG). Origin is at the top, anode at the bottom.

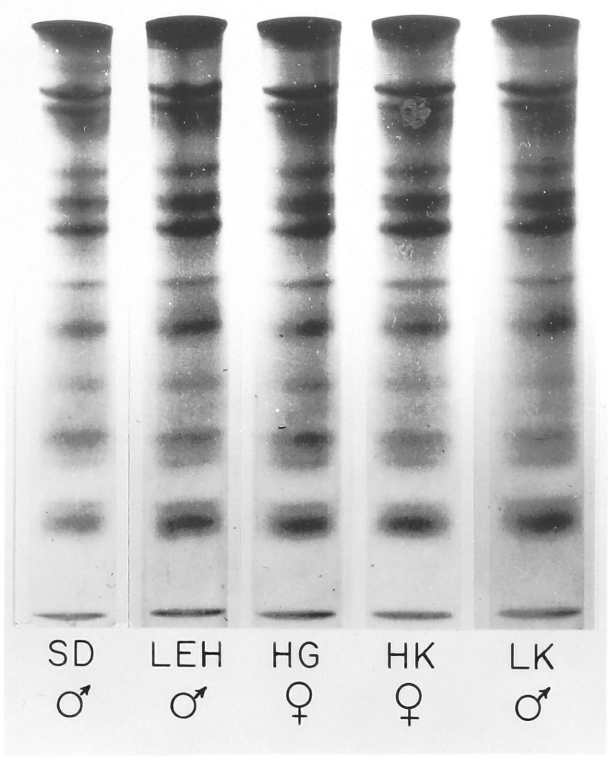

Figure 4. Analytical acrylamide gels of brain extracts from different strains of rats. SD, Sprague-Dawley; LEH, Long-Evans Hooded, HG and HK, two different Berkeley lines inbred for high acetylcholine; LK, Berkeley strain inbred for low acetylcholine. Origin is at the top; anode at the bottom.

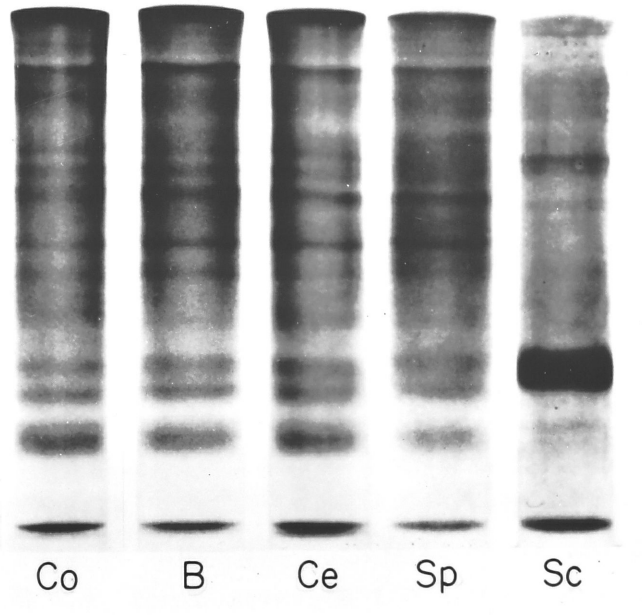

Figure 5. Analytical acrylamide gels of extracts from different regions of rat nervous system. Co, cortex; B, brainstem; Ce, cerebellum; Sp, spinal cord; Sc, sciatic nerve. Origin is at the top; anode at the bottom.

Peripheral nerve (sciatic), however, appeared to be quite different. Some of the difference in soluble protein components of sciatic nerve may be attributed to two additional variables: 1) the presence of large amounts of connective tissue in peripheral nerve and 2) the difficulty in preparing an extract of peripheral nerve. It is extremely difficult to homogenize, and the extract applied to the gel shown in Figure 5 was prepared by sonication. This pattern is therefore not strictly comparable to the others.

In the case of human brain, smaller and more precisely defined areas could be examined. Several samples of gray matter, white matter, "normal" and tumor (astrocytoma) infiltrated tissue from a single brain were obtained at autopsy. Examination of extracts of each of these on acrylamide gels showed no qualitative and no obvious quantitative differences.

3. Fractionation of Soluble Proteins by Isoelectric Precipitation

Rat brain extracts were brought to pH 5.0 either by dialysis against sodium acetate buffer, pH 5.0, ionic strength 0.1, or by addition of 0.075 volume sodium acetate pH 4.7, ionic strength 1.0. The precipitate formed was separated by centrifugation. The pH 5 insoluble proteins could be redissolved by suspension in phosphate buffer, and raising the pH to 8 or 9 with the addition of NaOH. They then remained in solution after dialysis against pH 7.4 phosphate buffer.

The pH 5 soluble and insoluble fractions of the soluble brain proteins showed several interesting properties. Whole RBE was compared on polyacrylamide gels with pH 5 soluble and insoluble fractions (Figure 6). With the exception of two rapidly migrating bands, all of the discrete bands seen in RBE are present in the pH 5 soluble fraction, and completely absent in the pH 5 insoluble fraction. Furthermore, the material in RBE that does not enter the gel (see section 2a) is absent from the pH 5 supernate. This material, and also some protein that does enter the gel, but is not resolved into discrete bands, makes up a major proportion of the pH 5 insoluble fraction. In attempts to fractionate the pH 5 insoluble proteins by gel filtration on Bio-Gel (Bio Rad Laboratories, Richmond, Calif.) it was found that most of this material was bound tightly to the gel and could not be eluted. Since Bio-Gel is composed of acrylamide,

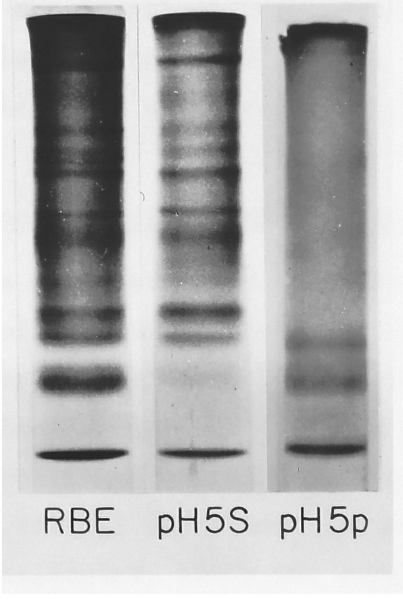

Figure 6. Analytical acrylamide gels comparing whole rat brain extract (RBE), pH 5 supernate (pH 5S) and redissolved pH 5 precipitate (pH 5 p). Origin is at the top; anode at the bottom.

this result indicated that the failure of a large amount of protein to enter the polyacrylamide gels during electrophoresis may be due to specific binding to polyacrylamide rather than to size or to lack of net charge.

Extracts of cortex and brainstem were compraed with respect to amount of protein in each of these fractions (Table II). While the content of pH 5 soluble proteins was identical in both regions, brainstem contained 30% less pH 5 insoluble proteins than cortex. The pH 5 insoluble fraction accounts for 55% of the total soluble protein in cortex.

D. Amino Acid Incorporation Into Soluble Brain Proteins In Vivo

These experiments were performed in order to determine whether the rate of biosynthesis of some brain proteins was preferentially altered as a result of the changes in the electrical activity of the brain occurring during spreading cortical depression. Before presenting these results some studies will be described of the kinetics of amino acid incorporation into fractions of the soluble proteins in cortex and brainstem. Antigen α was not examined in particular, since the purpose was to identify additional nerve proteins of potential significance. The antigen was, however, present in the fractions studied.

1. Kinetics of ^{3}H-Leucine Incorporation The change with time in specific activity of protein and in TCA soluble radioactivity in "cortex" is shown in Figure 7. The specific activity of protein increased linearly for about 30 minutes, then increased more slowly for an additional 30 minutes, and leveled off thereafter. TCA soluble radioactivity attained a maximum value within 15 minutes and declined thereafter. Incorporation periods shorter than 15 minutes or longer than three hours were not examined. Due to considerable variation between individual animals within a given incorporation period, the curves shown in Figure 7 are only approximate.

When "brainstem" proteins were compared with "cortical" proteins of the same animal, no differences were found in the shape of the kinetic curve or in the magnitude of specific activity. Also, no differences were detected between pH 5 soluble and insoluble proteins.

Some preliminary experiments were performed in which the ^{3}H-leucine labeled pH 5 soluble proteins obtained after several different incorporation periods were separated by electrophoresis on large polyacrylamide gels.

TABLE II

Solubility of Rat Brain Extract Protein as a Function of pH

(Mg/gm Wet Weight)

	A Total	B pH 5 Soluble	C pH 5 Insoluble
Cortex	43.5	19.4	24.6
Brainstem	36.4	19.6	17.2

Protein was determined with the phenol reagent according to the method of Lowry *et al*. (1951). For the pH 5 insoluble proteins, the modification for TCA precipitates was used.

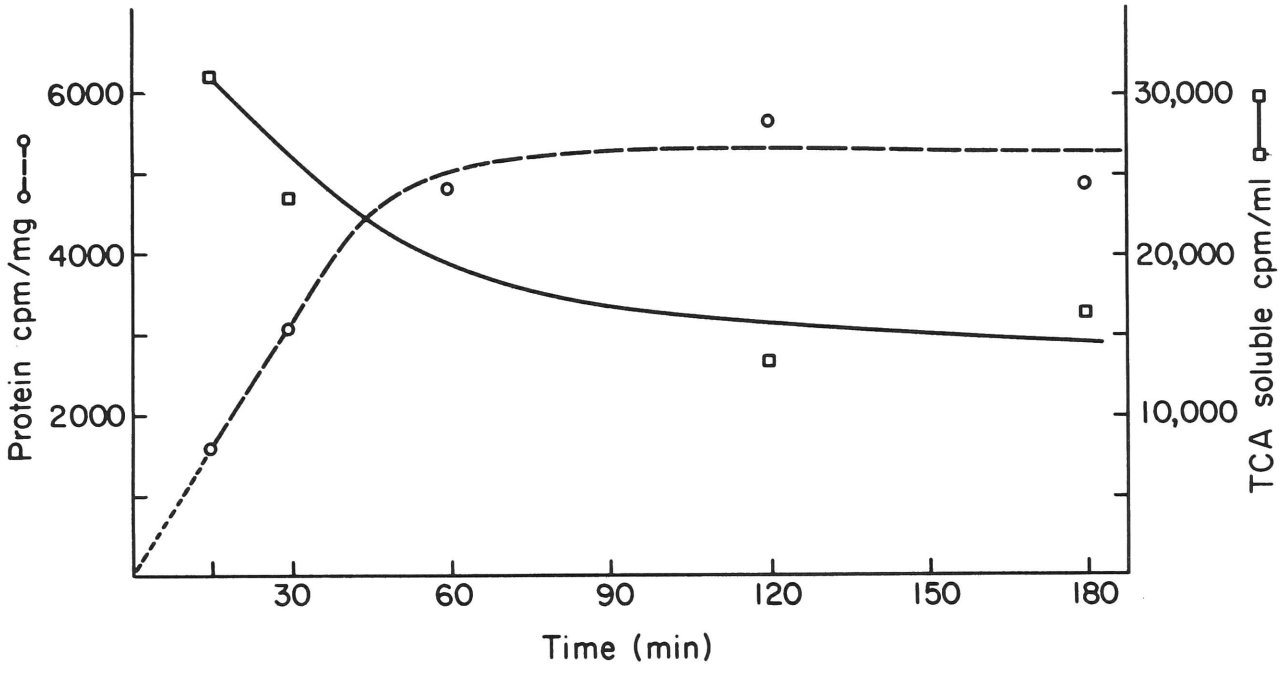

Figure 7. Change with time in specific activity of total soluble proteins (o--o) and in acid soluble radioactivity (□—□) of rat cortex, following intraperitoneal injection of 3.5μc/gm body weight of ^{3}H-Leucine. Each point is the mean of from two to seven animals.

The gels were fixed in 5% acetic acid, but not stained, and then cut into slices for counting. Separate experiments showed that stained large gels gave the same band pattern as the analytical size gels. Protein content of each slice was not determined, but if the intensity of staining is assumed to reflect the protein concentration, specific activity could be estimated by comparing the counts in each slice with the dye intensity of the corresponding area of a stained gel. Although the analysis was severely limited by this assumption and by the low absolute value of counts per minute in each slice, there was no indication of any particular subfraction of the pH 5 soluble proteins whose specific activity or rate of synthesis differed greatly from the average value obtained for the total pH 5 soluble proteins.

2. Effect of Spreading Cortical Depression on ^{3}H-Leucine Incorporation

For these experiments, rats with cranial cannulas were used, prepared as described in section B. Unilateral spreading depression was elicited by filling one cannula with a 10% KCl solution. The test for the presence of spreading depression was the loss of the placing response in the contralateral hind limb (Bures, 1959). The isotope was not injected until the existence of spreading depression had been verified by this test--usually about 20 minutes after application of KCl.

Results for cortical proteins are given in Table III. In initial experiments a two-hour incorporation period was chosen in order to obtain a maximal protein specific activity, and the animals divided into two groups. For group A, both cannulas were rinsed with saline. For group B, the left cannula was rinsed with saline and the right with 10% KCl. Both groups were otherwise treated identically, including an occasional test of the placing response in both hind limbs. When saline was applied to both hemispheres (A), cortical proteins had an equal specific activity in both hemispheres, as shown by a right-to-left specific activity ratio of 1. In animals with unilateral spreading depression (B), the specific activity of cortical proteins on the depressed side was only 86% of that on the control side. The values of the protein specific activity ratio for these animals were significantly different from 1 and also from the corresponding ratio for group A. Differences between pH 5 soluble and insoluble proteins were not significant. TCA soluble radioactivity was

TABLE III

Effect of Spreading Depression on Incorporation of ^{3}H-Leucine into Cortical Proteins

	Specific Activity - Right* / Specific Activity - Left			CPM/ml Extract - Right / CPM/ml Extract - Left
	Total Protein in Extract	pH 5 Insoluble Protein	pH 5 Soluble Protein	TCA Soluble
A. NaCl on right and left cortex N = 6 2 hr incorporation	1.01 ± 0.06 $p > .6$	1.02 ± 0.07 $p > .4$	1.01 ± 0.06 $p > .6$	0.97 ± 0.04 $p > .3$
B. KCl on right cortex NaCl on left N = 7 2 hr incorporation	0.86 ± 0.05 $p < .01$	0.87 ± 0.05 $p < .01$	0.84 ± 0.08 $p < .01$	1.03 ± 0.04 $p > .1$
C. KCl on right and left cortex N = 1 2 hr incorporation	0.99	1.00	0.95	1.05
D. KCl on right cortex NaCl on left N = 2 30-min incorporation	0.87	0.82	0.92	1.04

*Specific activity in CPM/mg. R/L Ratios calculated for each animal, and results expressed as mean ± standard deviation.

p values obtained using t-test for significant differences of R/L ratios from the value of 1.

N = number of animals.

the same in both hemispheres, in both groups of animals. In one additional animal (C) under bilateral spreading depression, there was also no difference in protein specific activity between right and left cortex, indicating that neither hemisphere is preferentially susceptible to the effects of spreading depression on protein metabolism. Two animals (D) under unilateral spreading depression were sacrificed after only 30 minutes of incorporation. The magnitude of the effect was the same as after two hours (B).

The results for brainstem proteins are given in Table IV. Both fractions of brainstem extract proteins have essentially the same specific activity on the depressed and control sides, as shown by a specific activity ratio close to 1. It was not possible in these experiments, however, to distinguish between a lack of effect on brainstem, and a bilateral effect.

Although the equal magnitude of the effect of spreading depression on pH 5 soluble and insoluble cortical proteins made it unlikely that any one protein subfraction was preferentially affected, the pH 5 soluble proteins were further examined on acrylamide gels. No preferentially affected sub-fraction could be detected.

E. Antigenic Components of Rat Brain Extracts

1. Antisera to whole RBE The qualitative analysis of extracts of brain and other tissues on acrylamide gels showed that there were many soluble proteins in brain, and indicated that some of these might not be present in other tissues. In order to identify nerve specific proteins with certainty, antisera were prepared against whole rat brain extract.

Several rabbits were immunized with RBE. Eight to 10 mg of protein was used for each injection. No precipitating antibody activity could be demonstrated with either the capillary precipitin or agar diffusion methods of assay, until the animals had received three or more injections. With repeated injections, sera from all rabbits did show precipitating antibodies against RBE, although the activity always remained extremely low. Serum from unimmunized rabbits did not react with RBE. The number of components was determined by immunoelectrophoretic analysis, and an example

TABLE IV

Effect of Spreading Cortical Depression on Incorporation of ^{3}H-Leucine into Brainstem Proteins

	Specific Activity - Right* / Specific Activity - Left			CPM/ml Extract - Right / CPM/ml Extract - Left
	Total Protein In Extract	pH 5 Insoluble Protein	pH 5 Soluble Protein	TCA Soluble
A. NaCl on right and left cortex	0.96 ± 0.06	0.90 ± 0.07	0.97 ± 0.06	1.02 ± 0.06
N = 4	$p > .2$	$p < .05$	$p > .3$	$p > .5$
B. KCl on right cortex. NaCl on left cortex	0.95 ± 0.03	0.95 ± 0.11	1.00 ± 0.03	0.95 ± 0.05
N = 4	$p > .1$	$p > .5$		$p > .1$
C. KCl on right and left cortex	0.91	0.93	0.93	0.95
N = 1				

* Specific activity in CPM/mg. R/L ratios calculated for each animal, and results expressed as mean ± stand deviation.

p values obtained using t-test for significant differences of R/L ratios from the value of 1.

N = number of animals.

is shown in Figure 8. The number of components varied with antisera from different rabbits. Upon close examination, from 8 to 12 precipitin arcs could be discerned. Most of the components were not specific to nerve tissue, as indicated by the presence of precipitin arcs at the same position with brain extracts and with extracts from other tissues (kidney in Figure 8). The absence of a reaction between RBE and rabbit antiserum to rat serum showed that perfusion of the animals before removing their brains did indeed eliminate serum proteins.

Analysis of antisera to RBE from several different rabbits did, however, indicate that there may be one or two components in brain extract that are not present in other tissues. These components always appeared to have a high electrophoretic mobility, i. e. were "acidic" proteins. Because of the number of nonspecific antibodies and the weak precipitin arcs, a further analysis of brain specific components was not carried out with antisera to whole RBE. Instead, the antigens in RBE were fractionated, and rabbits immunized with the fractions.

2. Fractionation of Antigens

a. pH 5 precipitation RBE proteins were separated into pH 5 soluble and insoluble fractions, and each fraction was then tested against antiserum to RBE by immunodiffusion. Most of the antigenic components present in RBE were also present in the pH 5 soluble fraction, although some antigenic activity could be demonstrated in the pH insoluble fraction as well.

b. Zone electrophoresis on starch The immunoelectrophoresis of RBE showed that the antigenic components were distributed among a range of electrophoretic mobilities. It was also apparent that separation was better at pH 7.4 than at pH 8.6. The electrophoretic separation of RBE proteins was further examined on an analytical scale on cellulose acetate strips (Oxoid, Oxo Ltd., London, England). The stained patterns showed that the best resolution of discrete bands was obtained at pH values between 6.4 and 7.4. This preliminary analysis also showed the existence in RBE of proteins more acidic than those found in extracts of other tissues.

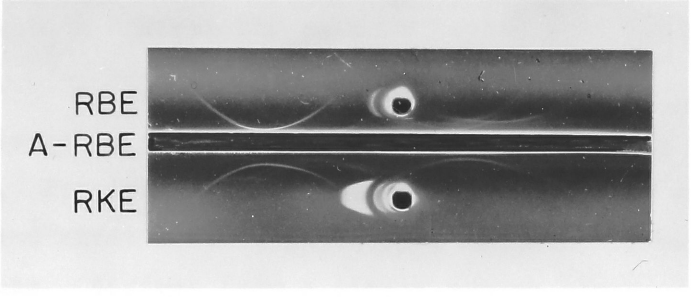

Figure 8. Immunoelectrophoresis of rat brain extract (RBE) and rat kidney extract (RKE). Antiserum to rat brain extract (A-RBE). Anode is at the right.

On the basis of these results RBE proteins were separated on a preparative scale by zone electrophoresis on starch in pH 7.4 phosphate buffer, $\Gamma/2 = 0.1$. The protein concentration profile of the eluates is shown in Figure 9. Protein concentration in this case was determined by the method of Lowry et al. (1951). An identical profile is obtained by measurement of the absorbancy of the solution at 280 mμ (A_{280}). The eluates were pooled according to the peaks in protein concentration. This resulted in six fractions, numbered according to their electrophoretic mobility, the fraction nearest the cathode having been assigned the number I.

c. Antisera to starch block fractions Antisera were prepared to each fraction. Precipitating antibody was detected in all sera after two injections, and after a third injection, the rabbits were exsanguinated by cardiac puncture. Antigen from a single starch block electrophoresis was used for all injections. The antisera were examined by immunoelectrophoresis, using whole RBE and kidney extract as antigen (Figure 10). The antisera are numbered to correspond to the starch block fraction used as antigen. It can be seen that a separation of antigens according to electrophoretic mobility was achieved. Furthermore, the precipitin arcs are considerably stronger than those obtained with antiserum to whole RBE (Figure 8). Antiserum V did contain one component, which could be seen in simple immune diffusion, but could not be demonstrated easily in immunoelectrophoresis. Antisera I to IV contained several components, most of which reacted with both brain and kidney. A more detailed analysis of these antisera was not attempted. Antiserum VI, however, gave two precipitin arcs in the shape of a "gull wing" with RBE, neither of which were obtained with kidney. As will be shown in section F4, these two bands are due to a single antigen, hereafter referred to as antigen α, which is specific to nerve tissue.

F. Isolation and Characterization of an Antigen Found in Nerve Tissue

1. Purification of Antigen α The various steps used in the purification of antigen α from rat brains are shown in Figure 11. The individual steps are described below, and are lettered to correspond to Figure 11.

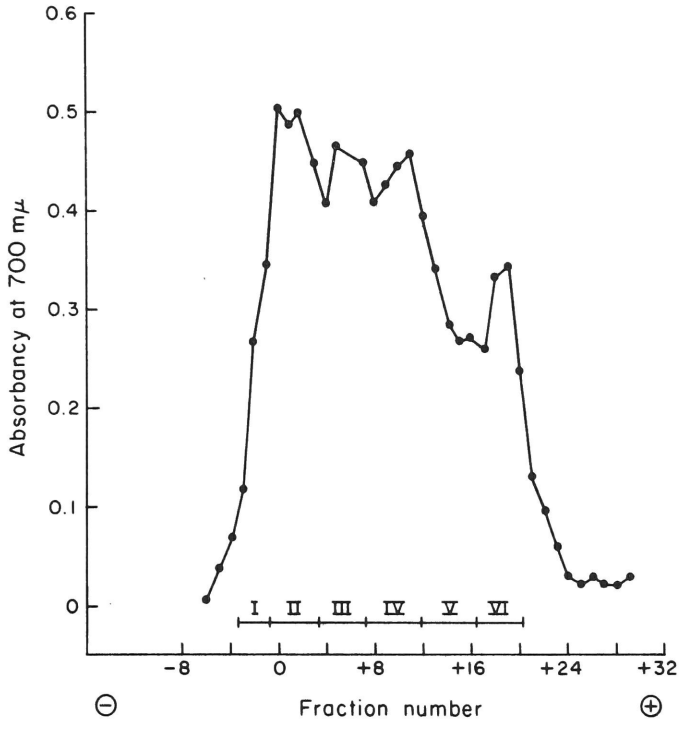

Figure 9. Fractionation of rat brain extract by zone electrophoresis on starch. 0, origin; (-) cathode; (+) anode. I-VI, fractions pooled for immunization of rabbits.

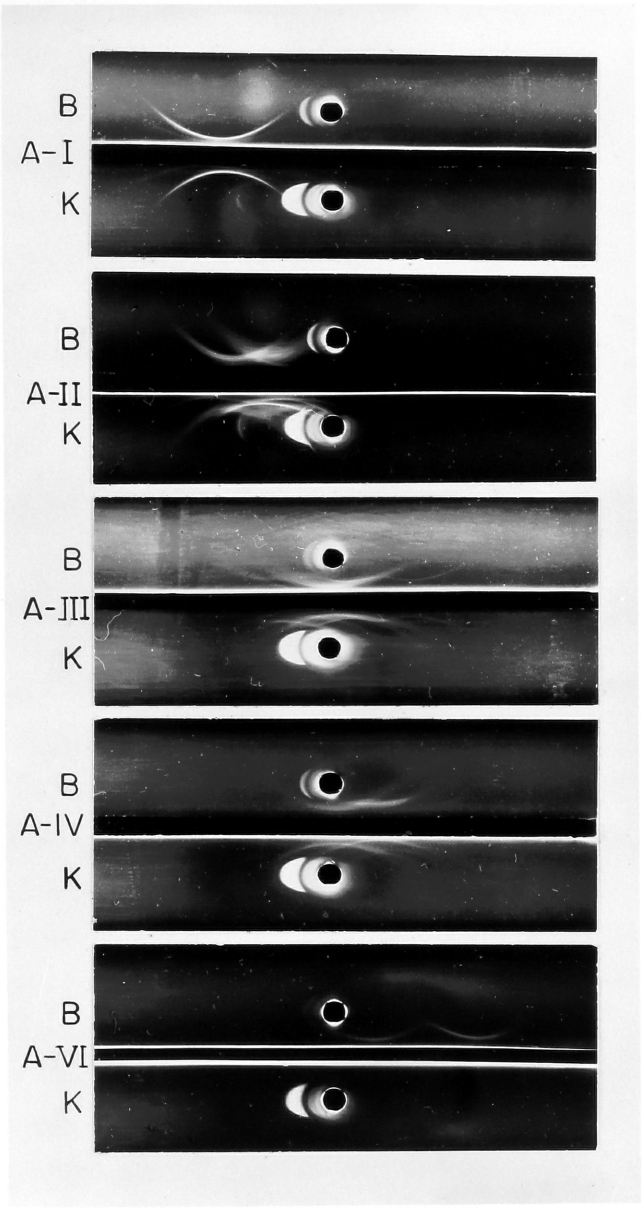

Figure 10. Immunoelectrophoresis of rat brain extract (B) and rat kidney extract (K). Antisera to starch block fractions of rat brain extract. Anode is at the right.

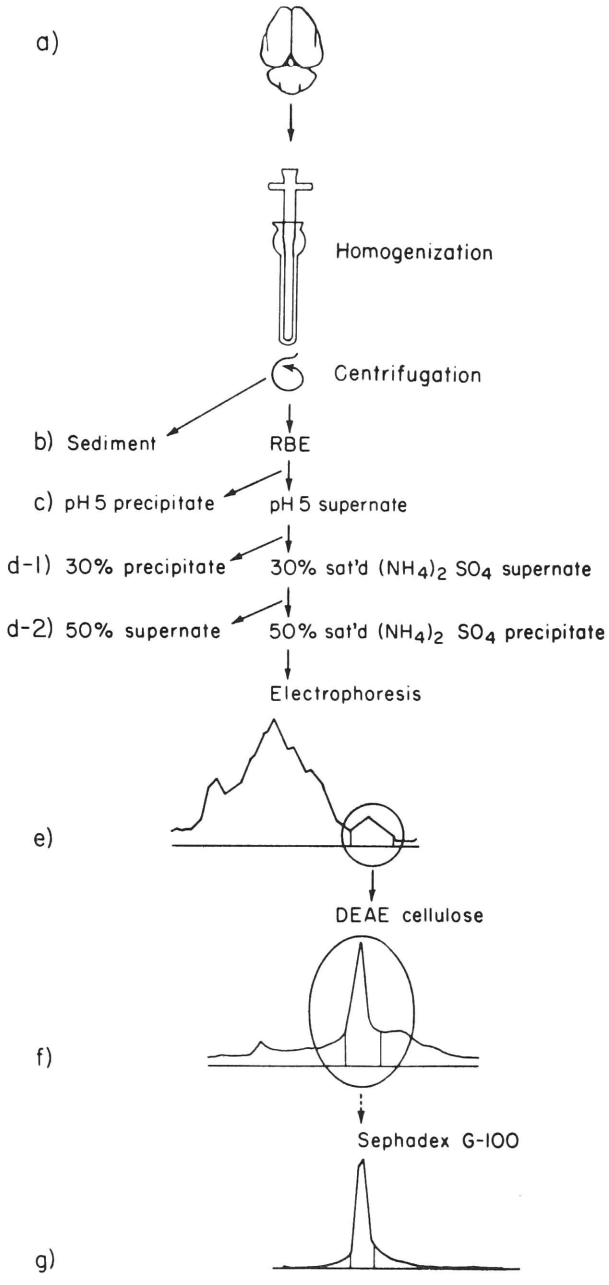

Figure 11. Purification scheme for antigen α. The individual steps a to g are described in the text.

a. Preparation of brain tissues In order to obtain a large amount of starting material (whole rat brain) with maximum efficiency, it was first established that complete removal of blood from the brain was unnecessary for the purification. Zone electrophoresis on starch was performed simultaneously with two identical starch blocks 2" x 18" x 1/4" in size. Rat serum was applied to the origin of one block (A), pH 5 supernate fraction of brain extract from animals which had not been perfused with saline prior to removal of the brain was applied to the origin of the other (B). Protein concentration profiles of the eluates of both starch blocks are shown in Figure 12. The component in (A) having a peak at fraction +10 corresponds to albumin. The eluates from starch block (B) were tested by immune diffusion for antigen α activity against antiserum VI, and for serum proteins against antiserum to rat serum. The presence of antigen α in two regions of the starch block will be discussed below. Since only the more acidic peak (fractions 16 and 17) was used for further purification, it was sufficient to establish that there were no serum proteins in this region. This was shown by the fact that fractions 16 and 17 from both (A) and (B) did not react with anti-rat serum. Fraction 16 from starch block (A) also did not contain any protein components visible on analytical acrylamide gels. Furthermore, the only fractions between +7 and +17 from (B) that reacted with anti-rat serum at all were 9 and 10--those corresponding to the albumin peak. Also, the overall profile of B did not differ qualitatively from the profile obtained with pH 5 supernate from the brains of perfused animals, indicating that whatever serum proteins were present in the brains of animals not perfused amounted to only a negligible fraction of the total protein. This is further demonstrated by the fact that the protein concentration of RBE from perfused and unperfused animals was the same. It was thus concluded that perfusion of animals was not necessary for the purification of antigen α from the acidic peak.

b. Preparation of rat brain extract The perfusion was therefore omitted from the procedure described in Section B for obtaining brain tissue. RBE was then prepared using 2 ml phosphate buffer per gram wet weight whole brain. This yielded a protein concentration of 15 mg/ml in RBE.

c. pH 5 precipitation After pH 5 precipitation of RBE, antigen α could be demonstrated in the pH 5 soluble fraction, but not in the washed, redissolved pH 5 insoluble fraction (Figure 13a). Although it is possible

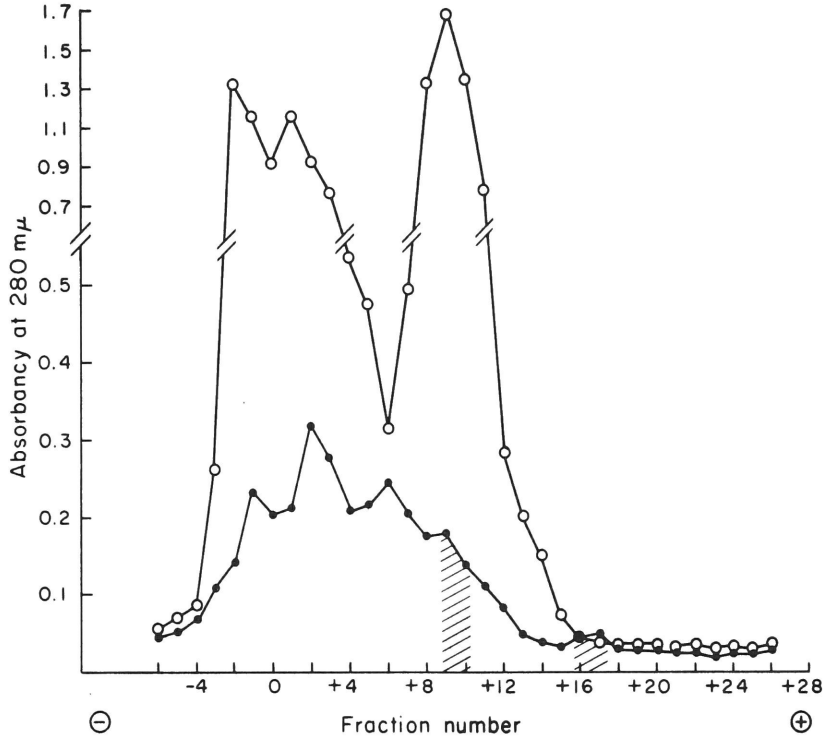

Figure 12. Fractionation of rat serum (o—o = A) and pH 5 supernate from blood-contaminated rat brain extract (●—● = B) by zone electrophoresis on starch at pH 7.4. O, origin; (-), cathode; (+), anode. Shaded area of A represents region containing antigen α.

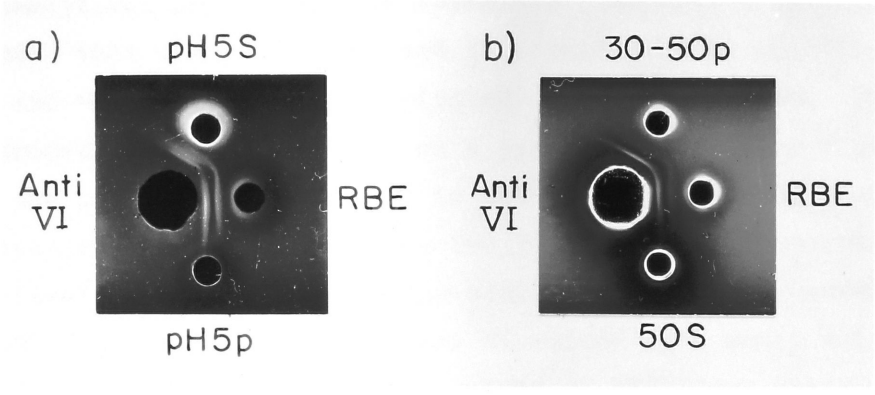

Figure 13. Immunodiffusion showing distribution of antigen α among a) pH 5 fractions, and b) ammonium sulfate fractions of pH 5 supernate. A-VI, antiserum VI; RBE, whole rat brain extract; pH 5 s, pH 5 supernate; pH 5 p, pH 5 precipitate concentrated four fold; 30-50 p, redissolved 30-50% saturated ammonium sulfate precipitate; 50s, dialysed 50% saturated ammonium sulfate supernate, concentrated ten fold.

that some antigen α did precipitate at pH 5, and was irreversibly modified so that antigenic activity was lost, the separation of pH 5 insoluble proteins was necessary to remove components that could not otherwise be separated from α.

d. Ammonium sulfate fractionation The primary objective of the ammonium sulfate fractionation of the pH 5 supernate was to decrease the total volume to the 15 ml which could be applied to the starch block. A preliminary analytical scale ammonium sulfate fractionation carried out at pH 5.0 showed that antigen α remained in solution until 30% saturation was reached, and was completely precipitated at 50% saturation. These results determined the procedure used on a preparative scale. Figure 13b demonstrates the presence of antigen α in the 30-50% saturated ammonium sulfate precipitate, and its absence in the 50% supernate, concentrated ten-fold by ultrafiltration. In a large-scale preparation, however, in which the 0-30% saturated precipitate was dissolved in a small volume of phosphate buffer (5% of the original volume of RBE) some antigen α could be demonstrated in the 0-30% saturated fraction by immunodiffusion against antiserum VI. By testing serial dilutions of the redissolved 0-30% and 30-50% saturated fractions, it could be estimated that the 0-30% precipitate contained less than 10% as much antigen as the 30-50% precipitate. Table V shows the distribution of total pH 5 soluble protein among the three ammonium sulfate fractions.

e. Zone electrophoresis on starch The redissolved 30-50% saturated precipitate was subjected to zone electrophoresis on starch and the protein concentration profile of the eluates is shown in Figure 14. A comparison of similar starch block electrophoreses performed directly on RBE (Figure 15a) and on the pH 5 supernate (Figure 15b) shows the effect of removing pH 5 insoluble proteins on the acidic antigen α region. After pH 5 precipitation, this region becomes a separate peak and contains a relatively smaller amount of protein. From the bands obtained on acrylamide gel electrophoresis of the acidic α region (Figure 16), it can be seen that pH 5 precipitation reduced the amount of a major component that has the same electrophoretic mobility on starch as α.

The presence of antigen α in a second, more slowly migrating region in zone electrophoresis was consistently observed regardless of whether

TABLE V

Ammonium Sulfate Fractionation of pH 5 Soluble Protein

	Mg/100 Mg pH 5 Soluble
0-30% saturated precipitate	15
30-50% saturated precipitate	33
50% saturated supernate	43

Protein determination according to method of Lowry, after redissolving precipitates. BSA standard; see Materials and Methods.

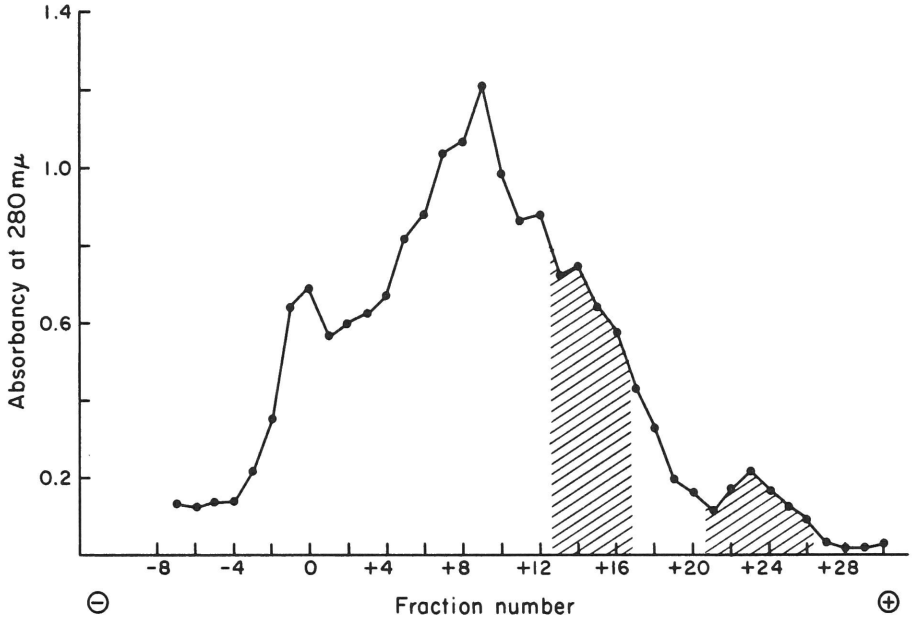

Figure 14. Fractionation of redissolved 30-50% saturated ammonium sulfate precipitate by zone electrophoresis on starch. O, origin; (-), cathode; (+), anode. Shaded area contains antigen α activity. Fractions 21 to 26 were pooled for DEAE-cellulose chromatography.

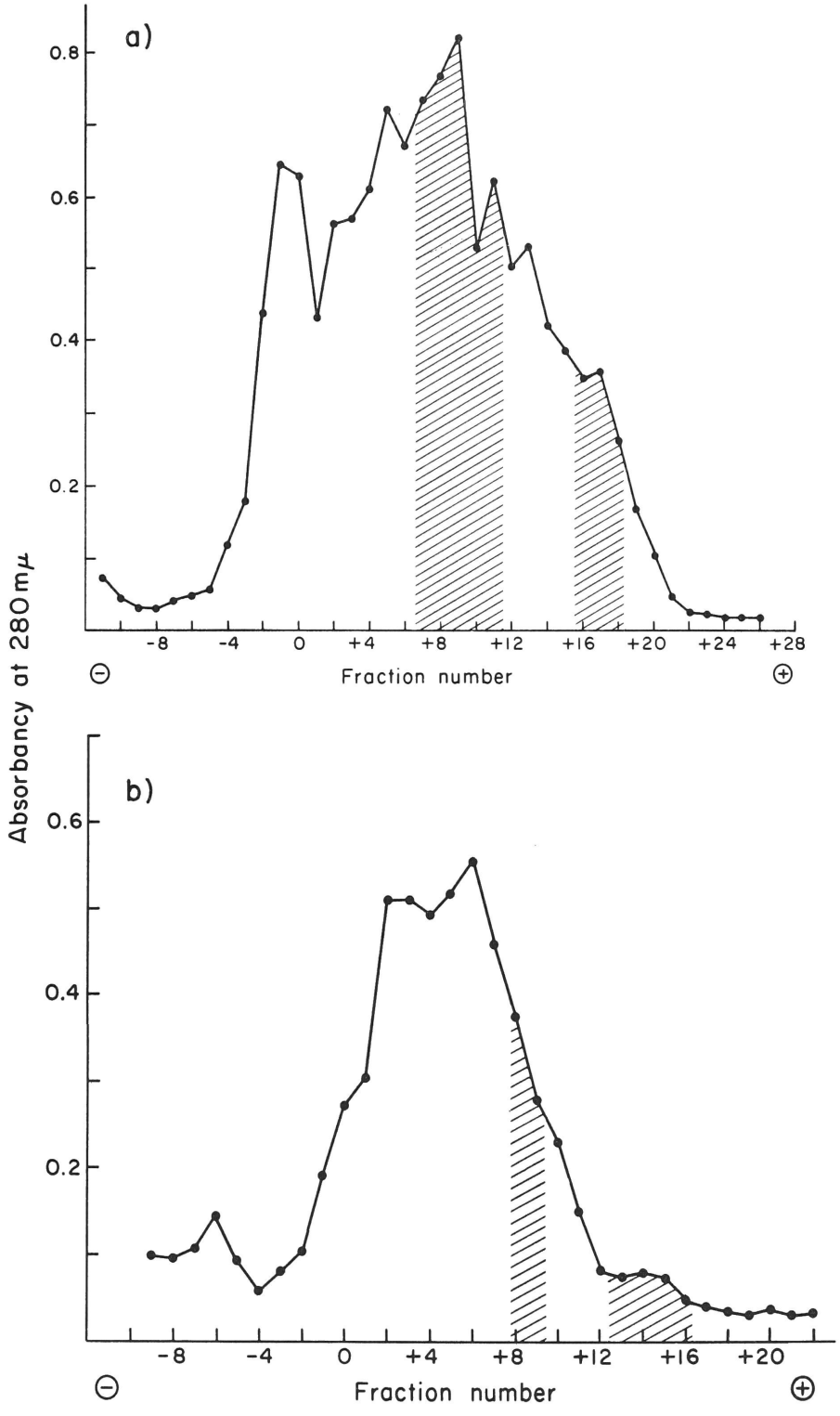

Figure 15. Fractionation of a) whole rat brain extract, and b) pH 5 supernate by zone electrophoresis on starch. 0, origin; (-), cathode; (+), anode. Shaded area contains antigen α activity. Compare the acidic antigen peak of (a), fractions 16 to 18, with that of (b), fractions 13 to 16.

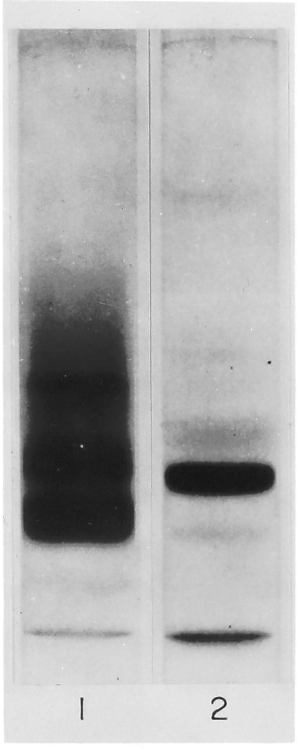

Figure 16. Analytical acrylamide gels of acidic antigen α region from zone electrophoresis on starch (see Figure 15), when whole RBE is applied to the starch block (1) and when the pH 5 supernate is applied (2).

the material applied to the starch block was whole RBE, pH 5 supernate, or 30-50% saturated ammonium sulfate fraction. This second region showed many protein components on analytical acrylamide gels, and contained several other antigens, when tested against antiserum IV (refer to Figure 10 for an immunoelectrophoretic analysis of antiserum IV). Antigen α could not be purified from this second region, using only the purification scheme of Figure 11.

f. DEAE-chromatography Ion exchange chromatography of the acidic starch block peak (fractions 21-26 in Figure 14) resulted in the chromatogram shown in Figure 17. The fractions containing the strongest antigen α activity were those in peak I, but fractions from peaks II and III also contained weak activity. These latter peaks contained several protein components as seen on analytical polyacrylamide gels.

g. Gel filtration on Sephadex G-100 When material from DEAE peak I was subjected to gel filtration on Sephadex G-100 (Figure 18), a single symmetrical peak was eluted, containing the antigenic activity, and about 90% of the applied sample. Thus, gel filtration did not result in further purification. Using the empirical formula of Determann and Michel (1966), the molecular weight of the material in this peak was calculated to be about 83,000.

h. Protein recovery The amount of protein obtained in the fraction containing antigen α at each step of the purification in a typical preparation is given in Table VI. The number of protein components obtained on analytical acrylamide gels after each step is shown in Figure 19. Evidence will be presented in section 2 to support the conclusion that the material obtained in DEAE Peak I and in the G-100 peak is virtually pure antigen, despite the apparent heterogeneity on these analytical gels. The two faint bands migrating between the dye marker (R_f = 1.0) and the major band at R_f = 0.75 probably are contaminants which might be removed by gel filtration on Sephadex G-200, although this has not been attempted.

2. Demonstration of Purity and Chemical Characterization of Antigen α

a. Reaction with antisera The material obtained from the G-100 peak contains no antigens other than antigen α. This is demonstrated in immunodiffusion by the lack of precipitin bands against any of the antisera

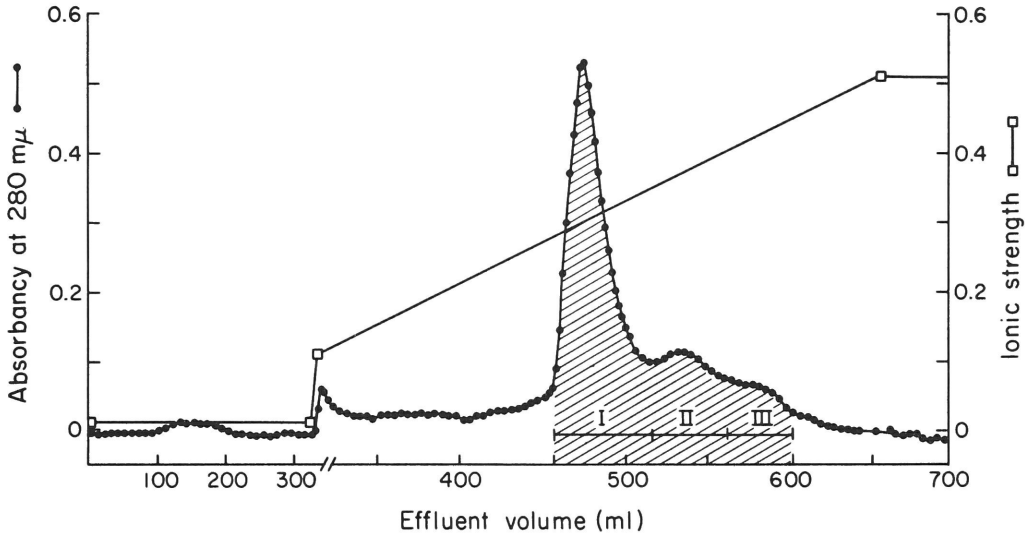

Figure 17. Chromatography on DEAE cellulose of acidic starch block peak (fractions 21-26 in Figure 14). ●—● A_{280}; □—□ ionic strength. Shaded area contains antigen α activity. I, II, III, pooled fractions. Fraction I was subjected to gel filtration on Sephadex G-100.

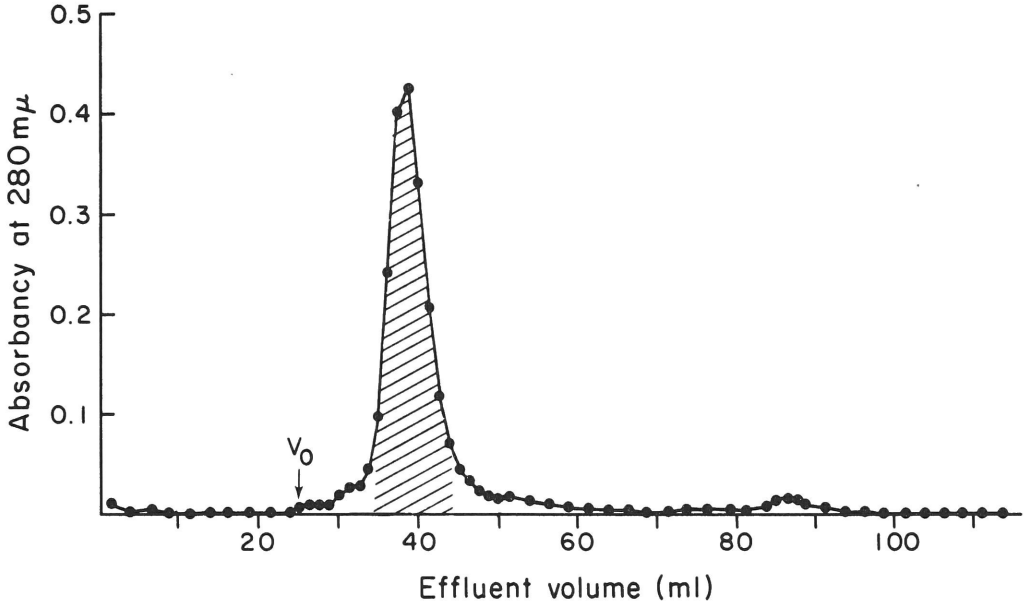

Figure 18. Gel filtration on Sephadex G-100 of DEAE peak I (see Figure 17). V_o, void volume of the 1 x 110 cm column. Shaded area contains antigen α activity. 0.05 M Tris, 0.85% NaCl, pH 8.0.

TABLE VI

Purification of Antigen α from Rat Brain

121 Brains - 220 Gm

	Step*	Volume(ml)	Mg Protein	% Protein**
Rat Brain Extract	b	350	4890	100
pH 5 Supernate	c	360	2550	52
30% - 50% Saturated $(NH_4)_2SO_4$ Precipitate	d-2	15	800	16
Starch Block				
Acidic α-Peak	e	170	45	0.92
DEAE - Fraction I	f	61	20	0.41

Protein determined by the method of Lowry using BSA standard.

*See fractionation scheme, Figure 11.

**Rat brain extract was considered the starting material, and the amount of protein therein defined as 100%. The protein recovered in the subsequent steps was calculated with respect to the whole brain extract.

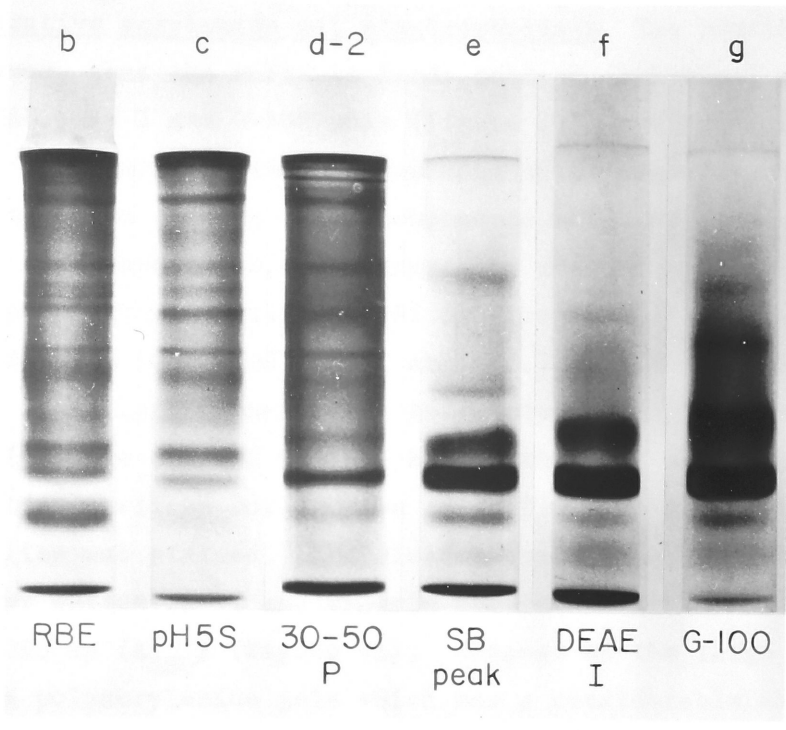

Figure 19. Analytical acrylamide gels of the fraction containing antigen α, from successive stages of purification. The steps b to g correspond to those of Figure 11.

to starch block fractions of RBE, except antiserum IV (Figure 20a) or against antisera to rat kidney and rat serum (Figure 20b). Both of these latter two antisera contained many strong antibody components with kidney and serum respectively. Furthermore, immunization of rabbits with purified antigen α resulted in no new antibody components.

b. Preparative acrylamide gel electrophoresis The possibility remained, however, that the multiple bands seen on analytical acrylamide gels of the DEAE peak I and G-100 peak (Figure 19) were non-antigenic contaminants. Since neither the ion-exchange chromatography nor gel filtration appeared to resolve these components which were resolved on acrylamide gel electrophoresis, an attempt was made to accomplish a separation by preparative acrylamide gel electrophoresis. Gels 12.5 mm in diameter and 10 cm long were used. About 0.7 to 0.8 mg of protein from DEAE peak I was applied per gel. After electrophoresis, a thin longitudinal slice was cut off the gel before the bulk of the gel was cut into cross sectional slices for elution in pH 8.0 Tris-NaCl buffer. The longitudinal slice was stained. The eluates were tested for antigenic activity against antiserum VI and protein concentration was estimated by absorbancy at 280 mμ (A_{280}) (Figure 21). Because of the large amount of material in the polyacrylamide gels which has a considerable absorbancy at 280 mμ, A_{280} gives only a rough approximation of protein concentration. The discrete band seen in the stained slice has an R_f of 0.7 - 0.75, corresponding to the major band seen on analytical gels (Figure 19). Discrete bands of slower mobility cannot be seen on the stained slice of the preparative gel, although there is a faintly stained smear. This may in part be due to the fact that the longitudinal slice of the preparative gel was much thinner than the diameter of the analytical gels.

Antigenic activity was found only in the eluates of slices 27 and 28, corresponding to the discrete band. Eluates of slices 21-30 were run on analytical acrylamide gels and the stained bands of some are shown in Figure 22. The antigenic material again resulted in three discrete bands. All eluates from slices 22 to 26 showed at least two bands. This result suggested that the multiple bands may be due to different aggregate states of a single protein.

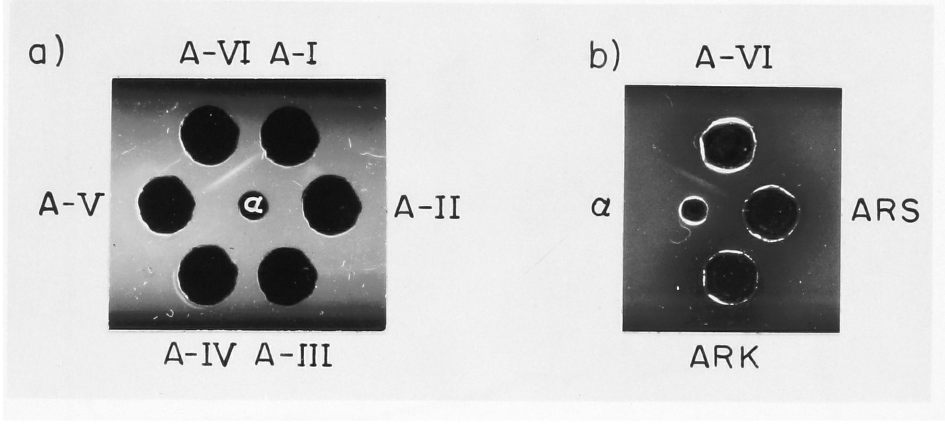

Figure 20. Immunodiffusion showing the absence of any brain, kidney, or serum antigens in the purified antigen α preparation. a) Antisera to starch block fractions of rat brain extract I to VI. b) Antisera to rat kidney, (ARK), rat serum (ARS), and starch block fraction VI of rat brain extract.

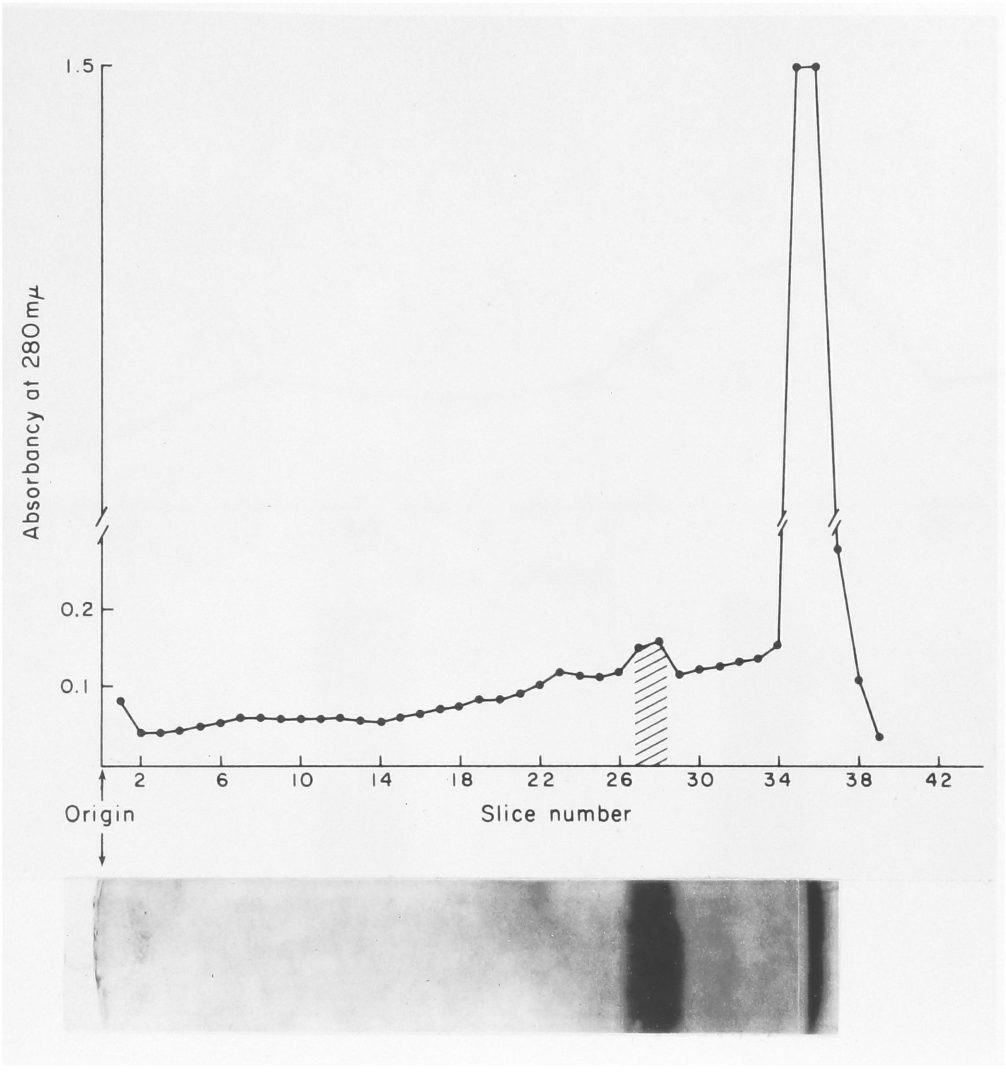

Figure 21. Preparative acrylamide gel electrophoresis of DEAE peak I material (see Figure 17). The A_{280} of the eluates of each of the cross sectional slices is shown in the curve (●—●). The points correspond in position to the stained longitudinal slice shown below.

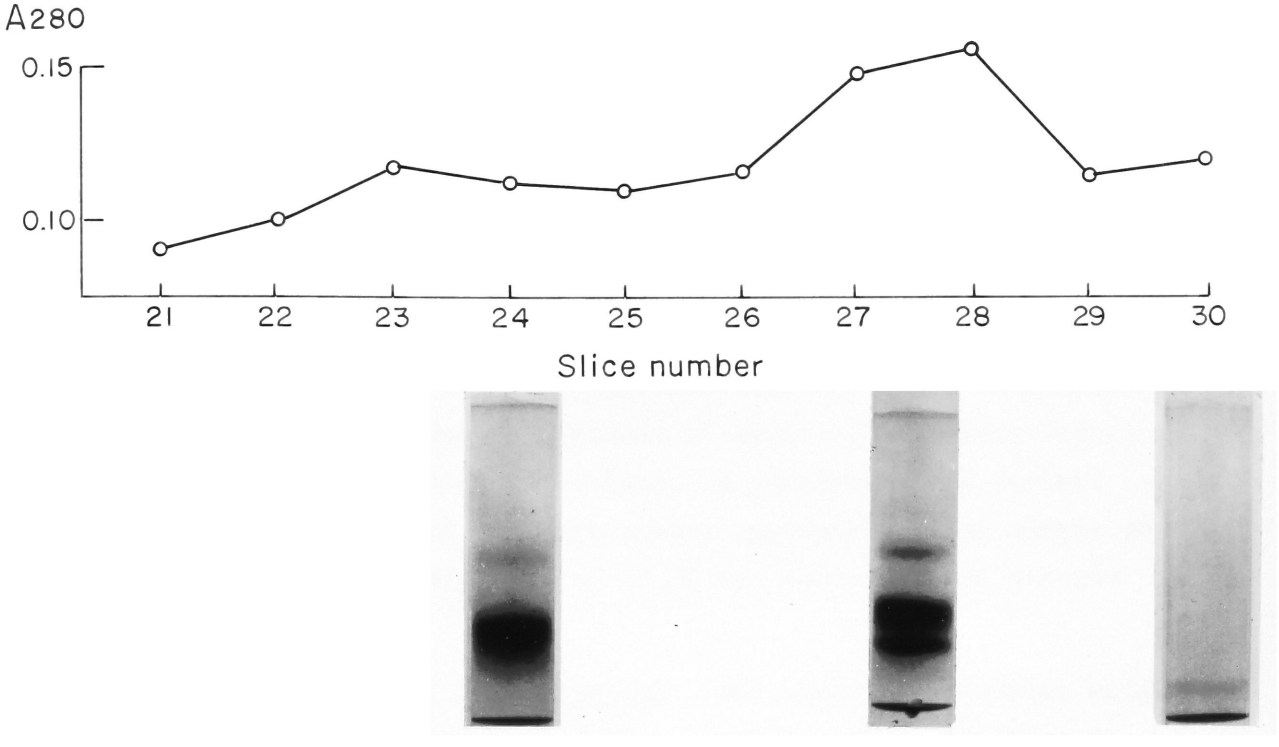

Figure 22. Analtyical acrylamide gels of eluate from slices 24, 27-28, and 30 of preparative gel shown in Figure 21. The curve is a portion of that in Figure 21, on an expanded scale.

This hypothesis was supported by examination of the material from DEAE peak I on starch urea gels and in the analytical ultracentrifuge.

c. Starch-urea gel After dialysis against 2% acetic acid followed by lyophilization, two 1 mg samples of DEAE peak I material were dissolved in 0.1 ml 8 M urea. One sample was applied directly to a starch urea gel, the other was first fully reduced and alkylated. The stained gel is shown in Figure 23. Before reduction and alkylation at least four bands can be seen. After complete reduction and alkylation only one major band can be seen. Thus breaking disulfide bonds appears to permit disaggregation into subunits.

d. Sedimentation velocity Sedimentation velocity experiments gave identical results for antigen α after both the ion-exchange chromatography and gel filtration steps of the purification (Table VII). At pH values between 7.0 and 8.0, the schlieren pattern showed a major component having a peak with a sedimentation coefficient of about 5.7 Svedberg Units, and a component of smaller molecular weight. A representative pattern is shown in Figure 24. At pH 3.0, the system aggregated. The single peak with a sedimentation coefficient of 12.7S was skewed toward heavier components (Table VII).

e. Molecular weight determination The apparent molecular weight of antigen α, as determined by equilibrium centrifugation, is given in Table VIII. In Tris-NaCl, the system was heterogeneous. This agrees with the heterogeneity apparent in sedimentation velocity experiments, done in the same solvent. In guanidine both with and without β-mercaptoethanol, the plot of log C vs $r^2/2$ was a straight line, indicating homogeneity, but the calculated molecular weight was considerably lower in the presence of the reducing agent. Figure 25 shows this plot for the solvent containing guanidine and β-mercaptoethanol.

f. Amino acid composition The amino acid composition of antigen α is presented in Table IX. It is expressed as moles per 39,000 gm, using the minimal molecular weight obtained from the analytical ultracentrifugation experiments, in order to indicate the number of residues per mole of subunit.

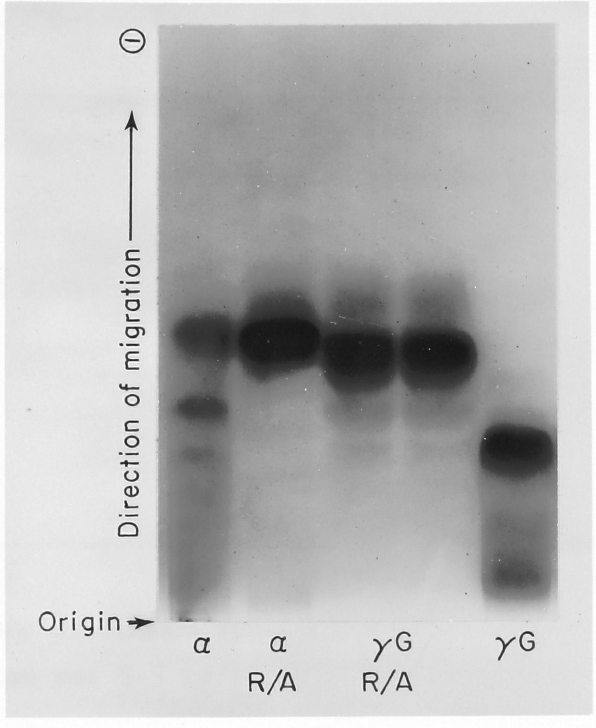

Figure 23. Electrophoresis on starch-urea gel of purified antigen α before (α) and after (α, R/A) complete reduction and alkylation, and human γ globulin before (γ) and after (γG, R/A) complete reduction and alkylation. (-), cathode.

TABLE VII

Sedimentation Velocity of Antigen α

Solvent	Stage of Purification	Approximate $s_{20,w}$ (Svedbergs)
Na-K-PO_4; T/2 =		
0.1; pH = 7.4	DEAE (f)	5.6
0.05 M Tris - 0.85% NaCl;		
pH = 8.0	G-100 (g)	5.7
0.1 M KCl, pH 7.0	G-100 (g)	5.7
0.1 M KCl, pH 3.0	G-100 (g)	12.7

All experiments done at 52,000 rpm.
Protein concentration was 3-5 mg/ml.

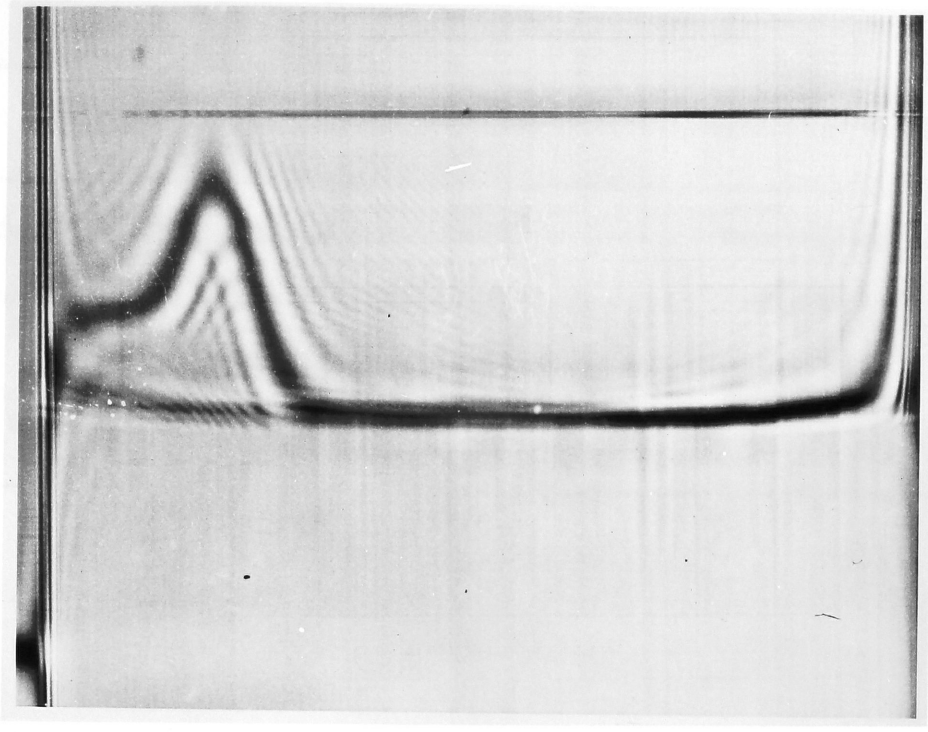

Figure 24. Sedimentation velocity experiment on antigen α. Direction of the field is to the right. Solvent, phosphate buffer, pH 7.4; protein concentration, approximately 3 mg/ml; phase plate angle, 45°; speed, 52,000 rpm; time of photograph, 33 minutes.

TABLE VIII

Molecular Weight of Antigen α

Solvent	Molecular Weight
0.05 M Tris - 0.85% NaCl, pH 8.0	Heterogeneous
6.3 M Guanidine HCl - 0.1 M Tris, pH 8.4	60,500
6.3 M Guanidine HCl - 0.1 M Tris, pH 8.4, 0.1 M β-mercaptoethanol	38,900

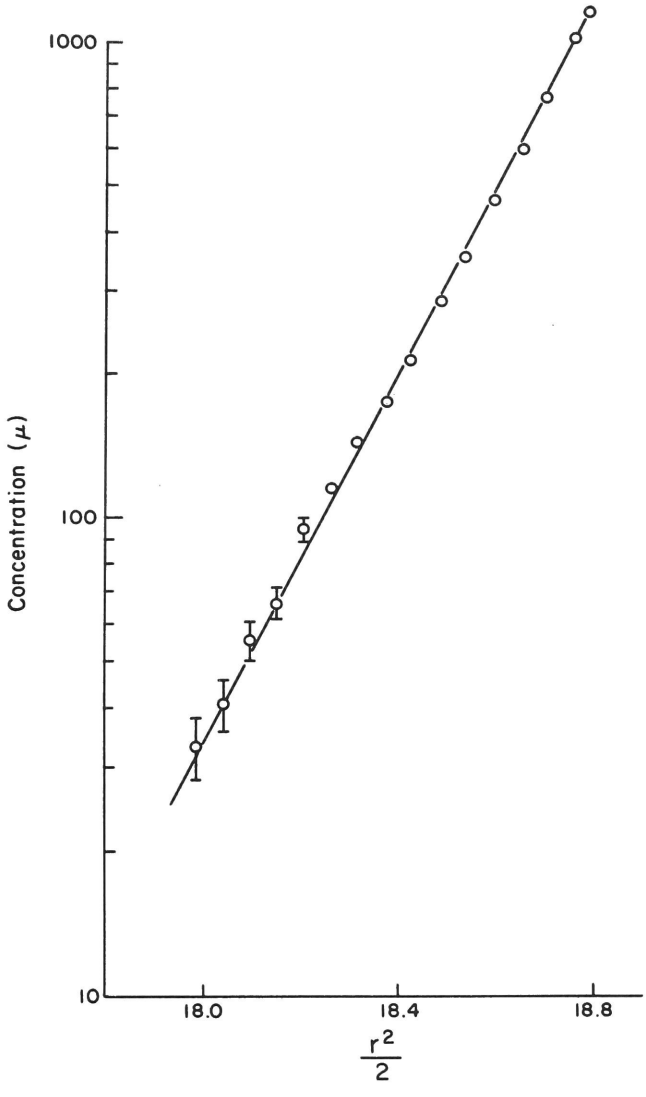

Figure 25. Equilibrium centrifugation of antigen α in a solvent containing guanidine plus β-mercaptoethanol.

TABLE IX

Amino Acid Composition of Antigen α

Amino Acid	Moles/39,000 gm protein
Lys	24
His	6
Arg	15
Asp	39
Thr	15
Ser	20
Glu	50
Pro	17
Gly	30
Ala	36
Cys	5
Val	25
Met	7
Ile	19
Leu	32
Tyr	8
Phe	11

The average of three analyses was used except as indicated in Materials and Methods. The error of the determinations was 5%. Values are shown to the nearest integer.

g. End group analysis The results of the starch urea gel and analytical ultracentrifuge experiments suggested that the purified antigen α preparation consisted of subunits of the same size. To establish whether different types of subunits were present, an attempt was made to determine the NH_2-terminal amino acid. No end group could be detected. Although the presence of histidine, cysteine, or tryptophan as NH_2-terminal amino acid could not be ruled out by the dansyl method, the failure to detect an end group was tentatively attributed to a "blocked" end group due to the presence of pyrroglutamic acid as NH_2-terminal residue, in view of the high glutamic acid content of the protein (see Table IX). Pyrroglutamic acid has been found in other proteins, especially in the amino-terminal position, and it can also be formed from glutamyl residues during the purification procedure (Blomback, 1967). The result was taken as provisional evidence for the existence of only one kind of subunit.

h. Peptide map The peptide map of antigen α (Figure 26) gave additional confirmation of the presence of a single kind of subunit, and also of the approximate molecular weight of 39,000 obtained from equilibrium centrifugation under dissociating and reducing conditions. The sum of arginine residues (15) and lysine residues (24) in a molecule of molecular weight of 39,000 (Table VIII) led to the prediction of 40 tryptic peptides. Twenty-nine spots could be identified with certainty, and three more became apparent in the photograph of the electropherogram after fixing the spots in copper nitrate (saturated aqueous $Cu(NO_3)_2$:10% aqueous HNO_3:acetone, 1:0.2:100).

i. Elemental analysis The nitrogen content of antigen α* is 14.6% of the dry weight, after correcting for 3.2% ash. This is in the range reported for proteins, but is slightly low, indicating the possible presence of non-protein component(s).

j. Carbohydrate content Total hexose was determined, and a value of about 5% (wt/wt) was obtained. Since the purification of antigen α included an electrophoresis on starch, some of the carbohydrate may be a contaminant. The amount of covalently bound carbohydrate, or the presence of other groups has not been determined.

* determined by T. Bella

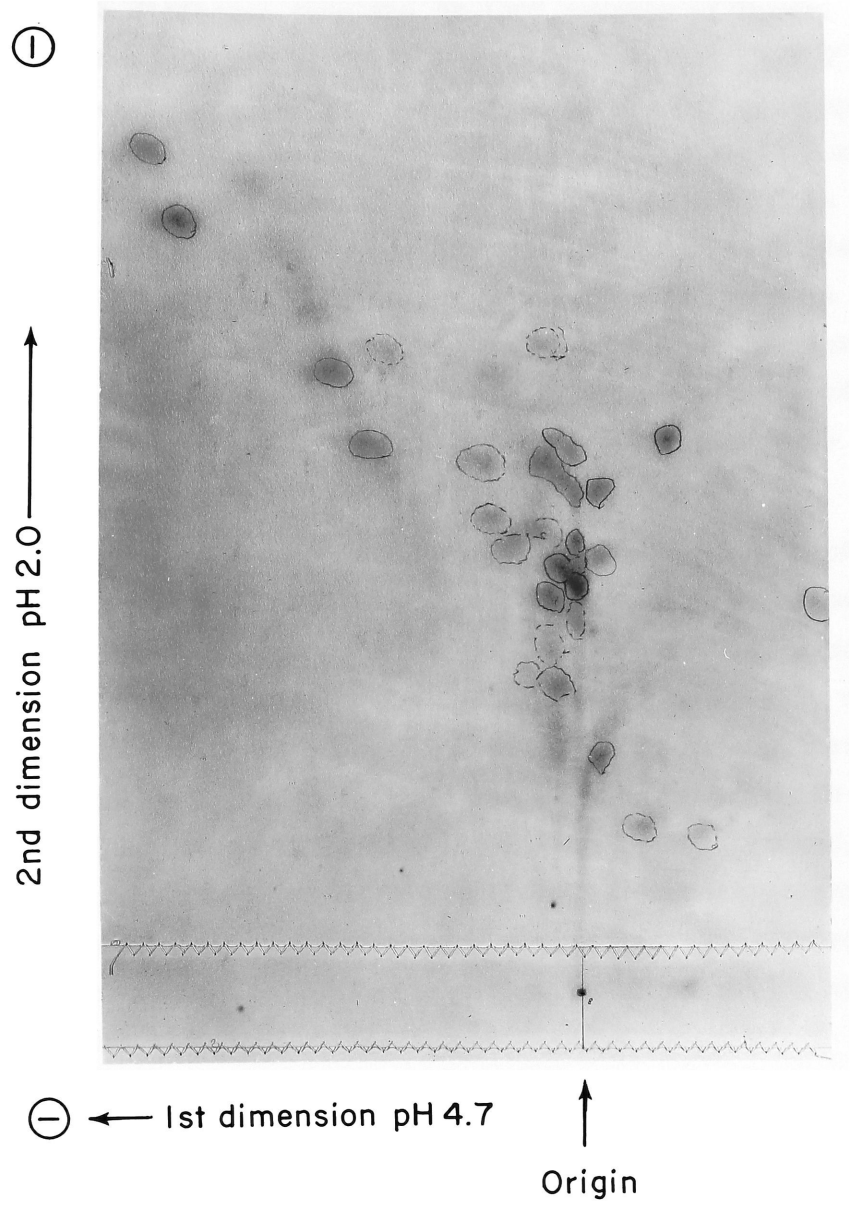

Figure 26. Peptide map of antigen α. See materials and methods for preparation of tryptic peptides.

3. Comparison of Antigen α with Other Proteins

a. "S-100" protein Using immunological methods, antigen α has been demonstrated to be different from the "S-100" protein of Moore. From the immunodiffusion shown in Figure 27a, it can be seen that "S-100" is present in RBE, but is clearly different from either of the components reacting with antiserum VI. Furthermore, purified antigen α does not react at all with antiserum to "S-100". The fate of "S-100" in the purification scheme for antigen α was also followed. "S-100" is present in both the pH 5 soluble and insoluble fractions of RBE, and is not completely precipitated in 50% saturated ammonium sulfate (Figure 27b). On zone electrophoresis, however, no "S-100" can be demonstrated in the acidic peak containing antigen α, but it is present in the more slowly migrating region of the starch block (Figure 27c).

b. Acid protease A proteolytic enzyme active at acidic pH and purified by Marks and Lajtha (1965) appeared to migrate with the same mobility on acrylamide gels as did antigen α. With the assay used by Marks and Lajtha (1965) antigen α did not have any proteolytic activity. The fate of acid protease activity was also followed through part of the purification scheme for antigen α. RBE and pH 5 supernate both contained activity (Table X). The pH 5 insoluble fraction was not tested, and ammonium sulfate fractionation was not performed. After subjecting the pH 5 supernate to zone electrophoresis, the eluates were assayed for acid protease activity (Figure 28). The only fractions containing any activity were well separated from antigen α.

4. Distribution of Antigen α

a. Tissue specificity The results of the immunoelectrophoresis shown in Figure 10 gave the initial suggestion that antiserum VI contained a component not present in kidney. Additional proof was obtained with an absorption experiment (Figure 29) using antiserum VI and antiserum I. Figure 10 indicated that antiserum I did not contain brain specific components. An aliquot of each antiserum was mixed with an equal volume of rat kidney extract and incubated at 37°C for 2 hours. After 12 hours at 4°C, the tubes were centrifuged. Any components in the antiserum reacting with kidney should have been removed. The absorbed antisera and unabsorbed antisera, diluted with an equal volume of phosphate buffer,

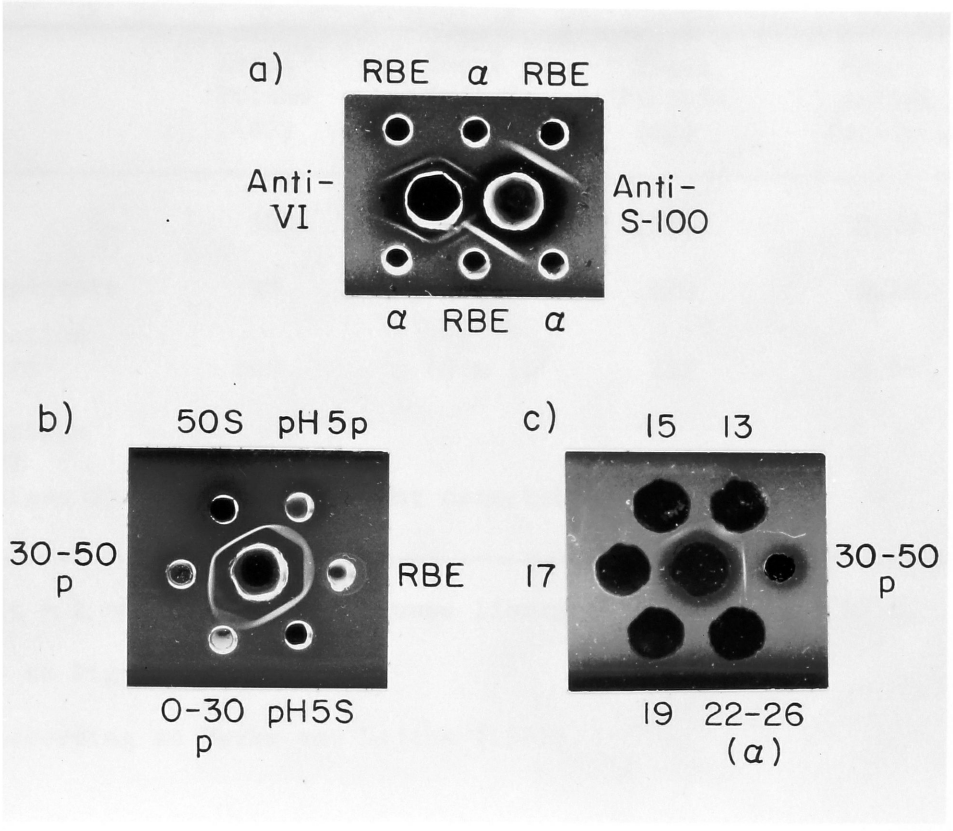

Figure 27. a) Immunodiffusion showing lack of identity between antigen α and the "S-100" protein. A-S-100, antiserum to "S-100." b) Distribution of "S-100" among pH 5 fractions (pH 5s and pH 5p) and ammonium sulfate fractions (0-30 p, 30-50 p, 50s) of rat brain extract, c) Migration of "S-100" in zone electrophoresis on starch (see Figure 14; numbers are fractions tested). Center wells of (b) and (c) contain antiserum to "S-100."

TABLE X

Acid Proteinase Activity

	Total Volume (ml)	Total Units*	Total Protein (mg)	Specific Activity (units/μg)
RBE	90	272×10^3	1350	0.20
pH 5 supernate	90	178×10^3	630	0.28
†SB fraction +1- +6	240	60×10^3	282	0.34
†SB fraction 20-22 (antigen α)	120	Not detectable	7	-

* 1 unit = 1 mμ mole α-amino groups liberated in 30 min. at 37°C.

† Refer to Figure 28.

Assay according to Marks and Lajtha (1965).

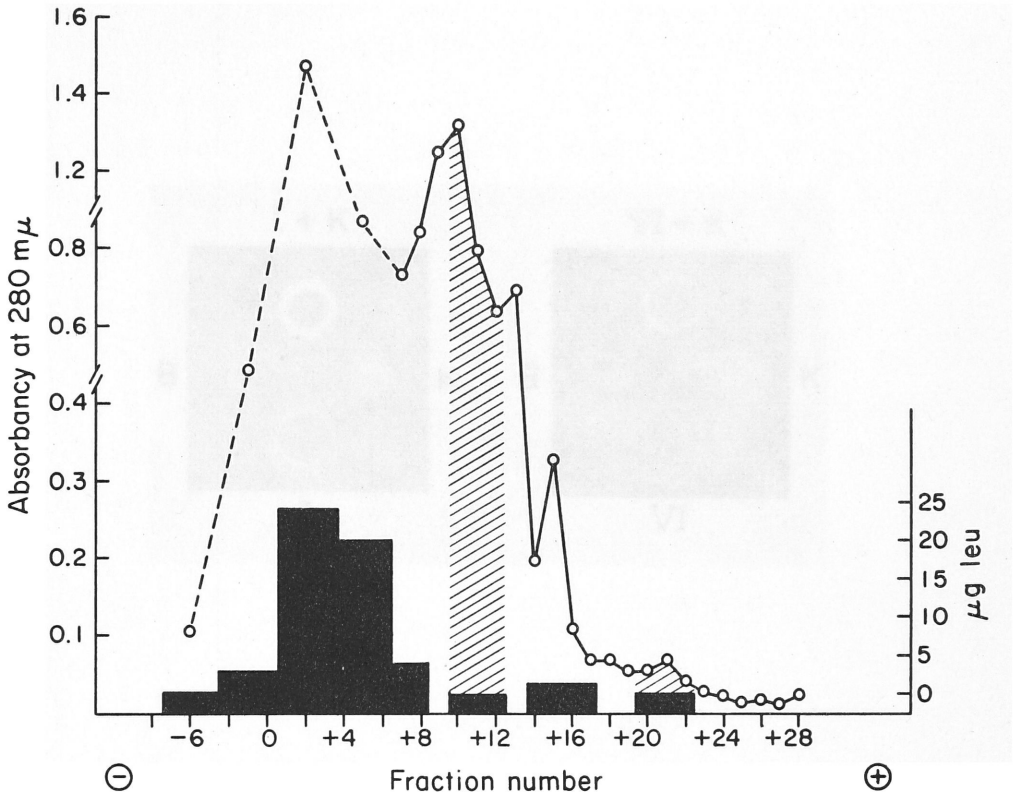

Figure 28. Localization of acid protease activity and antigen α in starch block eluates. O, origin; (-), cathode; (+), anode. o—o, A_{280}. Shaded area contains antigen α activity. Histogram: μg leucine equivalents released in assay for acid protease.

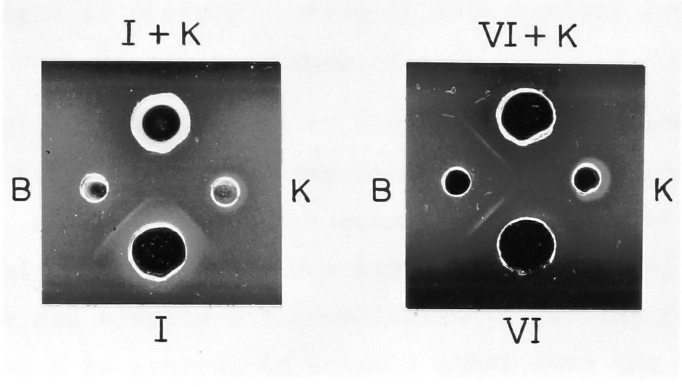

Figure 29. Immunodiffusion of rat brain extract (B) and rat kidney extract (K) against antiserum VI (VI), antiserum VI absorbed with rat kidney (VI + K), antiserum I (I), and antiserum I absorbed with rat kidney (I + K).

were tested against rat brain extract and rat kidney extract in the double diffusion shown in Figure 29. Both absorbed and unabsorbed antiserum VI gave a strong band corresponding to antigen α with brain, and none with kidney. Unabsorbed antiserum I formed a precipitin band with both brain and kidney which was removed from both by absorption of antiserum I with kidney. It was concluded that antigen α was not present in kidney in a detectable amount.

The absence of antigen α from other tissues is demonstrated in Figure 30. The antigen is clearly present in both central and peripheral nerve tissue, and absent in all non-neural tissue examined.

b. Animal specificity As shown in Figure 31, an antigen present in both whole mouse brain and human gray matter appears to be identical with rat antigen α. Although there was some reaction between antiserum VI and extracts of rabbit brain and various areas of beef brain, the precipitin bands were very weak and clearly not identical with rat antigen α. This suggests that antigen α is present in animals other than the rat, but that differences may exist in the tertiary structure of the molecule at the antigenic site.

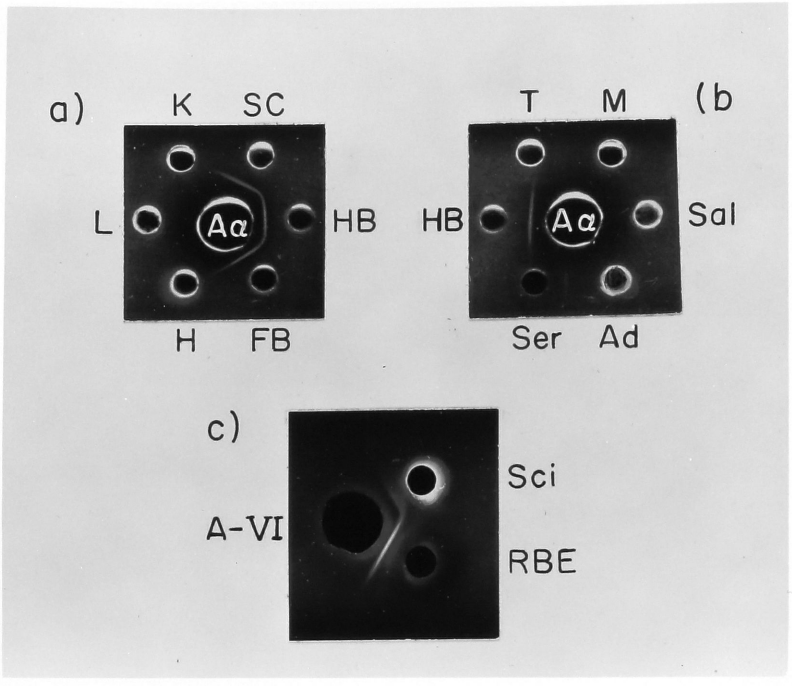

Figure 30. Immunodiffusion showing tissue specificity of antigen α. All tissues are from rats. HB, hindbrain; FB, forebrain; H, heart; L, liver; K, kidney; SC, spinal cord; T, testes; M, skeletal muscle; Sal, salivary gland; Ad, adrenal gland; Ser, serum, Sci, sciatic nerve. The center wells in (a) and (b) contain antiserum to antigen α (Aα).

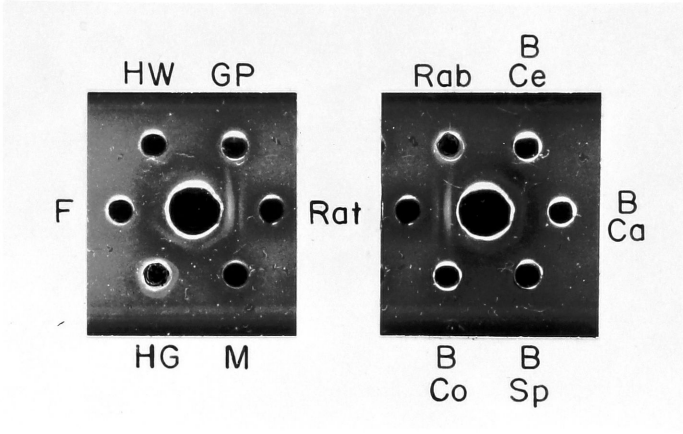

Figure 31. Immunodiffusion of antiserum to antigen α (antiserum VI) against brain extracts from various species. M, mouse; HG, human grey matter; F, frog; HW, human white matter; GP, guinea pig; Rab, rabbit; BCe, beef cerebellum; BCa, beef corpus callosum; BSp, beef spinal cord; BCo, beef cortex.

III. DISCUSSION

III. DISCUSSION

The experiments described in this thesis were undertaken to define and isolate proteins in the nervous system which might be of particular significance in the activity of neural tissue. This was done by developing fractionation schemes for the soluble proteins, and screening the fractions in two ways. One method was to prepare antisera to protein fractions and to use the antisera to identify nerve specific antigens. This led to the purification and partial characterization of the nerve specific protein, antigen α, and demonstrated the fruitfulness of such an approach. The second method was to develop a carefully controlled means of altering the activity of nerve tissue using the phenomenon of spreading cortical depression, and investigating the *in vivo* biosynthesis of individual protein fractions.

These two approaches will be discussed separately in the following pages, preceded by some general comments on the soluble proteins of nerve tissue.

A. Characterization of Soluble Brain Proteins in General

Examination of brain extracts on polyacrylamide gels showed that there is indeed a large number of different soluble proteins in nerve tissue. The superior resolving power of analytical disc electrophoresis made it a suitable method for comparing the soluble proteins of different types of neural and non-neural tissue to identify individual proteins of potential significance in nerve function.

The particular extraction procedure used in this study was quite arbitrary, the only aim being to limit the proteins to those easily soluble in mild aqueous media. This was done both to obtain the proteins in as close to their native state as possible and to facilitate subsequent fractionation. The value obtained for total protein content of brain (14% of wet weight) is somewhat higher than that frequently reported (10%) (Moore and McGregor, 1965; Rossiter, 1962). The difference is most likely due to the different procedures used to estimate total protein. About 30% of total protein was soluble in the phosphate buffer used, which is within the range reported for similar extraction procedures (20-50%) (LeBaron and Folch, 1959; Lajtha, 1964a). A difference in protein content of white and grey matter has been previously reported (LeBaron and Folch, 1959), and

thus the differences reported in Table II for cortex and brainstem may reflect the difference in myelin content. Table II also shows that the difference in total soluble protein content of cortex and brainstem is entirely due to the difference in the amount of pH 5 insoluble protein. Although these fractions of the soluble proteins are not directly comparable to any fractions obtained by other workers, it is interesting to note that LeBaron and Folch (1959) reported a very similar "albumin" content (defined as the fraction remaining soluble after dialysis against distilled water) in white and grey matter, but a large difference in "globulin" content (insoluble after dialysis against distilled water).

Previous qualitative analyses of different soluble protein fractions of nerve tissue by analytical electrophoretic separation have given conflicting results regarding differences between various animals and types of nerve tissue. For example, Bailey and Heald (1961a), using starch gel electrophoresis, reported distinct qualitative differences in soluble proteins from brains of different animal species, and consider the failure of earlier workers to detect such differences a measure of the poor resolving power of methods such as paper electrophoresis. They also found differences among genetically pure strains of mice, but not among inbred strains of rats, and differences between grey and white matter. The experiments reported here indicate that there are no qualitative differences in the soluble proteins of different lines of rats, of grey and white matter, or of different areas of brain.

The identical patterns obtained for different strains of rats is particularly interesting in view of the different performances of the strains in psychological tests. Although the chemical analysis of the brains of inbred strains is not yet complete (Bennett et al., 1966), some relevant points have been established. Those strains bred for high acetylcholinesterase do not appear to have higher levels of other enzyme activities or of total protein. This result, together with the acrylamide gel analyses suggest that any differences in relative protein content must be very subtle indeed.

Those analyses that did indicate qualitative differences in soluble proteins, the comparison of neural and non-neural tissue, and of brains from different animal species, showed so many differences, that the relevant

ones could not be identified without additional means of characterizing the individual components. Therefore two screening criteria, rate of synthesis and antigenicity were developed.

B. Effects of Alterations in Neural Activity on Synthesis of Soluble Brain Proteins

The results described in this thesis indicate that all fractions of brain proteins are affected by spreading depression to an equal extent. This suggests that examination of alterations in protein synthesis as a result of non-specific changes in electrical activity, such as spreading depression, electroconvulsive shock, or anesthesia, is not a fruitful approach towards defining a precise role for specific proteins in neural activity. This finding does, however, provide a baseline for further experiments designed to detect changes in the synthesis of particular proteins as a result of more specific alterations in neural activity such as learning and memory. The particular experimental design used in the present experiments is especially suitable for such further studies because several groups have reported experiments showing that the presence of unilateral spreading cortical depression restricts learning to the normal hemisphere (Bures, 1959; Russell and Ochs, 1963; Albert, 1966). This experimental system offers a situation in which both chemical and physiological changes can be well controlled, and could therefore be used to detect biochemical alterations in a "trained" hemisphere relative to the depressed hemisphere. It is therefore worthwhile considering the present results in some detail.

The results for total soluble proteins are entirely in agreement with the results reported by Ruscak (1964) for anesthetized animals. He obtained a 16% decrease in the specific activity of proteins on the depressed hemisphere thirty minutes after simultaneous administration of S^{35}-methionine intraperitoneally and 3 M KCl to the brain. The present experiments show in addition that the effects can be observed in conscious animals. The observation that the specific activity of brainstem proteins is the same on both sides cannot yet be correlated with the electrical concommitants of spreading depression. Physiological evidence indicates that, while spreading cortical depression elicited by topical application of KCl does not spread to subcortical areas, there are secondary effects

on some subcortical structures, such as thalamic nuclei, and these effects are not necessarily unilateral (Bures, 1959; Ochs, 1962).

Although it has not been possible to prove conclusively that the observed alterations in amino acid incorporation into protein are due to a decrease in the rate of protein biosynthesis, there are several indications that this is most likely the case. A lower specific activity of the leucine pool in the depressed hemisphere could lead to a decreased protein specific activity in the absence of any alteration in the rate of protein synthesis. Leucine has been shown to exchange rapidly between plasma and brain (Lajtha, 1959; Lajtha and Mela, 1961; Lajtha and Toth, 1962, Roberts and Morelos, 1965). Spreading depression is usually accompanied by vasodilation (Marshall, 1959). This would be expected to increase rather than decrease the rate of penetration of systemically administered isotope into the brain. Ruscak (1964) reported an increase in the amount of soluble radioactivity in the depressed hemisphere, which he attributed to the presence of differing amounts of blood remaining in the two hemispheres. In the present experiments, blood was eliminated, and the difference in trichloroacetic acid soluble radioactivity in the two hemispheres was insignificant and, if anything, higher in the depressed hemisphere than in the control. It is therefore considered unlikely that the results are due to differences in the rate of amino acid penetration into the depressed hemisphere.

Total acid soluble radioactivity is not, however, an accurate estimate of the radioactivity in free leucine. Roberts and Morelos (1965) have shown that conversion of leucine to other amino acids, especially dicarboxylic amino acids, occurs very rapidly in brain. Thirty minutes after an intravenous injection of C^{14}-leucine they were able to recover only about 30% of the cerebral radioactivity as leucine. It is therefore not possible to rule out differences in the specific activity of the amino acid pools without separation of the individual amino acids.

It also is conceivable that the decreased specific activity of cortical proteins during spreading depression is due to an increase in the rate of protein degradation. It was shown, however, that the specific activity of cortical proteins increases linearly for 30 minutes

after administration of isotope (Figure 7). In view of this, and the observation that the effect of spreading depression on protein specific activity can be demonstrated after only 30 minutes, it is unlikely that the degradation of labeled protein contributed to the results.

If, bearing in mind the limitations just mentioned, it is concluded that spreading depression does indeed result in a decrease in the rate of protein synthesis, it is interesting to speculate on the mechanism of this effect. The primary event in spreading depression is believed to be the loss of intracellular potassium, and rise of the extracellular levels of this cation (Brinley, 1963; Grafstein, 1963). A number of other biochemical alterations have also been reported. Glucose, glycogen and creatine phosphate levels fall, while the amounts of lactate, citrate, inorganic phosphate and total ninhydrin positive substances increase (Ruscak, 1961, 1962, 1963; Ruscak and Macejova, 1963; Krivanek, 1961). These changes are enhanced by ischemia and indicate a generalized alteration in the oxidative metabolism of cortical cells. In view of these changes and the present results, indicating an equal effect on all proteins, it seems most likely that the effect of spreading depression on protein synthesis is a non-specific, secondary consequence of the diversion of energy sources or amino acids or both into different metabolic pathways.

C. Immunological Analysis of Soluble Brain Proteins

A complete analysis of the tissue specificity of the various antigenic components of whole rat brain extracts was not attempted. Antisera to whole RBE were considered much too weak and too complex to make such an analysis significant. Kosinski and Grabar (1967), however, analyzed antisera to rat brain extracts prepared in an almost identical manner, and identified five brain specific components in immunoelectrophoresis by absorbing the antisera with extracts of various non-neural tissues. Three of these components were in the far-anodal "pre-albumin" region, which agrees with the results of the limited analysis in the experiments reported here.

Preparation of antisera to sub-fractions of RBE simplified the search for brain specific antigens considerably. The antiserum to the most acidic fraction (VI), on first inspection in immunoelectrophoresis appeared to have two acidic brain specific components, and no additional non-specific components. More complete analysis of this antiserum showed that the two arcs

visible in immunoelectrophoresis of whole RBE resulted from a single antigen (α) which migrated in two regions. Depending on the length of time of immunoelectrophoresis, the two arcs could be shown to be either entirely separate or to fuse and form a "gullwing". The second component in antiserum VI (β) was not clearly visible in immunoelectrophoresis of whole RBE, but could be demonstrated in simple immune diffusion. Antiserum V contained only component β, and it was possible to show with the combined use of antisera V and VI that α and β were two unrelated antigens. Antigen β was not extensively studied, but it did not appear to be specific to nerve tissue, and in zone electrophoresis on starch it migrated in a position between the two antigen α regions, extending into the more slowly migration region.

Of the remaining four antisera, those prepared against the two most cathodal starch block fractions, I and II, by absorption with kidney extract, were shown to contain no nerve specific antibodies. Antisera III and IV were not further examined.

In discussing the tissue specificity of antigens, the limits of resolution of the detection techniques must be borne in mind. The immunodiffusion methods employed in the present experiments can detect antigens at concentrations as low as 5 ug/ml (Kabat and Mayer, 1961), although this varies depending on the particular antigen-antibody system. Assuming that 50% of the antigen α present in RBE is lost during the purification, it can be estimated that the concentration of the protein in RBE is about 150 µg/ml or 1% of the total RBE protein. Thus, it is possible that the antigen is present in other tissues, but at a concentration less than 1/10 of that found in brain. If small amounts of antigen α are in fact present, in the case of liver and kidney the concentration is also too low to elicit antibody formation, as shown by the failure of antisera to these organs to react with antigen α. Moreover, small amounts of antigen α in some organs could be due to innervation. Trace amounts of the "S-100" protein in muscle were attributed to this source (Levine, 1967).

Antigen α was purified to the degree that it was virtually free of other protein contaminants, and a chemical characterization of the molecule was begun. Only one soluble protein, the "S-100" protein (Moore, 1965) has been previously purified and demonstrated to be nerve specific.

The combined results of the molecular weight determination in a reducing and dissociating solvent, the amino acid composition, and the peptide mapping are fairly good evidence for the existence in antigen α of a single type of subunit having a molecular weight of about 39,000 (Table VIII). Although the minimal size of the native protein cannot be stated with certainty as yet, most of the evidence at hand suggests that it is at least a dimer of two identical subunits. First, the position of the most rapidly migrating band (which is the one of smallest molecular size) of purified antigen α preparations on polyacrylamide gels corresponds to the position of serum albumin, which has a molecular weight of about 69,000 (Schachman, 1963). Further, the elution volume of antigen α on Sephadex G-100 corresponds approximately to a molecular weight of about 83,000. Both of these approximate values agree more closely with the molecular weight of the dimer than with that of the monomer. Finally, the lowest value of molecular weight was obtained from sedimentation equilibrium measurements only in the presence of β-mercaptoethanol which is known to cleave disulfide bonds. This further suggests that disulfide bonds are necessary for the formation of a dimer.

Antigen α showed a marked tendency to form higher order aggregates, as indicated by the appearance of multiple bands of larger molecular size on acrylamide gels, and in sedimentation velocity and equilibrium experiments under non-dissociating conditions. The difficulty in obtaining large quantities of antigen α has thus far prevented a complete investigation of the factors influencing the aggregation. Exposure to low pH seems to favor the formation of higher order aggregates, as shown by the increase in sedimentation coefficient, from 5.7S to 12.7S after dialysis of antigen α against 0.1 M KCl, pH 3.0. This aggregation may be irreversible, for attempts to return the preparation to neutral pH resulted in precipitation of antigen α. The chemical forces involved in the aggregation are not as yet clear. In equilibrium centrifugation experiments, the use of a dissociating solvent, guanidine, was sufficient to produce homogeneity as determined by the log c vs $r^2/2$ plot. This would indicate that the aggregates are formed through weak, noncovalent bonds. In starch gel electrophoresis in urea, however, cleavage of disulfide bonds was necessary before evidence of size homogeneity was obtained. While the results of these different experiments cannot be completely reconciled, there are several possible

explanations. Some heterogeneity in guanidine could easily be missed with the criteria used, especially since the experiment was done at only one initial concentration. Guanidine is a better denaturing agent for some proteins than urea (Tanford et al., 1967). Thus some noncovalent interactions may still have been intact in urea. The initial concentration of the antigen α preparation was much greater in the starch gel experiment (10 mg/ml) than in any of the other procedures. This preparation had also been dialyzed against 2% acetic acid, which, as indicated by the sedimentation velocity experiment at pH 3, may have produced entirely different aggregates from those observed at neutral pH.

Several observations suggest that antigen α may bind to other components in the brain extract. Such an interaction would be very interesting, for it raises the possibility that antigen α could regulate the activity of some other molecule, or, conversely, that there exists a component that might regulate the activity of the antigen. The evidence for this interaction is the appearance of antigen α in two regions in immunoelectrophoresis of whole rat brain extract, and in zone electrophoresis. Since purified antigen α gave only one band in immunoelectrophoresis, corresponding to the one having the greater anodal mobility, it is more likely that the slowly migrating antigen fraction is bound to other components of RBE, rather than to buffer salts. When the slowly migrating antigen fraction from zone electrophoresis was subjected to DEAE chromatography, antigenic activity again appeared at several discrete regions along the ionic strength gradient, including the region in which antigenic activity emerged when the acidic starch block peak was applied to the DEAE cellulose.

In considering the biological activity of antigen α, a comparison of the properties of the antigen with those of proteins of known function offers a means of eliminating some of the many possibilities. Since antigen α was found only in nerve tissue, it is unlikely that it is an enzyme common to all tissues, such as the enzymes of intermediary metabolism. None of the enzymes which seem to be present only in nerve tissue have been purified and characterized sufficiently to allow a comparison with antigen α on the basis of molecular weight or amino acid composition. The fact that acetyl cholinesterase is present in muscle as well as nerve and is also membrane bound and thus not easily solubilized, makes acetylcholinesterase an unlikely candidate for antigen α. Proteolytic activity

could not be discounted a priori, especially since the acid protease purified by Marks and Lajtha (1965) appeared to have a mobility on acrylamide gels similar to that of antigen α. For this reason, antigen α was tested for acid protease activity. The failure to find any protease activity either in a purified antigen preparation or in the starch block fractions containing antigenic activity is presumptive, but not conclusive proof that antigen α is not the protease purified by Marks and Lajtha. The enzyme could have been inactivated by any of the purification procedures used, to such a degree that any remaining activity was not detectable in the assay. However, the fact that some activity could be recovered in a different region of the starch block strengthens the argument that antigen α is not an acid protease. The proteases active at neutral pH were reported by Marks and Lajtha (1965) to be eluted from Sephadex G-100 columns in the void volume, and are therefore much larger proteins than antigen α.

A protein subunit which binds colchicine has recently been purified from microtubules (Shelanski and Taylor, 1967). Although the colchicine binding activity is not specific to nerve, a high activity has been demonstrated in brain and in squid axoplasm (Boresy and Taylor, 1967). The behavior of this microtubular subunit in sedimentation velocity experiments (6S), and its tendency to aggregate when the concentration is increased, suggested that it might be identical with antigen α. As evidence to the contrary, antigen α did not show any colchicine binding activity.* Furthermore, the microtubular protein is present in sperm tails in high concentration, but testes did not contain any antigen α activity.

The ease with which antigen α is obtained in soluble form makes it unlikely that the protein is one of the proteolipids of myelin. Protein components of myelin that are more easily solubilized, such as the encephalitogen, are all basic proteins, whereas antigen α ia acidic.

Since the "S-100" protein is also a soluble, acidic protein, a comparison of this nerve specific protein with antigen α was necessary. It was clearly shown by immunological techniques that the two proteins were unrelated. Additional evidence that antigen α is an entirely different protein is provided by a comparison of the amino acid compositions, which are listed in Table XI. Both proteins have a high glutamic and aspartic

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*This test was performed by Dr. S. H. Barondes, Albert Einstein College of Medicine.

TABLE XI

Comparison of Amino Acid Compositions of Antigen α and S-100
(moles per 100,000 gm)

Amino Acid	Antigen α	S-100*
Lys	61	76
His	15	33
Arg	38	13
Asp	100	80
Thr	40	23
Ser	52	23
Glu	127	166
Pro	44	3
Gly	77	47
Ala	92	50
Cys	13	13
Val	65	113
Met	18	?
Ile	49	27
Leu	83	67
Tyr	22	10
Phe	27	60

Tryptophan not included

*Calculated from data of B.W. Moore (1965).

acid content, but antigen α has a high proline content relative to "S-100" and a much lower valine and phenylalanine content. It must be mentioned that the amino acid composition listed for "S-100" is that for the rabbit protein, whereas the data for antigen α come from rat; it is unlikely that species differences account for the large differences in composition, especially since "S-100" from rabbit is very similar to that from beef (Moore, 1965).

Since antigen α and "S-100" are the only two soluble proteins apparently restricted to the nervous system, which have been obtained in pure form, it is interesting to compare some of their other properties. Some of the physicochemical properties of "S-100" have been reported. The elution volume from Sephadex G-100 suggests an approximate molecular weight of 30,000 (Moore, 1965), compared with about 80,000 for antigen α. Although purified S-100 was reported to give rise to several bands on mixed agarose-acrylamide gels (Gombos et al., 1966) it appeared homogeneous in sedimentation velocity experiments done at a concentration of 5 mg/ml, and in non-dissociating solvents having pH values between 6.8 and 8.8 (Vincendon et al., 1967). The $s_{20,w}$ was given as 2.0. Under similar conditions, antigen α appeared to have a heterogeneous size distribution. There have been no reports of any subunits in "S-100".

Both proteins are found in the peripheral and central nervous system. The regional distribution of "S-100" has been examined quantitatively by complement fixation (Levine and Moore, 1965; Levine, 1967). It has been found in considerably higher concentrations in white matter than in gray matter, and appeared to be confined primarily to glial cytoplasm (Hyden and McEwen, 1966). While quantitative data for the distribution of antigen α are not yet available, comparison of precipitin bands obtained with extracts of various regions of the central nervous system suggest that there are no large differences in antigen α content.

Again, using quantitative immunological techniques, Levine (1967; Levine and Moore, 1965) has pointed out the striking similarity of "S-100" in various animal species. The poor cross reaction of antiserum to antigen α with various brain extracts from different species would indicate that this is not true of antigen α. The fact that there was some

cross reaction, however, indicates that antigen α is most likely present in other species, but that the protein is sufficiently different at the antigenic site(s), to result in only partial recognition by antibodies to the rat antigen. This is the situation that prevails for most proteins having a given activity (Wilson et al., 1964).

Finally, antigen α, unlike "S-100", is antigenic in its native state. This raises the possibility that the antisera to nerve tissue which had effects on electrical activity (see for example, Mihailovic and Jankovic, 1961; de Robertis et al., 1966) contained anti-α components, and that, therefore, antigen α may have some role in excitability. This possibility can be tested more rigorously with a univalent antiserum to the protein. No reports have yet appeared indicating that antiserum to "S-100" has any physiological effects.

During the purification of antigen α it became apparent that at least one other protein could be purified without much difficulty. Such information was obtained by examination on acrylamide gels of the protein fractions obtained in the various stages in the purification of antigen α. This is the protein that migrates with the far-anodal antigen peak in zone electrophoresis, but is insoluble at pH 5. Zone electrophoresis of the redissolved pH 5 precipitate should result in the isolation of the protein. Antisera to this protein can then be prepared, and the tissue specificity determined.

Finally, further fractionation of each of the other starch block fractions, and immunization of rabbits with subfractions containing fewer individual protein components will certainly lead to the production of additional antisera containing sufficient amounts of antibodies to detect other nerve specific antigens.

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