IMMUNOLOGICAL STUDIES OF CHICKEN BRAIN: ISOLATION, PURIFICATION, AND LOCALIZATION OF A NEURAL SPECIFIC PROTEIN CNA-1

By

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ABSTRACT

The differentiation of any cell leads to the synthesis of certain proteins which are responsible for its specific function. In the nervous system this includes such functions as the conductance of action potentials, synaptic transmission and the establishment of specific connections. The developmental biology of proteins unique to the nervous system is therefore of great importance when studying the relationship of proteins to physiological function.

Although some neural specific components of adult chicken brain and their ontogeny in the chicken embryo have been previously described by others, they have not been extensively characterized. The present investigation was undertaken in order to enumerate, characterize and study the embryological appearance of antigens specific to the adult chicken brain that can be demonstrated by rabbit anti-brain sera. Particular interest was given to the isolation and purification of one such neural specific antigen in hopes of further elucidating its role in adult function and embryonic development.

Antiserum produced in response to a saline soluble extract of adult chicken brain (ABE), when absorbed with adult liver, serum and kidney extracts, yielded a neural specific antiserum (AABS). This polyvalent antiserum was capable of demonstrating at least eight adult neural specific antigens within ABE during immunoelectrophoretic

analysis. The sequential appearance of seven of these antigens during embryonic development (D2 to D11 of incubation) was established. An eighth antigen could not be detected within the embryonic brain extracts and was assumed to be associated with posthatching neural development. By immunochemical criteria, the majority of the neural specific antigens, but not all, were demonstrated to be restricted to avian brain. Similarly the majority of these antigens, but not all, were demonstrated to be protein in nature since reactions with trypsin and chymotrypsin resulted in loss of antigenicity.

Separation of the antigens within ABE, based on their net ionic charge and molecular weight, was attempted with each resulting fraction being analyzed by immunoelectrophoresis. One neural antigen (CNA-1), possessing alpha-1 globulin mobility was isolated from the other neural specific antigens. This thesis further describes a procedure developed for the purification of CNA-1, utilizing ammonium sulphate fractionation, ion exchange chromatography, gel filtration chromatography and preparative polyacrylamide gel electrophoresis. Highly sensitive and quantitative immunochemical techniques (crossed and fused rocket immunoelectrophoresis) were employed in monitoring the purification steps.

Studies on the purified antigen revealed it to be homogeneous by a number of criteria, having a native molecular weight of 65,000 daltons as determined by molecular exclusion chromatography and possessing two identical subunits (MW 30,000) as determined by SDS

polyacrylamide gel electrophoresis. The structure of CNA-1 was concluded to be protein on the basis of its sensitivity to trypsin and chymotrypsin.

Immunochemical studies utilizing a monovalent antiserum to CNA-1 revealed the protein to be avian specific and not present in brain extracts of mammalian species. Also both a high (> 1,500,000 daltons) and low (65,000 daltons) molecular weight component which shared the CNA-1 antigenic determinant were observed, indicating possible aggregation of the protein during the isolation procedure.

CNA-1 was first detectable by the seventh day of incubation and continued to accumulate during the period of embryonic neural development in which both neurogenesis and synaptogenesis are known to occur. Within the adult chicken cerebellum, CNA-1 was specifically localized to the cell surfaces of particular neuronal cell types (i.e. Purkinje cells, granule cells and neurons of the deep cerebellar nuclei) by immunohistochemical techniques. Glial elements were not labelled.

Organ specific brain antigens have been described by different investigators. The antigen described within this thesis appears to be completely different from all others described in the literature as revealed by a study of its physical and chemical properties. The function and role of CNA-1 on the cell surface remains to be elucidated. Its localization on specific cell types suggests that it may be associated with inhibitory synaptic function. For this reason,

further study of CNA-1 may prove to be important in unravelling some of the questions concerning cell-cell interaction and neuronal specificity during development of the central nervous system.

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INTRODUCTION

An antigen may be defined as any agent giving rise to antibody formation specific for that agent. The antigen may be protein, nucleic acid, carbohydrate, lipid, or a combination of these. In the field of embryology, particular attention has been given to those antigens which have been found in saline extracts of adult brain, and which are demonstrable by precipitin reactions in agar or agarose gels.

Biochemical analyses of saline extracts of brain have shown that the brain may contain several hundred different proteins (Bogoch *et al*., 1964; Moore and McGregor, 1965; Klose and von Wallenberg-Pachaly, 1976). However, most of these proteins do not appear to be antigenic, since immunochemical studies within a number of species have demonstrated only a limited number of antigens. The structural basis for this lack of antigenicity of the majority of brain proteins has not been completely elucidated.

Since the nervous system is characterized by a number of specialized functions, antigens (i.e. proteins), judged by immunochemical methods to be specific to the nervous system, may in some way be involved in its unique function and development. Isolation and characterization of neural specific antigens, following their initial demonstration, is therefore important in the study of the nervous system. The ontogenic appearance, characterization, isolation and purification of antigens specific to the avian nervous

system, with particular interest in acidic or slightly acidic antigens is the subject of this thesis.

A number of investigators have produced antiserum directed against either whole or fractioned saline soluble adult brain extracts. Subsequent absorption of the antiserum against organ extracts (i.e. liver, kidney, and serum) removed antibodies directed against antigens common to non-neural tissue, yielding antiserum specific for neural antigens. Immunoprecipitin analyses in agar or agarose gels have demonstrated acidic neural specific antigens within a number of species. These antigens have been demonstrated to be either, class and species non-specific (i.e. phylogenetically conserved), class but non-species specific, or class and species specific.

1. Mammalian Brain

a) Rodent

MacPherson and Liakopoulou (1966), in their early immunochemical studies of water soluble rat brain proteins demonstrated twelve antigens. One was both species specific and restricted to nervous tissue, while a second was neural specific, but common to brains of other mammalian species. Isolation of the species restricted antigen of rat nervous tissue (SRANT), was achieved by Liakopoulou and MacPherson (1970). SRANT was observed to be electrophoretically heterogeneous possessing both alpha-1 globulin and albumin mobilities, but homogeneous in size, having a molecular weight of 70,000 daltons.

Kosinski and Grabar (1967), obtained similar results from their crude saline extracts of rat brain. However, eleven antigens

were observed, with five being described as specific for the central nervous system. No further characterization of this antigen was undertaken by these investigators.

A second neural specific antigen was isolated and purified from rat brain extracts by Bennett and Edelman (1968). This acidic protein, designated antigen alpha, appeared as the most anodal migratory component during starch gel and immunoelectrophoresis at pH 7.4. However, heterogeneity within its electrophoretic mobility was also observed. Preliminary physicochemical studies revealed molecular weights of 83,000 daltons and a single subunit molecular weight of 39,000 daltons. Antigen alpha was detected within whole brain extracts of rat fetuses by the 17th day of gestation, at levels only 2% of those of adult brain (≈ 0.9% of the total soluble proteins) (Bennett and Vaguez, 1972).

The immunological and electrophoretic identity between antigens alpha and 14-3-2 (a bovine neural specific protein described by Moore and Perez, 1968) was confirmed by Bennett (1974). Slight differences were observed in their electrophoretic mobilities, but both proteins possessed the same, or very similar antigenic determinant. Similarly, Marangos _et al_. (1975a), isolated an acidic soluble protein from rat brain which also cross reacted with antiserum to bovine 14-3-2 protein. This acidic protein (nerve specific soluble protein of rat brain) was designated NSP-R, since this was a more functional classification than either 14-3-2 or antigen alpha (Marangos _et al_., 1975b). The physicochemical properties of NSP-R were described by Marangos _et al_. (1975a) and complemented those given by Bennett and Vaguez (1972).

The native NSP-R was shown to have an isoelectric point of 4.7 in the absence of denaturing agents and 5.0 in the presence of 2.0 M Urea. Sedimentation velocity and equilibrium data indicated a homogeneous component with a molecular weight of 78,000 daltons. Sedimentation of NSP-R in 6.0 M guanidine-HCl containing 0.02% glutathione yielded a molecular weight of 39,000 daltons indicating a dimeric structure for the native protein. Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis produced a sharp band with a relative mobility corresponding to a molecular weight of 48,000 daltons. Using a highly sensitive solid-phase radioimmunoassay for NSP-R, Marangos et al. (1975b), were able to show that NSP-R accounted for 1.4% of the total soluble protein of the adult rat brain.

NSP-R was found in cross reacting forms in all mammalian species tested and partially cross reacting forms in avian species. This indicated that NSP was not species specific and also partially non-class specific, although no cross reacting forms could be demonstrated in fish neural tissue (Moore and Perez, 1968). NSP-R was also purified from cat and human brain as well as rat and bovine brain mentioned above. NSP from each species was compared using electrophoretic and immunological criteria. Both physicochemical properties and amino acid composition of NSP from these species were found to be similar, with the predominant feature being the high content of glutamic and aspartic acid (Marangos et al., 1977).

Based on indirect evidence such as decreasing levels of NSP in bovine brain areas during nerve degeneration (Cicero et al., 1970a),

higher concentrations in human grey matter than in white matter (Moore, 1972) and higher concentrations within synaptosomal preparation obtained from rat brain (Grasso and Chen, 1974), NSP was stated to be of neuronal origin. The sole presence of NSP within some neurons and their processes, but not all neurons, was confirmed by the immunohistochemical localization studies of Pickel et al. (1976.) Grasso et al. (1977), and Ronnback et al. (1977), demonstrated NSP in both axons and dendrites, as well as synaptic junctions by immunoelectron microscopy. NSP was shown to accumulate in not only postsynaptic membranes, but also the presynaptic densities. However, NSP was never demonstrated in the membranes of the Golgi complex, in the inner membranes of mitochondria or in the nucleoplasm of neurons, nor was it found in astrocytes, oligodendrocytes or in non-neuroectodermal tissue elements (Persson et al., 1978). Cimino et al. (1977), studied localization of NSP during fetal development by immunofluorescence. Three classes of neurons, were distinguished: neurons that had NSP early in development and throughout the life of the animal; neurons in which the protein was present only for a limited period of time during development (eg. Purkinje cells); and neurons which never contain NSP (eg. cortical motor neurons).

Levels of NSP have been determined during embryonic and fetal development. Detectable levels were observed by the fourth day of incubation within the optic tectum of chicken embryos, at about the time the first differentiated cells can be recognized morphologically. However, little increase in concentration was observed until after

hatching (21 days) (Cicero et al., 1970b). Since practically all cell division in the optic tectum is completed by 14 days of incubation, this suggested that NSP was correlated with functional rather than morphological development (Moore, 1973).

Bock and Dissing (1975), demonstrated enolase (2-phospho-D-glycerate hydrolyase, E.C. 4.2.1.11) activity connected to NSP immunoprecipitin peaks during quantitative immunoelectrophoresis. The two electrophoretically different forms (first observed by Bennett and Edelman, 1968), were observed to be immunochemically partially identical and were both shown to be brain specific isozymes of enolase. The coincidental chromatographic elution of NSP and two of the three brain enolase isozymes confirmed this observation (Marangos et al., 1976; Fletcher et al., 1976). The isozymes were shown to be dimers, $\gamma\gamma$ and $\gamma\alpha$, made up of the brain specific γ subunit and the α subunit known to be common to liver enolase. The $\gamma\gamma$ enolase corresponded to the rapid anodal migrating protein isolated by Bennett and Edelman (1968), and the $\alpha\gamma$ hybrid enolase was a slower migrating and cross reacting isozyme (Bock et al., 1978). NSP has recently been renamed neuron specific enolase (NSE) by Schmechel et al. (1978), to encompass its functional role as both a neuron specific marker and a brain specific glycolytic enzyme.

Antigen-antibody crossed electrophoresis has been employed by Bock et al. (1971), to study water soluble antigens of rat brain. This sensitive immunochemical procedure demonstrated at least 25 to 62 water soluble tissue antigens (Bock et al., 1974). Five of these antigens

were shown to be brain specific. Two were found to be highly acidic proteins (Antigen 1 and 3), and a third antigen possessed both alpha-1 globulin and albumin mobilities (Antigen 7). The two remaining brain specific antigens were shown to behave as an alpha-1 globulin (Antigen 17) and an alpha-2 globulin (Antigen 27) respectively (Bock, 1972). The species restricted nature of Antigen 7 led these authors to suggest its identity with the SRANT antigen isolated by Liakopoulou and MacPherson (1970). Antigens 3 and 17 were later shown to be the two brain specific enolase isozymes discussed above (Bock et al., 1975).

The presence of these brain specific antigens within rat brain synaptosomes and synaptic vesicles was also investigated by Bock et al. (1974). Antigens 3, 7 and 17 were found among synaptosomal antigens, while only trace amounts of antigen 3 and 17 were found among the synaptic vesicle antigens. Polyvalent antiserum induced against both rat brain synaptosomal membranes and synaptic vesicles demonstrated several antigens not previously demonstrated within the whole brain extract. Four additional neural specific proteins, C1 (synaptin), D1, D2, and D3 were revealed (Bock et al., 1974; Jorgensen and Bock, 1974). Although these antigens were absent from astroglial cell cultures, and were neuronal membrane associated (Bock et al., 1975; Jorgensen, 1976), their precise physiological function has not been elucidated.

b) Bovine

Saline soluble extracts of beef brain have been immunochemically studied similarly to that described for rat brain. At

least seventeen antigens were observed during immunoelectrophoretic analyses. Two were both species and neural restricted, while five additional neural specific antigens were common to other mammalian species (Hatcher and MacPherson, 1969). One antigen (α BASNT), a member of this latter group, was isolated and purified by Hatcher and MacPherson (1970). α BASNT was found in only one molecular form, with a molecular weight of 84,000 daltons and possessed an electrophoretic mobility of an alpha globulin. The antigen was shown to appear as early as 7 months gestation in the bovine fetal brain, and by 3 months in the human fetal brain. Preliminary studies suggested a glial cell origin since enriched glial cell preparations contained high concentrations of α BASNT. The immunological and physical properties of α BASNT described by Hatcher and MacPherson (1970), were clearly distinct from those of bovine NSP (14-3-2) which have been discussed previously.

A third neural specific bovine antigen, S-100, initially isolated by Moore (1965), was so-named because of its solubility in saturated ammonium sulphate at pH 7.0, a property which together with a very acidic isoelectric pH has been exploited during its purification. This small molecule (MW 21,000 - 24,000) was found to be of low immunogenicity and coupling of the S-100 protein to methylated bovine serum albumin was required to induce antibody production (Levine and Moore, 1965).

S-100 has been the focus of extensive research during the past decade. Its immunological identity has been conserved throughout

phylogeny, being present in all classes of vertebrates, and in brains of several invertebrates (Levine and Moore, 1965; Moore et al., 1968). The S-100 protein has been isolated and characterized from: human brain (Uozumi and Ryan, 1973); sheep brain (Uyemura et al., 1971), pig brain (Uyemura et al., 1971; Abe et al., 1974); and chicken brain (Kato et al., 1977). Amino acid analyses of the S-100 proteins were similar, thirty percent of its total amino acid content being glutamic and aspartic acid residues (Moore and McGregor, 1965).

Evidence has been reported which suggests that S-100 is not a single homogeneous protein, but rather a heterogeneous mixture of polypeptide chains, possessing similar physicochemical properties (Uyemura et al., 1971; Stewart, 1972a; Abe et al., 1974). Two S-100 protein components (phenylalanine rich acidic proteins, PAP1-a and PAP1-b) have been demonstrated to exhibit similar molecular weights and amino acid compositions with a common amino acid sequence within a portion of the molecule (Isobe et al., 1977). PAPI-b protein was observed to be a dimer of two identical polypeptide chains. The amino acid sequence showed regions of strong clustering of hydrophobic, basic and acidic amino acids, with an apparent calcium-binding site in the acidic cluster (Isobe and Okuyama, 1978). In the presence of calcium ions, S-100 was previously observed to undergo conformational changes which exposed the hydrophobic groups of the molecule, thus making the protein capable of interacting with liposomes, and facilitating the membrane transport of monovalent cations (Calissano and Bangham, 1971; Calissano, 1973; Calissano et al., 1974).

S-100 protein has been demonstrated both in the central and peripheral nervous system. Generally the levels in white matter were found to be higher than in grey matter (Moore, 1972). However, some controversy still surrounds the localization of S-100. Most authors have demonstrated S-100 within glia (Cicero et al., 1970a; Perez et al., 1970), particularly in astrocytes and oligodendroglia (Ludwin et al., 1976), in the absence of neuronal localization. Other studies however, have suggested S-100 in neurons (Hydén and McEwen, 1966; Sviridov et al., 1972; Tabuchi et al., 1976; Miani et al., 1972 and Michetti et al., 1974). Support for the neuronal distribution came from the demonstration of S-100 as both a soluble and a membrane bound protein (Rusca et al., 1972; Donato and Michetti, 1974; Donato et al., 1975; Hyden and Ronnback, 1975 and Hydén and Rönnbäck, 1978) and its detection in isolated nerve endings (synaptosomes). S-100 was also observed by Miani et al. (1972), to undergo proximo-distal axoplasmic flow. However, caution has been expressed in the interpretation of this data, since synaptosomal fractions have been found to contain glial subcellular particles (Cotman et al., 1971), and S-100 has been found in schwannomas by Pfeiffer et al., (1972), which may indicate that schwann cells are the source of S-100 localized in the peripheral nervous system.

Developmental studies of S-100 have been conducted in several mammalian species (Zuckerman et al., 1977). These studies have shown low levels of S-100 at birth, followed by an increase during postnatal development until adult levels are reached. Similarly Cicero et al.,

(1970b), found that S-100 increased at a time when cell division had ceased in the chicken embryonic optic tectum. This observation was supported by tissue culture studies utilizing rat C_6 glioma cells (Pfeiffer *et al.*, 1970), and monolayer cultures of rat astrocytes (Labourdette and Mandel, 1978), which also demonstrated S-100 synthesis during the exponential growth phase, but its levels increased only after cell division had stopped.

Despite the extensive research cited above, the precise physiological role of S-100 protein(s) within the nervous system has remained elusive and will require additional investigation prior to the complete realization of its function.

c) Human

Caspary and Field (1963), using double immunodiffusion analysis of saline soluble extracts of human brain, demonstrated three precipitin lines when developed with anti-brain antiserum. Absorption of this antiserum with kidney and liver extracts produced only one precipitin line against brain extract. This brain specific antigen was further shown to be destroyed by proteolytic digestion.

Buffer soluble extracts of adult cortical grey matter demonstrated at least thirteen distinct tissue antigens during double immunodiffusion and immunoelectrophoretic analyses performed by Rajam and Bogoch (1966). Five antigens were observed within a basic fraction and a progressively acidic group contained seven to eight antigens. Furthermore these authors demonstrated that three or four of these

antigens were resistant to 100°C for 1 hour (thermostable) and were relatively soluble in ethanol. Preliminary studies indicated that two or three such thermostable antigens were not shared by either liver or kidney extracts (Rajam et al., 1966). At least two brain specific antigens with alpha-1 globulin mobility could be demonstrated by immunoelectrophoresis developed with fully absorbed antiserum (Rajam et al., 1969).

Burbaeva (1972), described similar results after immunoelectrophoresis of phosphate buffered extracts of whole human brain developed with anti-brain antiserum absorbed with serum and liver extracts. However, this author found three brain specific antigens, one with alpha-1 globulin mobility and two with alpha-2 globulin mobility. Also, one antigen within this latter group was described as species specific, and was not observed in any other mammalian brain extracts tested.

Immunochemical analyses of soluble extracts of human brain white matter were also performed by Warecka and Bauer (1967). Their anti-brain antiserum, being previously absorbed with serum, liver and kidney extracts, demonstrated a single brain specific antigen possessing alpha-2 globulin mobility. PAS staining in agar and cleavage by neuraminidase indicated this was an acidic alpha-2 glycoprotein. Studies with human fetal brain extract (Warecka and Muller, 1969), demonstrated the appearance of this antigen between 24 and 28 weeks gestation. This was found to be strongly correlated with the morphological appearance and differentation of glia during

neurogenesis. Although present mainly in the white matter of the cerebral hemispheres, the spinal cord and optic nerves, traces of alpha-2 glycoprotein were also demonstrated in grey matter and bulk prepared neurons, but absent in peripheral nerves. This data suggested a predominantly glial localization for this protein (Warecka *et al.*, 1972).

The presence of alpha-2 glyocoprotein within various brain tumors has been studied by Wadrecka (1975). Although the antigen was present in benign astrocytoma tumors, it could not be demonstrated in malignant gliomas (i.e. glioblastoma multiforma). This indicated that alpha-2 glycoprotein was an attribute of mature astroglia and of astrocytoma cells, but not of immature undifferentiated glioblasts.

Immuno-affinity chromatography (Warecka *et al.*, 1972), and concanavalin A binding (Brunngraber *et al.*, 1975), have both been employed during purification of the alpha-2 glycoprotein. A multiplicity of protein monomers of various molecular weights was visualized during SDS polyacrylamide gel electrophoresis of the purified antigen. However, a major band corresponding to a molecular weight of 45,000 - 50,000 daltons was demonstrated to be PAS positive (Brunngraber *et al.*, 1974).

An acidic protein isolated by Eng *et al.* (1971), from pathological human neural tissue (i.e. multiple sclerosis plaque), rich in fibrous astrocytes was designated glial fibrillary acidic protein (GFA). The protein was shown to contain high proportions of glutamic acid, aspartic acid, arginine, alanine and leucine, but no cysteine

and negligible amounts of lipid and carbohydrate. GFA migrated as two bands with an average molecular weight of 43,000 daltons during SDS polyacrylamide gel electrophoresis, but was excluded from Sephadex G-200. This suggests the GFA protein occurs in solutions as an oligomer with a molecular weight exceeding 100,000 daltons.

GFA was shown to be immunogenic in rabbits and subsequent immunochemical studies revealed this antigen to be specific to the central nervous system (CNS), not being present in peripheral nerves or non-neural tissues (Uyeda et al., 1972). GFA is not species specific or class specific; cross reaction has been observed with several mammalian and non-mammalian species. The antigen migrated as an alpha-2 globulin during immunoelectrophoretic analyses of all species tested. However, only partial identity toward non-mammalian species was exhibited during double immunodiffusion. Unlike glial S-100, GFA could not be detected in invertebrate species (Dahl and Bignami, 1973).

Selective localization of GFA within astrocytes has been demonstrated by a number of investigators employing immunofluorescence (Bignami et al., 1972; Bignami and Dahl, 1974; Braak et al., 1978), and immunoperoxidase staining (Ludwin et al., 1976). These observations were confirmed during ultrastructural localization studies by Schachner et al. (1977a). The antigen was detected in astroglia and their processes, but not in neurons or their processes, or in oligodendroglia. The immunoperoxidase immunochemical reaction product appeared both as a diffuse cytoplasmic label and as elongated strands, similar in distribution and frequency to glial filaments. This

supported the hypothesis that GFA was a major component of these filaments (Eng *et al.*, 1971).

The glial filaments, 8-10 nm in diameter, are morphologically similar to neurofilaments and intermediate filaments found in a variety of cells and tissues (Shelanski and Liem, 1979). Recent comparative studies of GFA and neurofilament proteins (Yen *et al.*, 1976), and the isolation of GFA-like proteins from peripheral nerves where glial filaments are absent (Dahl and Bignami, 1976), indicated similarities between the protein subunits of glial and nerve filaments. However, antisera raised against neurofilaments from peripheral nerves (Schlaepfer and Lynch, 1977), or from brain (Matus *et al.*, 1979), were shown to stain only neuronal processes in both the peripheral and central nervous system, and failed to stain glial cytoplasmic filaments. These observations suggested that neuronal and glial 10 nm filaments were not identical, but have been co-purified during some preparations (Bignami and Dahl, 1977; Dahl and Bignami, 1979).

The developmental appearance of GFA was shown by immunofluorescence to coincide with a period when bundles of glial filaments become visible by electron microscopy (Bignami and Dahl, 1973). The ontogenic appearance of GFA within the chicken embryo spinal cord has also been studied. GFA was observed on the 12th day of incubation, and gradually increased during the following days (Bignami and Dahl, 1975). These observations indicated a close temporal and spatial correlation between the appearance of myelin and of GFA protein in chicken spinal cord during a period of cell multiplication. Similar results were

found in the mouse (Jacque *et al.*, 1976). Quantitatively, a peak of GFA concentration was observed between Days 10 and 14 postnatally, corresponding to the outbreak of astroglial differentiation at the time of myelination.

In spite of the data presented above, little is known of the function of GFA in astrocytes, or why it should be specific to the CNS while other fibrillary proteins such as neurotubulin are found in other tissues.

2. Avian Brain

Considerably less work on neural specific components in avian species has been reported in comparison to that of mammalian species. Preliminary studies by Burke *et al.* (1944), using both complement fixation and precipitin tests demonstrated at least four antigenic components in saline extracts of avian brain. Two were shown to be organ specific after absorption of the antiserum with extracts of testes, ovary, kidney and liver. Developmental studies revealed the appearance of at least one brain specific antigen in the chicken embryo between the eleventh and thirteenth day of incubation. Apparently the second brain specific antigen or group of antigens was limited to posthatching neural development.

Ebert (1950), using precipitin testing, reported that anti-sera produced in rabbits in response to saline extracts of brain and other organs from adult chicken were class specific and quantitatively organ specific. His anti-brain antiserum failed to produce precipitin reactions against brain extract when the antiserum had been previously

absorbed with extracts of either heart or spleen.

McCallion and Langman (1964), by means of double immunodiffusion, demonstrated that there were at least three, and possibly as many as five antigenic substances in adult chicken brain. These antigens were both class and neural specific, occurring only in the brain, spinal cord, neural retina and peripheral nerves. The first of the brain specific antigens appeared in the embryo by the end of the fifth day of incubation and all were present by the end of the twelfth day of incubation. There was no evidence from this study, of adult neural specific antigens appearing in the embryo during the early stages of neural development. This observation was supported by subsequent studies using immunodiffusion (McCallion and Trott, 1964), and immunoelectrophoresis (McCallion and Trott, 1965) with antiserum directed against nine day chicken embryonic brain absorbed with adult serum. Using this antiserum, the first neural specific antigen was detected by the sixth day of incubation. A second neural specific transient antigen, appeared by the eighth day of incubation in the embryo but was absent in brain extracts of prehatching chicken embryos. These results suggested that changes in the antigenic pattern of the embryonic brain was due to the appearance and disappearance of components during development.

With regards to the electrophoretic mobility, the adult neural specific antigen or group of antigens observed during this study expressed little, if any anodal migration during immunoelectrophoresis. This observation differed from the earlier studies by McCallion and Langman (1964), who demonstrated neural specific antigens which

migrated toward the anode during continuous flow electrophoresis. Similarly, studies by Schalekamp (1963), using rabbit anti-chicken brain serum absorbed with either liver or kidney extracts demonstrated three antigens that were neural specific and class specific by double immunodiffusion and immunoelectrophoresis. These adult antigens were observed to appear sequentially in the embryo on Days 2, 7, and 14 of incubation. The early appearing antigens were shown to possess alpha-2 globulin mobilities, the latter possessing beta-globulin mobility. Discrepancies between the ontogenic appearance of these neural specific antigens within the chicken embryo and differences expressed in their electrophoretic mobilities, suggested that there were many more antigens specific to the adult avian brain which were not revealed by these studies.

Antisera induced in response to adult chicken brain in all studies cited above were found to be class specific, failing to demonstrate cross reacting antigens within non-avian species, particularly mammalian. However, immunochemical studies of purified mammalian neural specific proteins (S-100, NSP and GFA, and alpha-2 glycoprotein) have demonstrated immunologically related or partially related antigenic components within the avian nervous system. The absence of precipitating antibodies found in earlier studies which are not class specific, demands further investigation.

A non-precipitating, complement fixing anti-chicken brain antiserum was produced by Friedman and Wenger (1965a). This antiserum,

absorbed with liver and yolk powder was demonstrated to be both neural specific and non-class specific reacting with brain extracts of a number of mammalian species. However, no qualitative distinction between neural specific antigens could be obtained from this serum (Friedman and Wenger, 1965b).

One approach to the study of development has been to relate the onset of differentiation to the production of antigens (i.e. proteins) that are unique to a single cell or tissue type. The basis for such studies is the concept that differentiated forms and functions of a specialized cell may be expressed, in part, by means of the properties of the individual antigens. One method of determining the significance of antigens in normal embryonic development has been to expose developing embryos to antisera against adult tissue. Ebert (1950), evaluated the effect of various tissue antisera (heart, spleen, brain) on the development of predifferentiated chicken embryos *in vitro*. The embryos were found to become rapidly disorganized and their growth was inhibited. Using progressively diluted antisera he obtained largely mesodermal deficiencies with anti-heart and anti-spleen sera and largely neural defects with anti-brain serum at moderate dilutions. Only growth retardation was demonstrated with greater dilutions of anti-brain serum. Specific neural defects have been described in chicken embryos treated *in ovo* at 33-36 hours of incubation with antibodies directed against neural specific antigens (McCallion, 1971; Barson, 1972). Degeneration and abnormal growth were restricted to neural tissues. Non-neural tissue showed no sign of degeneration.

Treatment at later stages of development was found to be ineffective. One possible reason suggested was that the target antigen was no longer available to the specific antibody. These studies suffered however, from poorly characterized polyvalent antiserum and crudely purified immunoglobulin fractions. Although demonstrating disorganizing and disruptive effects in neural tissue, studies of this kind shed little light on the physiological function or specific role played in the differentiation of the nervous system by these neural specific antigens.

A second method of determining the significance of neural specific antigens in normal embryonic development has been to selectively isolate antigens/proteins demonstrated to be neural specific and subsequently induce monovalent antiserum directed against them. The isolation and purification of several neural specific proteins from a number of mammalian species has been previously discussed.

The induction of antisera which is both monovalent and neural specific has become a powerful tool for the developmental neurobiologist. A number of immunochemical and immunohistochemical investigations have been undertaken on the ontogenic appearance and localization of several neural specific proteins within the chicken embryo (S-100, Cicero et al., 1970b; Friedman and Wenger, 1970; NSP, Cicero et al., 1970b; GFA, Bignami and Dahl, 1975). The nervous system of the chicken embryo offers the practical advantages of accessibility and accurate developmental staging (i.e. Hamburger and Hamilton, 1951).

In addition, much is known about the temporal origin and arrangement of particular cell types within specific morphological parameters. In each of the immunological studies cited above, monovalent antiserum directed against the purified mammalian neural specific protein was used. However, it has been observed by Friedman and Wenger (1970), that the complement fixation reaction of anti-beef brain S-100 serum with chicken brain extract was much more variable and less sensitive than with the homologous antigen. They also calculated that approximately 170 times more chicken brain protein was required to fix complement than was required to fix beef brain protein. Similarly, Kato _et al_. (1977), observed that precipitin reactions required approximately 12 times as much purified chicken S-100 (designated CBA-1) to form lines of comparative strength against antiserum to bovine S-100. These observations indicated that there was significantly less CBA-1 within chicken brain than was found for S-100 in bovine brain. Slight dissimilarities were also observed in the physicochemical characteristics of the avian and bovine acidic protein. The molecular weight of CBA-1 was calculated to be lower (MW 14,500) compared to S-100 (MW 16,000), but the amino acid composition of both proteins showed similar amounts of acidic residues. However, no structural similarities existed on peptide mapping and N-terminal amino acids were detected for CBA-1 but not for S-100. In the presence of reducing reagents, CBA-1 failed to separate into subunits, while bovine S-100 was previously shown to separate into three (Dannies and Levine, 1971) or four (Stewart, 1972b) subunits. In spite of these differences

in physicochemical properties, CBA-1 contained a closely related antigenic determinant to that of bovine S-100 protein. Kato *et al.* (1977), concluded however, that the chicken S-100 was clearly different from purified S-100 protein obtained from mammalian sources.

It is quite apparent that studies utilizing avian systems and monovalent antiserum induced in response to cross reacting proteins, isolated and purified from mammalian species must be interpreted with caution in the light of differences in molecular structure between supposedly analogous proteins of avian and mammalian species. The need to characterize the saline soluble antigens specific to the chicken nervous system, both class and non-class specific, is therefore apparent.

Although some neural specific components of adult chicken brain and their ontogeny in the chicken embryo have been previously described by a number of authors, they have not been extensively characterized.

The specific objectives of the present investigation have been four fold: (1) to enumerate, characterize and study the embryological appearance of saline soluble adult chicken brain antigens that can be detected by immunoprecipitation with rabbit anti-brain antiserum; (2) to isolate and purify one such brain specific antigen employing sensitive and quantitative immunochemical techniques; (3) to localize this antigen within the adult chicken brain utilizing monovalent antiserum and immunohistochemical techniques; and (4) to correlate its detection within the embryo with structural events occurring during the time of appearance and accumulation.

MATERIALS AND METHODS

1. Preparation of Antigen Extracts

Heads of freshly killed adult leghorn chickens, obtained at a commercial poultry processing plant, were packed in ice and transported to the laboratory, where they were immediately frozen and stored at -25°C.

Adult brain extract (ABE) was prepared using the following protocol and all procedures were carried out at 4°C. The heads were thawed, the whole brain removed, washed in 0.85% NaCl, freed of surrounding membranes and homogenized for 60 seconds in an equal volume of 0.85% saline in a Brinkman Polytron tissue homogenizer. The homogenate was then centrifuged for 30 minutes at 2,600 x g (Sorval RC-5 superspeed refrigerated centrifuge) to remove cellular debris. The precipitate was washed twice by resuspension in equal volumes of 0.85% saline and centrifuged after each washing. The initial supernatant plus the two washings were pooled and the precipitate discarded. This pooled supernatant was further centrifuged for 1 hour at 100,000 x g (IEC/B-60 ultracentrifuge). The clear supernatant was concentrated to approximately 30.0 mg/ml total protein by positive pressure ultrafiltration (Amicon Diaflow; PM-10 filter) and stored at -25°C in small aliquots.

Extracts of chicken embryonic brain from Day 5 to 20 and whole embryos from Day 1 to 4 of incubation, and extracts of adult liver

and kidney were prepared using the above protocol. Extracts of mammalian brain from adult rabbits, mice and hamsters were similarly prepared.

2. Preparation of Antisera

I) Antibody Induction

Antiserum to ABE was obtained in New Zealand white rabbits. Eight rabbits were injected subcutaneously and intramuscularly at each of two sites (shoulders and hips) with 2.0 ml of a 1:1 solution of ABE and Freund's complete adjuvant once weekly for four weeks. The rabbits were subsequently boosted every six weeks. Blood was obtained for testing ten days after each injection via the median artery of the ear. After at least four such trials the rabbits were anaesthetized with sodium pentabarbital and exsanguinated by carotid catheter.

The blood was allowed to clot in 50.0 ml glass centrifuge tubes at 37°C for 2 hours prior to refrigeration at 4°C for 1 hour to contract the clot. This was followed by centrifugation at 2,600 x g for 15 minutes to obtain clear serum.

Anti-brain antisera (ABS) from six rabbits which exhibited identical immunoprecipitin patterns were pooled and stored in glass vials at -25°C.

II) Absorption of Antisera

Antiserum was absorbed with adult chicken serum, and liver and kidney extracts to remove antibodies to common tissue antigens.

Absorption was accomplished by mixing anti-brain serum, kidney and liver extracts and chicken serum, incubating the mixture at 37°C for 1 hour, centrifuging at 10,000 x g for 15 minutes and repeating the procedure until no further cross reaction with antigens of non-neural tissues was obtained during immunoprecipitin testing.

III) Isolation of Gamma-globulins

Crude IgG was prepared from absorbed antiserum to adult brain by precipitation with ammonium sulphate. The serum was diluted 1:1 with a 0.85% NaCl solution. Saturated ammonium sulphate was added dropwise with stirring to an equal volume of diluted serum to make the final mixture half-saturated with ammonium sulphate. The mixture was stirred mechanically for 45 minutes at room temperature, then centrifuged at 4,000 x g. The precipitate was resuspended in the original volume of saline and again precipitated with equal volume of saturated ammonium sulphate. This procedure was repeated twice. After the final centrifugation the precipitate was dissolved in a small amount of distilled water and dialyzed overnight against distilled water. The lipoprotein precipitate which formed was removed by centrifugation at 10,000 x g for 15 minutes. The clear supernatant was further dialyzed against 0.85% saline, concentrated to half the original serum volume, and stored in 0.5 ml aliquots at -25°C. This IgG enriched serum fraction was designated absorbed anti-brain serum (AAβS).

3. Immunoelectrophoresis and Immunodiffusion

Immunoelectrophoretic analyses (Grabar and Williams, 1953), were run according to the micromodification of Scheidegger (1955). The agarose gel was made by dissolving 1.5 gm of agarose (Sigma, Type I Low EEO) in 50.0 ml of electrophoresis buffer (0.1 M barbitone acetate pH 8.6 + 0.1% NaN_3) and 50.0 ml of distilled water. Hot agarose (3.0 ml) was pipetted onto each microscope slide (25.0 x 75.0 mm). The slides were allowed to cool and stored in gel buffer at 4°C until use. Electrophoresis was carried out at a constant current of 6 mA/slide for 1.5 hours. Diffusion of antiserum from the central trough was allowed to proceed for 24-36 hours at 37°C in a humidified plexiglass container. Slides were then washed in 0.85% saline for 24 hours and photographed.

Double immunodiffusion analyses were performed by the method of Ouchterlony (1953) on glass slides prepared as for microimmunoelectrophoresis and diffusion was carried out under identical conditions.

4. Protein Determination

The Bio-Rad protein assay (Bio-Rad Laboratories) was used to measure relative total protein concentrations, with lyophilized bovine gamma-globulin as a standard. This assay is modified from the method of Bradford (1976), based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 to 595 nm when binding to protein occurs.

A modified assay procedure was utilized. Several dilutions of protein standard containing 10.0 to 50.0 μg total protein in 50.0 μl of 20.0 mM potassium phosphate buffer, pH 7.6 and appropriately diluted samples were placed in clean test tubes (13.0 x 100.0 mm). With vortexing, 2.5 ml of diluted (1:4) dye reagent was added, and allowed to stand for about 15 minutes for the reagent-protein complex to develop a stable colour. The optical density was measured at 595 nm on a Gilford 620 spectrophotometer.

5. Enzymic Digestion

The proteolytic activity of trypsin and chymotrypsin was utilized to determine the nature of the antigenic determinants found to be neural specific.

To 10.0 μl of ABE (20.0 mg/ml), 10.0 μl of either trypsin (1.0 mg/ml, 10,000.0 units/ml) or chymotrypsin (1.0 mg/ml, 34.0 units/ml) in 50.0 mM Tris-HCl, pH 7.6 containing 20.0 mM $CaCl_2$ were added. The hydrolysis was allowed to proceed for 2, 4 and 6 hours at 37°C. The digestion was terminated by the addition of 20.0 μl of aprotinin (23.0 trypsin inhibitor units/ml). The samples were then analyzed by double immunodiffusion developed with AABS. A control sample consisted of 10.0 μl of ABE, 10.0 μl of buffer without enzyme, and 20.0 μl of aprotinin. Total inhibition of the proteases by aprotinin within the samples was monitored using the Bio-Rad protease detection kit (Bio-Rad Laboratories).

6. Ammonium Sulphate Fractionation

Adult brain extract (ABE) was prepared as previously described (section 1), except the final ultrafiltration of the supernatant was omitted.

The ABE solution was brought to 35% $(NH_4)_2SO_4$ saturation by slow addition of the solid (20.9 gm/100ml) with continuous stirring for 30 minutes at 22°C. The resulting suspension was centrifuged at 10,000 x g for 30 minutes at 22°C. The 35% supernatant was then adjusted to 65% $(NH_4)_2SO_4$ saturation (20.0 gm/100ml) and continuously stirred for 30 minutes. The precipitate was resuspended in a small volume of 0.85% saline, designated P1 and dialyzed against 0.85% saline at 4°C. The 65% saturated suspension was centrifuged at 10,000 x g for 30 minutes. The supernatant was adjusted to 100% $(NH_4)_2SO_4$ saturation (27.5 gm/100ml) and stirred for 30 minutes. The precipitate, designated P2, was resuspended as before and dialyzed against 0.85% saline. The 100% saturated suspension was centrifuged at 10,000 x g for 30 minutes. The supernatant was decanted, the pH adjusted from the 6.7 to 4.0 by the slow addition of 85.5% sulphuric acid and mechanically stirred for 30 minutes. The precipitate was resuspended in 0.85% saline, dialyzed against 0.85% saline and designated P3. The 100% $(NH_4)_2SO_4$ saturated suspension (pH 4.0) was centrifuged at 10,000 x g for 30 minutes and the supernatant was

discarded. The final precipitate, designated P4 was processed as stated above for the other precipitates.

All protein solutions were then dialyzed twice, each for 12 hours at 4°C against buffer (20.0 mM potassium phosphate, 2.0 mM EDTA, 0.1 mM 2-mercaptoethanol, 0.1 M KCl pH 7.6), centrifuged at 10,000 x g at 4°C and each supernatant stored at -25°C until required. The fractions were tested by double immunodiffusion and immunoelectrophoresis developed with AABS.

7. Diethylaminoethyl (DEAE)-Cellulose Chromatography

DEAE-cellulose chromatography was performed in a 2.5 x 45.0 cm column using DE-52 (Whatman preswollen) cycled through acid (0.5 N HCl) and base (0.5 N NaOH) and equilibrated in 20.0 mM potassium phosphate buffer containing 0.1 mM 2-mercaptoethanol, 2.0 mM EDTA, and 0.1 M KCl, pH 7.6.

About 6.5 gm total protein (P2) was applied to each column followed by washing with three bed volumes of starting buffer. Bound protein was eluted using step elutions of 0.2 M, 0.3 M, and 1.0 M KCl in starting buffer. Three bed volumes of each ionic strength were utilized during each step. All columns were run at 4°C, at a flow rate of 30 ml/hour, fractions (5.0 ml) were collected and absorbance at 280 nm monitored using a Gilford 620 spectrophotometer.

The fractions which corresponded to the four elution peaks were pooled, concentrated by ultrafiltration (30 mg/ml) and stored at -25°C.

Eluted proteins from each peak were subsequently analyzed by immunoelectrophoresis developed with AABS to determine the distribution of neural specific antigens.

8. Sephadex Chromatography (gel filtration)

Sephadex G-150 SF (10-40 μ), and Sephadex G-100 SF (10-40μ) (Pharmacia Fine Chemicals) were employed for purification and molecular weight determinations.

The Sephadex beads were swollen in buffer and packed according to specifications in a column 2.5 x 98.0 cm for G-150 SF and a column 1.5 x 87.0 cm for G-100 SF. The buffer used consisted of 20.0 mM potassium phosphate, 0.1 M KCl, 2.0 mM EDTA, and 0.1 mM 2-mercaptoethanol, pH 7.6. The columns were washed and equilibrated with three times their volumes with buffer solution. All columns were run at 4°C.

The protein sample to be purified (P2F2) was thoroughly dialyzed against buffer, concentrated by ultrafiltration and applied with care to the top of the G-150 SF column. The volume of sample applied varied from 2.0 to 3.0 ml. Eluted fractions of 2.0 ml were collected at a flow rate of 10.0 ml/hour. The protein profile of elution was measured by monitoring the absorbance at 280 nm of every second fraction, and neural specific antigens were assayed by double immunodiffusion in agarose gels developed with AABS.

9. Diethylaminoethyl (DEAE)-Sephadex Chromatography

DEAE-Sephadex (A-50) (Pharmacia Fine Chemicals) was swollen and equilibrated by decantation with 50.0 mM potassium phosphate buffer containing 0.1 mM 2-mercaptoethanol, and 2.0 mM EDTA, pH 7.0 (starting buffer). The slurry was packed into a column 0.5 x 5.0 cm.

The pooled fractions containing a single neural specific antigen as determined by immunoprecipitation (P2F22) were equilibrated with starting buffer and applied on the column at a flow rate of 30.0 ml/hour. The protein was eluted at the same flow rate with a parabolic salt gradient developed by a three-chambered system consisting of 100 ml of starting buffer in the first two chambers and 100 ml of 0.1 mM potassium phosphate buffer containing 1.0 M KCl, 0.1 mM 2-mercaptoethanol, 2.0 mM EDTA in the last chamber. All columns were run at 4°C. Eluted fractions of 2.0 ml were collected, the absorbance measured at 280 nm, and antigens assayed by double immunodiffusion developed with AABS. Those fractions containing the single neural specific antigen were pooled (P2F221), dialyzed against gel filtration buffer, (pH 7.6) and concentrated by ultrafiltration.

The P2F221 protein solution was carefully applied to the top of the G-100 SF column prepared as described above. The volume of the sample applied, was 1.0 ml. Eluted fractions of 1.0 ml were collected at a flow rate of 2.0 ml/hour. The protein profile of elution was measured by monitoring the absorbance at 280 nm and the neural specific antigen was assayed by immunodiffusion developed with AABS. These fractions were pooled (P2F2211) and concentrated by ultrafiltration.

10. Detection of Protease

The Bio-Rad protease detection kit (Bio-Rad Laboratories) was utilized to detect protease activity during the steps in protein purification. This assay is adapted from the method of Bjerrum, *et al.* (1975). Briefly, 2 tablets containing agarose and protease substrate (casein) were added to 10.0 ml of water, heated in boiling water bath until the agarose had completely dissolved, and poured onto a 5.0 x 7.0 cm plate. Sample wells punched into the agarose-substrate plate were filled with 15.0 μl of each protein sample to be tested. The plates were incubated at room temperature for 2 to 24 hours. Termination of protease digestion was performed by overlaying the plate with a solution of 3% acetic acid for 10 minutes, followed by rinsing in water and storing at 4°C.

11. Protease Inhibitors

A number of protease inhibitors were added to 10 μl of protease containing fraction P2F221 (27.6 μg total protein), incubated at 37°C for 2 hours, and tested using the protease detection method described above. Inhibitors used in their final concentrations were: phenylmethylsulfonylfluoride (2.0 to 6.0 mM); N-ε-p-tosyl-L-lysine chloromethyl ketone HCl (1.67 to 3.3 mM); L-1-tosylamide-2-phenylethylchloromethyl ketone (1.67 to 3.3 mM); ε-amino-n-caproic acid (16.7 to 33.3 mM); soybean trypsin inhibitor (1.3 to 3.3 mg/ml); and aprotinin (0.05 to 0.11 trypsin inhibitor units).

Sample volumes of 15.0 μl were placed in the wells, and the plates incubated at room temperature for approximately 3 hours. At this time sufficient protease activity could be detected.

12. Preparation of Monovalent Antiserum

Antiserum against fraction P2F221, containing a single neural specific antigen (CNA-1) was induced anamnestically in one rabbit. The anti-CNA-1 antiserum (ACNA-1) obtained was prepared following the protocol described in section 2. Since antibodies directed against common tissue antigens were observed during double immunodiffusion, absorption of the ACNA-1 with extracts of liver, kidney, and serum was undertaken. The absorbed IgG enriched serum fraction, designated AACNA-1 was further analyzed and found to be monovalent by double immunodiffusion and immunoelectrophoresis.

13. Quantitative Immunoelectrophoresis

The procedures used for quantitative immunoelectrophoresis were essentially those described by Weeke (1973). The methods were modified to accommodate available equipment. All electrophoretic procedures were carried out at 4°C using a 0.1 M barbitone acetate buffer, 0.1% NaN_3 pH 8.6 in the buffer vessels and half-strength buffer (0.05 M) in the agarose gels.

I) Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis (Laurell, 1965; Clarke & Freeman,

1968), is superior to the classical immunoelectrophoresis technique of Grabar and Williams (1953), by providing increased resolution due to the combination of electrophoretic separation of proteins in agarose gel followed by a perpendicular electrophoresis into an antibody containing gel.

The first dimension electrophoresis was performed on 7.5 x 7.5 cm plates covered with 10 ml of a 1.5% agarose, at 18 mA/plate (constant current) for 2 hours. Sample wells were filled with diluted protein solution containing 3.5 to 85.0 μg total protein. The second dimension electrophoresis was performed into a polyvalent antibody containing gel (reference gel), after having turned the electric field 90°. The electrophoresis was continued for 8 hours at 18 mA/plate.

The antigen-antibody complexes precipitate in arches delimiting an area proportional to the amount of antigen and inversely proportional to the concentration of antibodies. Non-precipitated proteins were removed by washing for 24 hours in 0.85% saline. After washing, the plates were overlayed with Whatman No. 1 filter paper, towels and a suitable weight (i.e. books) to squeeze the liquid phase from the gel. Final drying was completed under a stream of hot air. Dryed plates were stained for 5 minutes in a solution of 0.5% Coomassie brilliant blue R, 40% ethanol and 10% acetic acid, and destained in 30% ethanol and 12.5% acetic acid.

II) Fused Rocket Immunoelectrophoresis

Fused rocket immunoelectrophoresis (Svendsen, 1973), is a modified version of the rocket immunoelectrophoresis. Small aliquots (5-15 1) from fractions obtained in separation experiments are

transferred to a row of sample wells in an antibody-free agarose gel, where they are allowed to briefly diffuse (5-10 minutes) into the gel. The proteins are then electrophoresed in one dimension into a reference gel containing polyvalent antiserum (i.e. ABS or ACNA-1) at 18 mA/plate for 8 hours. The plates were washed, dried, stained and destained as described above.

Fused rocket immunoelectrophoresis yields an immunochemical elution profile for each individual protein or protein complex which appears as a continuous precipitin line indicating the fraction in which the protein is eluted, the relative distribution of the protein in the fraction and the distribution of contaminating proteins. The method makes it possible to pool fractions containing particular proteins for further fractionation with maximum yield and minimum contamination. More information is therefore obtained by this method than by ordinary ultraviolet absorption monitoring.

III) Immunoelectrophoresis with Intermediate Gel

Crossed immunoelectrophoresis and fused rocket immunoelectrophoresis with intermediate gels (Svendsen & Axelsen, 1972), were performed as described above. However, an intermediate gel containing monovalent antiserum (AACNA-1) was interposed between the first dimensional and the second dimensional gel (reference gel containing polyvalent ABS or ACNA-1). By this arrangement it was possible to compare: a) the electrophoretic mobility of CNA-1 with respect to other antigens in a complex mixture (i.e. ABE); b) the specificity of the absorbed antibody (AACNA-1); and c) the elution profile of the

CNA-1 antigen during various separation techniques, as well as the profile of contaminating antigens present in CNA-1 rich fractions.

IV) Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis (Laurell, 1972), represents a simple and reproducible method to determine antigen concentration within a number of samples. The diluted samples (i.e. embryonic extracts) to be compared are applied in circular wells side by side. The electrophoresis is performed in one dimension into agarose gel containing a monovalent antiserum (AACNA-1). The identification of the protein is given by the rocket shaped precipitin formed, and quantitation is based upon measuring the height of the precipitate.

Briefly 12.0 ml of 1.5% agarose containing a suitable amount of AACNA-1 was poured onto a 7.0 x 10.0 cm plate and allowed to cool. The electrophoresis, 10 mA/per plate for 16 hours (slow rocket), was started prior to the addition of samples to the wells. This was done to prevent radial diffusion. 15.0 μl of each diluted embryonic extract was placed in one of six wells punched at the cathodal end, the total protein concentration of each sample having been determined previously. Standard dilutions of purified CNA-1 containing 0.26 μg, 0.52 μg, and 0.78 μg in 15.0 μl were placed in the remaining three wells. This was to ensure the quantitative accuracy of each individual plate. After the run, the plates were removed, washed in 0.85% saline, stained, and destained as previously described.

Standard curves were produced for each plate, and the peak height used to quantitate the amount of CNA-1 present in each sample.

In this way, the percentage of CNA-1 with respect to total soluble protein concentration was determined for all embryonic extracts. The percent of CNA-1 within fractions obtained during each step of isolation and purification of CNA-1 was also calculated in this way.

14. <u>Disc Polyacrylamide Gel Electrophoresis</u>

Polyacrylamide gel electrophoresis was used in the purification of CNA-1 and as one of the criteria for the homogeneity and purity of the protein.

Stock solutions of the following reagents were prepared: solution A, 48.0 ml of 1 N HCl , 36.3 gm Tris, 0.46 ml of TEMED (N,N,N^1N^1-tetramethylethylenediamine) and water to give a final volume of 100.0 ml; and solution B, 30.0 gm of acrylamide monomer, 0.8 gm Bis (N,N^1-methylene-bis-acrylamide monomer) and water to give a final volume of 100.0 ml.

The gel solution was prepared from one part of solution A, two parts of solution B, one part water, and four parts of ammonium persulphate solution (0.14 gm/100 ml).

The glass tubes used for protein purification were 1.4 cm internal diameter and 14.0 cm in length. The tubes used for the determination of protein purity had an internal diameter of 0.5 cm and a length of 7.5 cm. Before use the tubes were soaked in cleaning solution (acid bath consisting of HCl and HNO_3), rinsed thoroughly and dried in an oven.

After preparation of the polyacrylamide gel solution a few drops of water were layered on top of the gel before it hardened. This water layer was removed prior to sample application.

The buffer used during electrophoresis consisted of 28.8 gm glycine and 6.0 gm Tris in a final volume of one litre (pH 8.8).

For protein purification, each gel received 3.2 to 4.8 mg total protein in a volume of 125 to 160 μl. In addition a drop of glycerol and 3 μl of bromophenol blue dye (0.5% in H_2O) was added to each sample. A current of 10 mA/tube was applied for about 5 hours. At that time the tracking dye had travelled 11.0 cm. All gels were run at 4°C. For criteria of purity, each gel received 20.0 to 50.0 μg total protein in 100 μl. A current of 2 mA/tube was applied.

The gels were removed by rimming the glass-gel surface with an 18 gauge needle attached to a water source. In the case of preparative electrophoresis, a gel was sectioned longitudinally and one strip stained in 0.25% Coomassie brilliant blue R, in 25.0% isopropanol, and 10.0% acetic acid overnight. Destaining was accomplished in 10.0% isopropanol and 10.0% acetic acid in a Bio-Rad diffusion destainer (Bio-Rad Laboratories). The remainder of the unstained gel and the other simultaneously run gels were sectioned into 0.5 cm discs for the entire length of the gel. The corresponding gel segments were homogenized in a polytron tissue homogenizer in three volumes of 50.0 mM potassium phosphate buffer, containing 0.1 mM 2-mercapto-ethanol, 2.0 mM EDTA and 0.15 M KCl, pH 7.0.

CNA-1 was assayed in each fraction by fused rocket immunoelectrophoresis with an intermediate gel containing AACNA-1. The fractions which demonstrated the presence of CNA-1 were further eluted several times until no further cross reaction was observed by immunodiffusion developed with AACNA-1. The diluted fractions, containing CNA-1 were dialyzed against buffer twice for 12 hours each, prior to the final DEAE-Sephadex chromatography.

15. Final DEAE-Sephadex Chromatography

DEAE-Sephadex (A-50) gel and column were prepared as described in section 9, with the addition of 0.15 M KCl to the equilibrating and starting buffers.

The CNA-1 containing fraction (P2F22E1) was applied on the column at a flow rate of 12.0 ml per hour. The proteins were eluted at the same flow rate with a linear salt gradient developed by a two-chambered system consisting of 30.0 ml of starting buffer in the first chamber and 30.0 ml of 50.0 mM potassium phosphate buffer containing 0.3 M KCl, 0.1 mM 2-mercaptoethanol, 2.0 mM EDTA, pH 7.0 in the second chamber.

Eluted fractions of 1.0 ml were collected and CNA-1 assayed by fused rocket immunoelectrophoresis with an intermediate gel containing AACNA-1. The fractions which demonstrated CNA-1, without the presence of contaminating antigens in the reference gel, were pooled, concentrated by ultrafiltration, and stored in small aliquots at -25°C.

16. Characterization of CNA-1

Preliminary characterization of the purified CNA-1 consisted of enzymic digestion studies employing trypsin and chymotrypsin with the protocol previously described (section 5) for the digestion of ABE. Also the monovalent AACNA-1 antiserum was tested against mammalian brain extracts (i.e. mouse, hamster, and rabbit) by double immunodiffusion and immunoelectrophoresis, to determine the class-specificity of the antigenic determinant of CNA-1.

17. Molecular Weight Determination

I) Molecular Exclusion Chromatography

Molecular exclusion chromatography utilizing Sephadex G-100 SF (10-40μ) and Sephacryl S-300 SF (40-50μ) (Pharmacia Fine Chemicals) was performed to calculate the molecular weight, or more specifically, the stokes radius of CNA-1 according to the method of Andrews (1965).

a) The preparation of the Sephadex G100 SF and the 1.5 x 87.0 cm column have been described earlier (section 8). The column was calibrated using the blue dextran (1.0 mg), bovine serum albumin (5.0 mg), ovalbumin (5.0 mg), chymotrypsinogen A (5.0 mg), and ribonuclease A (5.0 mg), dissolved in 1.0 ml of 20.0 mM potassium phosphate buffer, containing 0.1 ml mM 2-mercaptoethanol, 20 mM EDTA, 0.1 M KCl pH 7.6. The flow rate was 2.0 ml per hour, and 1.0 ml fractions of the eluant were collected.

The elution volume Ve for blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A was measured. The

distribution coefficient, Kav, of each was calculated using the equation:

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Vo = Void volume of blue dextran
Ve = Total volume of column
Vt = Total bed volume

and plotted as a function of log molecular weight.

A sample containing CNA-1 was applied as a second run of the column. Fractions were assayed for the presence of CNA-1 by fused rocket immunoelectrophoresis with AACNA-1 in the antibody containing agarose. The molecular weight of CNA-1 was calculated by intrapolating the Kav value of the protein on the ordinate axis against log molecular weight on the abscissa.

b) The Sephacryl S-300 SF (Pharmacia Fine Chemicals) obtained preswollen, was diluted with 2.0 mM potassium phosphate buffer containing 0.1 mM 2-mercaptoethanol, 2.0 mM EDTA, 0.1 M KCl, pH 7.6, packed into a column 2.5 x 98.0 cm and further equilibrated with three bed volumes of starting buffer. The column was calibrated using blue dextran (2.0 mg), thyroglobulin (2.0 mg), ferritin (0.5 mg), catalase (5.0 mg), aldolase (2.0 mg), bovine serum albumin (7.0 mg), ovalbumin (7.0 mg), chymotrypsinogen A (3.0 mg), and ribonuclease A (10.0 mg) in a sample volume of 1.0 ml. The flow rate was adjusted to 10.0 ml per hour, and 2.0 ml fractions were collected.

A sample of the high molecular weight fraction P2F21 which also contained the CNA-1 antigenic determinant, was applied to the

calibrated column. Fractions were assayed for the presence of CNA-1 by double immunodiffusion developed with AACNA-1 in the central well. The molecular weight was determined directly, by comparison of the elution profile of the high molecular weight CNA-1 with that of blue dextran (2,000,000 MW) and the other protein standards.

II) SDS Polyacrylamide Gel Electrophoresis

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was performed using precast Bio-Phore gels (Bio-Rad Laboratories) and the discontinuous method adapted from Yamada and Weston (1974).

a) Preparation of gels. 10.0% SDS polyacrylamide gels were purchased precast in 5.5 mm ID, 7.0 mm OD, x 125.0 mm long glass tubes containing a low ionic strength SDS-Tris-glycine buffer at an operating pH of 8.3. Gels were removed from the container and the tops rinsed with distilled water. The gels were then placed in the upper chamber of a model 150A electrophoresis cell (Bio-Rad Laboratories) and the upper and lower chambers filled with buffer (25.0 mM Tris, 190.0 mM glycine, 0.1% SDS, and 1.0 mM EDTA, pH 8.3).

b) Preparation of protein solutions. Purified CNA-1 obtained as indicated in the previous procedures, and the marker proteins, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories), were diluted to a concentration of 2.5 μg each in 100.0 μl of 50.0 mM potassium phosphate buffer, 1.0 mM 2-mercaptoethanol, 2.0 mM EDTA, 0.5 M KCl, pH 7.0. The protein samples were incubated at 100°C for 5 minutes after the addition of 10.0 μl of an SDS-mercaptoethanol solution (0.6% SDS in 50.0% 2-mercaptoethanol). The samples were

allowed to cool to room temperature prior to the addition of 3 μl of tracking dye (0.05% bromophenol blue in water) and one drop of glycerol. After mixing, the protein solutions were layered on the gels. A current of 3 mA/gel was applied with the positive electrode in the lower chamber. The electrophoretic run was carried out at room temperature and required approximately 3 hours to complete.

c) Staining and destaining. The gels were removed from the glass tubes by tube elimination. They were stained in 0.25% Coomassie brilliant blue R in 25.5% isopropanol and 10.0% acetic acid overnight. The gels were then rinsed in water and placed in destaining solution made of 10.0% isopropanol and 10.0% acetic acid in a Bio-Rad diffusion destainer, Model 172A (Bio-Rad Laboratories). Complete destaining required 24 to 48 hours.

d) Calculation of mobility and molecular weight. The mobility was expressed as a percentage relative to the mobility of bromophenol blue as calculated by:

$$\% \text{ Mobility} = \frac{\text{Distance moved by the protein} \times 100}{\text{Distance moved by the tracking dye.}}$$

The mobilities were plotted against the known log molecular weights. Interpolation of the mobility of CNA-1 on the log molecular weight axis gave the log of the molecular weight of CNA-1 or its dissociated subunits.

18. Staining for Enolase

Enolase activity in isoenzyme zones after electrophoresis in agarose was demonstrated by means of the specific staining method of Dave et al. (1966). The gels were incubated at room temperature with a solution consisting of: Tris 0.022 M, $MgSO_4$ 0.1 M, KCl 0.4 M, ADP 2.3 mM, NADH 1.4 mM, glycerate 2-P 2.8 mM, lactic dehydrogenase approximately 30 U/ml, pyruvate kinase approximately 6 U/ml, pH 7.6. Zones containing enolase activity showed up as dark areas on a fluorescent background in UV light, due to oxidation of NADH to NAD^+.

19. Immunohistochemical Localization

I) Preparation of Tissue for Immunohistochemistry

Brains from freshly killed adult chickens were quickly removed, freed of surrounding membranes, washed in PBS (10.0 mM sodium phosphate buffer, 0.85% saline, pH 8.0) at 4°C and grossly sectioned into blocks. The tissue blocks were immediately frozen using a dry ice-acetone bath. The tissue was embedded and mounted in Ames O.C.T. embedding compound. Cryostat sections (10μ) were cut in an American Optical Cryostat at -20°C and placed on untreated glass slides. The tissue sections were allowed to air dry for approximately 30 minutes and stored at -20°C.

II) Indirect Peroxidase-labelled Immunoglobulin Technique

Tissue slides taken directly from -20°C were placed in 2.0% formalin in PBS for 30 minutes, then washed in three changes of PBS for 5 minutes each. Endogenous peroxidase activity was blocked by

incubation with 3.0% hydrogen peroxide for 5 minutes followed by three changes of PBS. The slides were then immersed in 10.0% ovalbumin for 30 minutes and then washed as described above. This step was required to prevent excessive non-specific immunohistochemical staining (Zehr, 1978).

The sections were sequentially incubated at room temperature for 40 minute steps in the following reagents: a) monovalent rabbit antisera (AACNA-1); b) peroxidase-labelled swine anti-rabbit IgG (Bio-Rad Laboratories) diluted 1:5 in PBS. Each step was followed by washing in three changes of PBS for 5 minutes each.

To develop the peroxidase reaction, sections were first incubated for 30 minutes in Karnovsky's diaminobenzidene solution (0.05% 3-3' diaminobenzidene in 50 mM Tris-HCl pH 7.6 without hydrogen peroxide) (Graham and Karnovsky, 1966), and then incubated for an additional 5 minutes in complete Karnovsky's solution containing 0.005% hydrogen peroxide. Slides were washed in PBS followed by a rinse in distilled water, dehydrated in a graded ethanol series, cleared in xylene, and mounted in permount.

III) Indirect Immunofluorescence Technique

Tissue slides taken directly from -20°C were placed in 2.0% formaldehyde-PBS solution for 30 minutes, then washed in three changes of PBS for 5 minutes each. The sections were incubated for 40 minutes with monovalent antisera (AACNA-1), then 40 minutes with swine anti-rabbit IgG conjugated FITC (Bio-Rad Laboratories) diluted 1:5 in PBS. Each step was followed by three washings in PBS for 5 minutes each.

Slides were covered by a drop of glycerol-PBS (1:1) solution and overlayed with a cover slip. The fluorescence slides were viewed under a Zeiss universal microscope with an epifluorescence UV light source.

IV) Specificity Controls

Control of the specificity of the immunohistochemical staining, both immunoperoxidase and immunofluorescence was undertaken with one of the following serum controls. The primary antiserum AACNA-1 was replaced by: a) normal rabbit serum (preimmune); b) PBS only or c) AACNA-1 which had been completely absorbed with CNA-1 and showed no cross reaction during immunodiffusion.

As tissue controls, adult chicken liver and adult mouse cerebellum and pons were sectioned and treated by immunohistochemical techniques described above.

20. Histological Staining

Cryostat tissue sections (10μ) were obtained as described above for immunohistochemistry. Slides taken directly from -20°C were placed in 10% formalin in PBS for 60 minutes, then washed in two changes of PBS for 5 minutes each and one change of distilled water for 5 minutes. Tissue sections were stained for 1 hour in a freshly made solution of 0.01% cresyl violet acetate (Allied Chemical) dissolved in 0.1 M acetate buffer, pH 3.5. Sections were differentiated by rinsing quickly in 70% and 95% ethyl alcohol, then dehydrated in two changes

of 100% isopropanol (3-4 minutes each), cleared in xylene, and mounted in permount. Using this technique Nissl substance within nerve cells stained deep blue.

RESULTS

1. Production of Antiserum and Identification of Neural Specific Antigens

Anti-brain serum (ABS) pooled from six rabbits injected with adult chicken brain extract (ABE) showed extensive precipitin reactions with antigens of both neural and non-neural origin, when tested by double immunodiffusion in agarose gels (Figure 1A). The non-specific polyvalent nature of the ABS was exemplified by the indecipherable array of precipitin lines during immunoelectrophoretic analysis of the ABE (Figure 1C).

Absorption of ABS with extracts of non-neural tissue (i.e. liver, kidney and serum) was used for the preparation of a neural specific antiserum. The double immunodiffusion pattern developed with absorbed anti-brain serum (AABS) demonstrated only three distinct precipitin lines (Figure 1B). The neural specificity of the AABS was also apparent from the absence of cross reactivity with non-neural antigens.

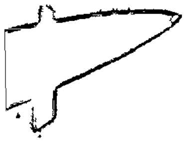
Immunoelectrophoretic analysis of ABE developed with AABS (Figure 1D) demonstrated at least eight distinct arcs with varying degrees of anodal migration. The polyvalent nature of the AABS observed during double immunodiffusion was also apparent.

Figure 1A Photograph of double immunodiffusion plate containing rabbit ABS (a) in the central well and the following tissue extracts in the peripheral wells: liver (LIV), brain (ABE), serum (SER), and kidney (KID).
Note numerous precipitin lines common to both neural and non-neural tissue extracts.

Figure 1B Photograph of double immunodiffusion plate containing rabbit AABS (aa) in the central well and the following tissue extracts in the peripheral wells: liver (LIV), brain (ABE), serum (SER), and kidney (KID).
Note reaction of antiserum with brain extract only, and no precipitin lines with extract of non-neural origin.

Figure 1C Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit ABS (a).
Note innumerable array of precipitin arcs displaying anodal migration at pH 8.6.

Figure 1D Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit AABS.
Note eight precipitin arcs demonstrating eight distinct neural specific antigens along the axis of migration.



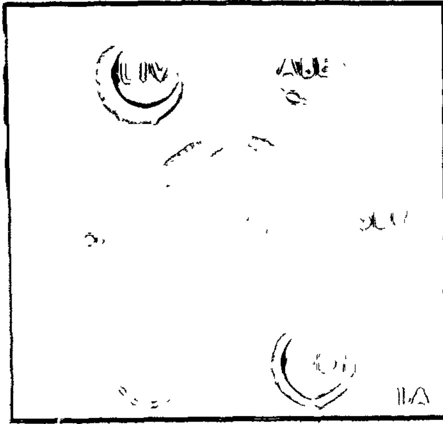
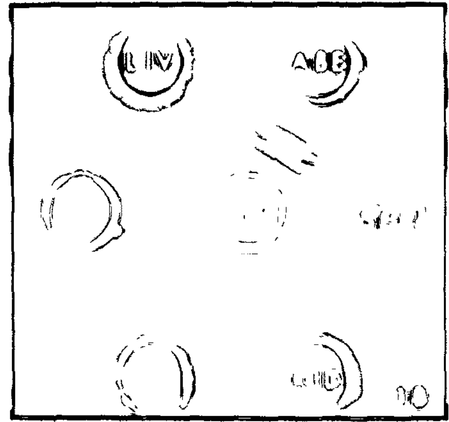
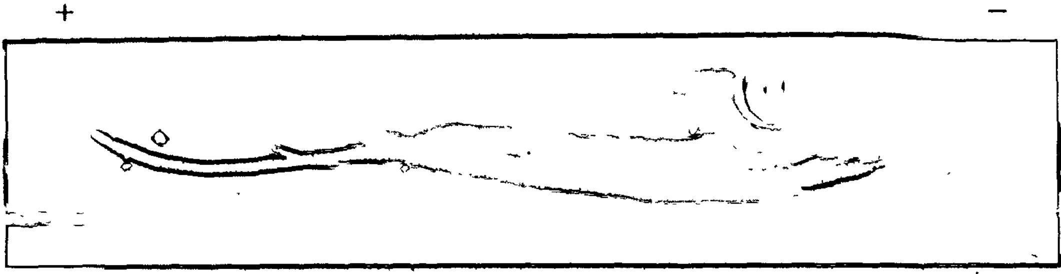
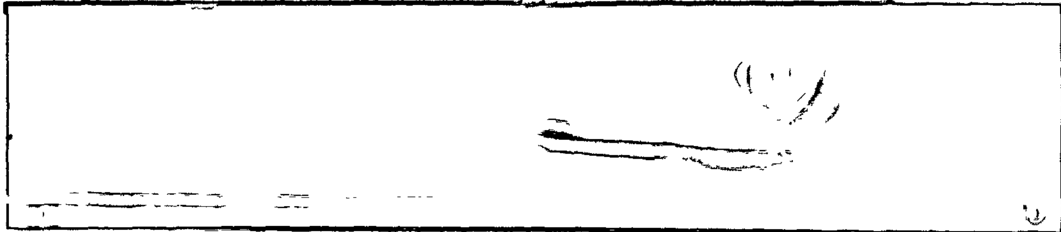





Figure 2A Photograph of immunoelectrophoretic plate with extract of whole embryos from Day 1 of incubation (D1) in the upper well, and the trough containing rabbit AABS (aa).
Note absence of precipitin lines.

Figure 2B Photograph of immunoelectrophoretic plate with extract of whole embryos from Day 2 of incubation (D2) in the upper well, and the trough containing rabbit AABS (aa).
Note appearance of a single faint precipitin arc.

Figure 2C Photograph of immunoelectrophoretic plate with extract of whole embryos from Day 3 of incubation (D3) in the upper well, and the trough containing rabbit AABS (aa).
Note the appearance of two precipitin lines.

Figure 2D Photograph of immunoelectrophoretic plate with extract of whole embryos from Day 4 of incubation (D4) in the upper well, and the trough containing rabbit AABS (aa).
Note the appearance of a third but faster anodal migrating antigen.

Figure 2E Photograph of immunoelectrophoretic plate with brain extract from embryos of 5 days incubation (D5) in the upper well, and the trough containing rabbit AABS (aa).

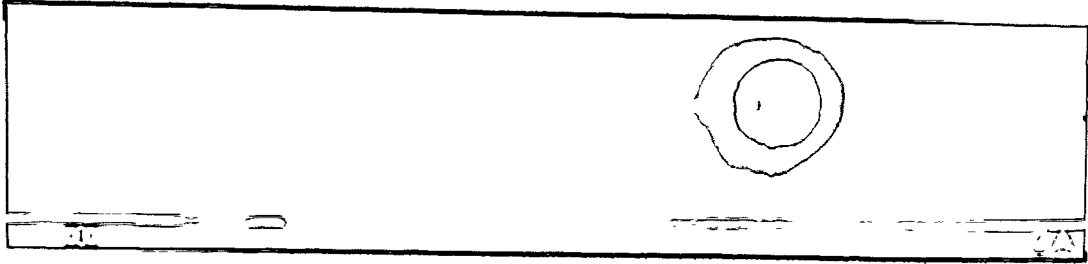
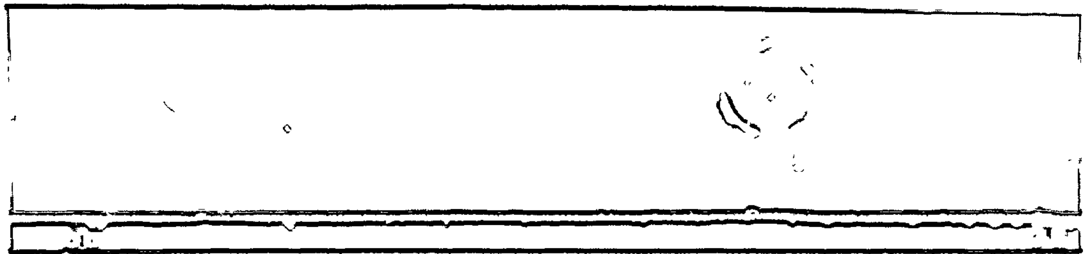
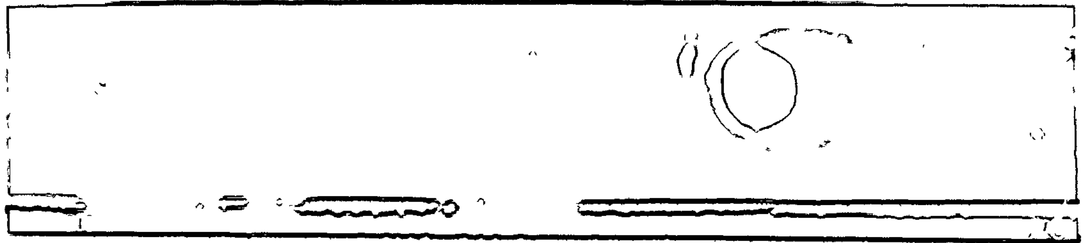
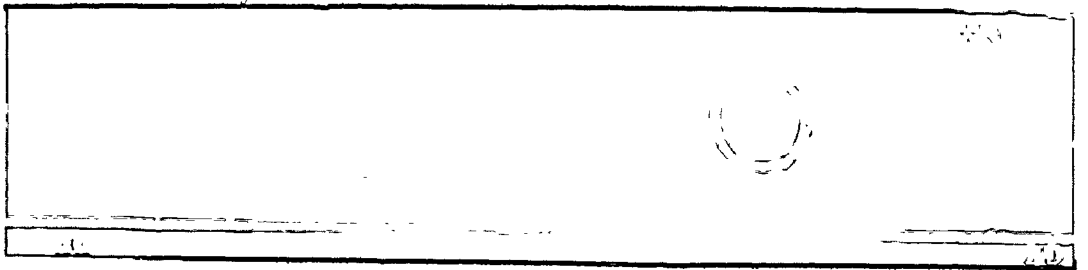
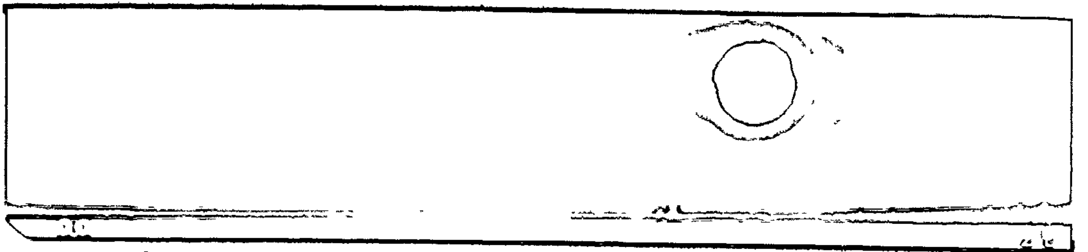






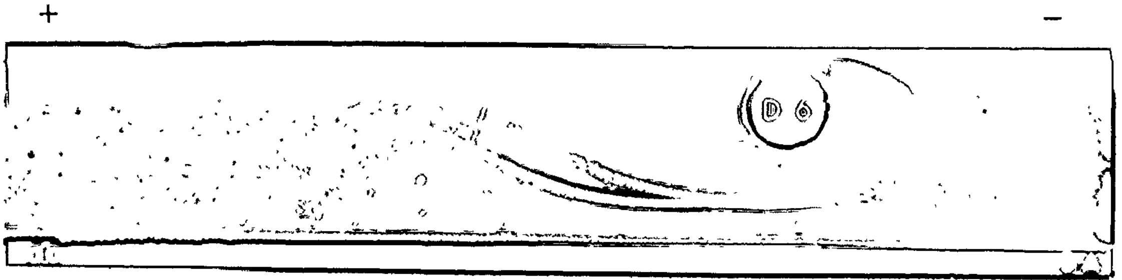
Figure 3A Photograph of immunoelectrophoretic plate with brain extract from embryos of 6 days incubation (D6) in the upper well, and the trough containing rabbit AABS (aa).

Figure 3B Photograph of immunoelectrophoretic plate with brain extract from embryos of 7 days incubation (D7) in the upper well, and the trough containing rabbit AABS (aa).
Note the appearance of at least five neural specific antigens along the axis of migration.

Figure 3C Photograph of immunoelectrophoretic plate with brain extract from embryos of 8 days incubation (D8) in the upper well, and the trough containing rabbit AABS (aa).
Note the appearance of a sixth neural specific antigen.

Figure 3D Photograph of immunoelectrophoretic plate with brain extract from embryos of 9 days incubation (D9) in the upper well, and the trough containing rabbit AABS (aa).

Figure 3E Photograph of immunoelectrophoretic plate with brain extract from embryos of 10 days incubation (D10) in the upper well, and the trough containing rabbit AABS (aa).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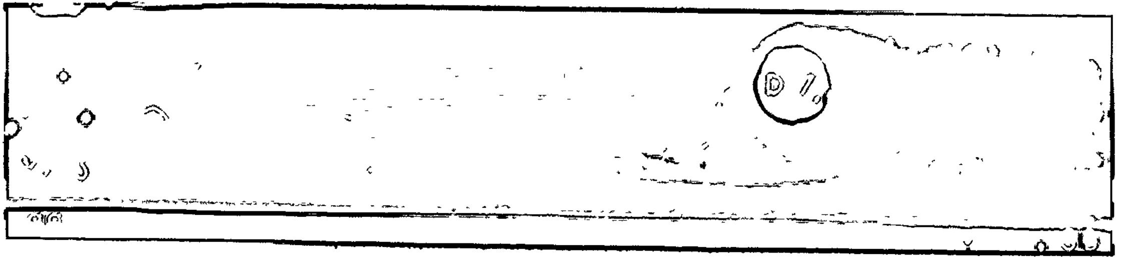
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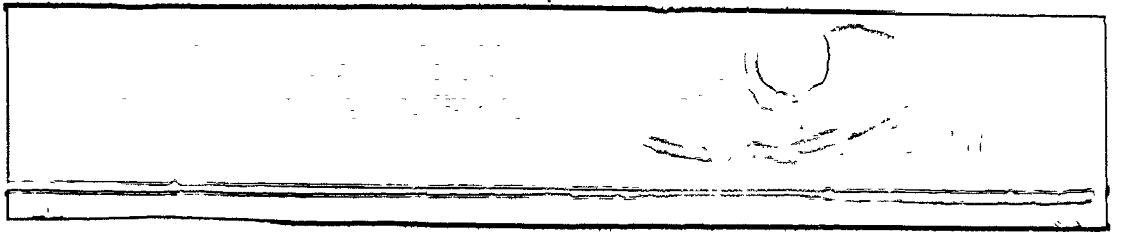
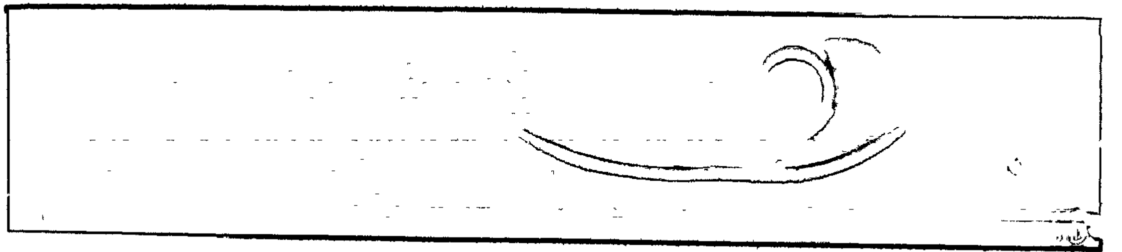
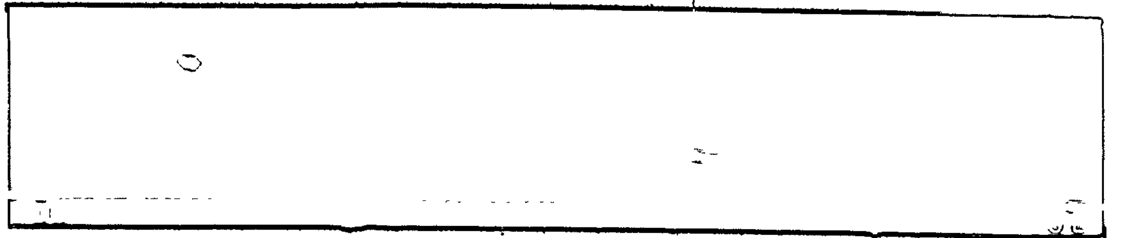






2. Ontogenic Appearance of Neural Specific Antigens

Antigens within extracts of embryonic whole brain from Days 5 to 20, and whole embyros from Days 1 to 4 of incubation were compared immunoelectrophoretically against ABE using AABS. The immunoelectrophoretic pattern obtained from embryonic extracts is shown in Figures 2 through 5.

No precipitin reaction with the Day 1 extract could be visualized (Figure 2A). However, a faint single arc was observed during double immunodiffusion of this extract. This was assumed to be evidence of an earlier appearance of the first antigen which was visualized in the immunoelectrophoretic analysis of the Day 2 embryonic extract (Figure 2B). It was quite apparent that a sequential appearance of adult brain antigen occurs within the embryo during subsequent neural development. The second antigen appeared by Day 3 (Figure 2C), the third by Day 4 (Figure 2D), the fourth and fifth by Day 7 (Figure 3B), the sixth by Day 8 (Figure 3C), and the seventh neural specific antigen by the eleventh day of incubation (Figure 4A). However, one antigen appeared to be limited to posthatching neural development since it was not observed in any of the embryonic extracts.

Figure 6A depicts, diagrammatically, the relative positions held by the adult neural specific antigens within the electromagnetic field, under constant experimental conditions (see Materials and Methods, section 3). The antigens were assigned letters A through H

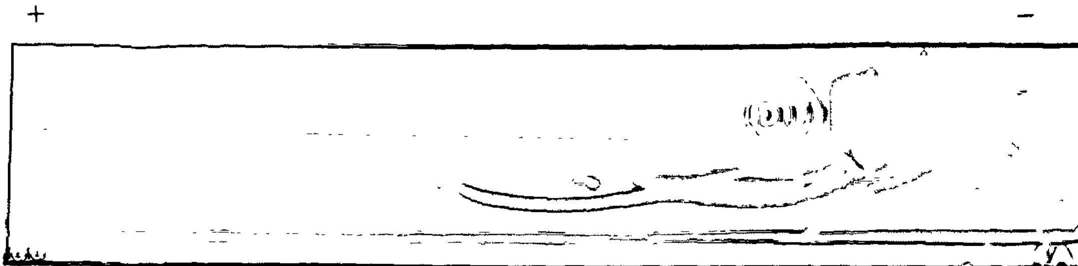
Figure 4A Photograph of immunoelectrophoretic plate with brain extract from embryos of 11 days incubation (D11) in the upper well, and the trough containing rabbit AABS (aa).
Note the appearance of a seventh neural specific antigen.

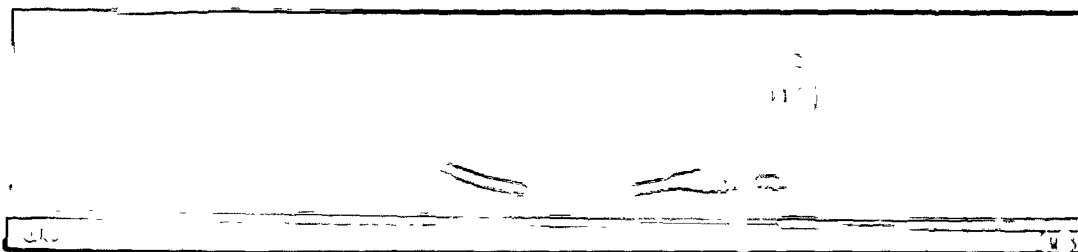
Figure 4B Photograph of immunoelectrophoretic plate with brain extract from embryos of 12 days incubation (D12) in the upper well, and the trough containing rabbit AABS (aa).

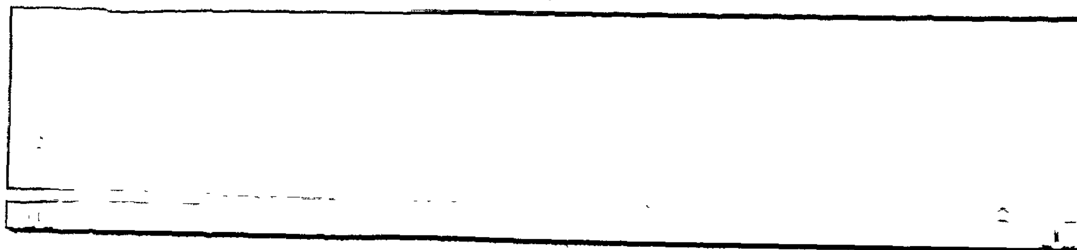
Figure 4C Photograph of immunoelectrophoretic plate with brain extract from embryos of 13 days incubation (D13) in the upper well, and the trough containing rabbit AABS (aa).

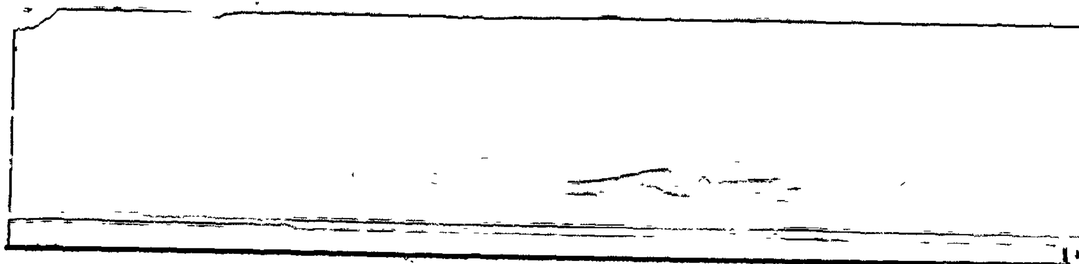
Figure 4D Photograph of immunoelectrophoretic plate with brain extract from embryos of 14 days incubation (D14) in the upper well, and the trough containing rabbit AABS (aa).

Figure 4E Photograph of immunoelectrophoretic plate with brain extract from embryos of 15 days incubation (D15) in the upper well, and the trough containing rabbit AABS (aa).









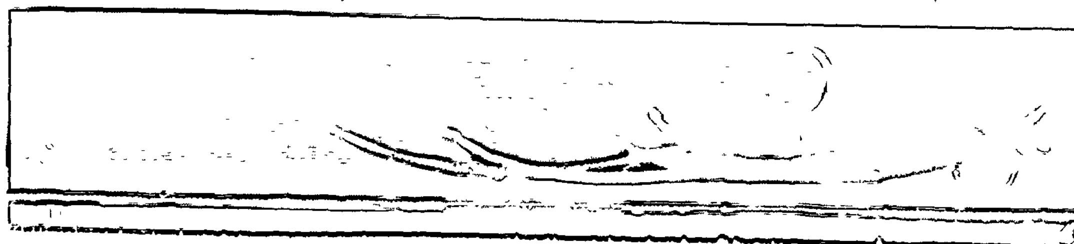


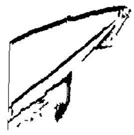
Figure 5A Photograph of immunoelectrophoretic plate with brain extract from embryos of 16 days incubation (D16) in the upper well, and the trough containing rabbit AABS (aa).

Figure 5B Photograph of immunoelectrophoretic plate with brain extract from embryos of 17 days incubation (D17) in the upper well, and the trough containing rabbit AABS (aa).

Figure 5C Photograph of immunoelectrophoretic plate with brain extract from embryos of 18 days incubation (D18) in the upper well, and the trough containing rabbit AABS (aa).

Figure 5D Photograph of immunoelectrophoretic plate with brain extract from embryos of 19 days incubation (D19) in the upper well, and the trough containing rabbit AABS (aa).

Figure 5E Photograph of immunoelectrophoretic plate with brain extract from embryos of 20 days incubation (D20) in the upper well, and the trough containing rabbit AABS (aa).



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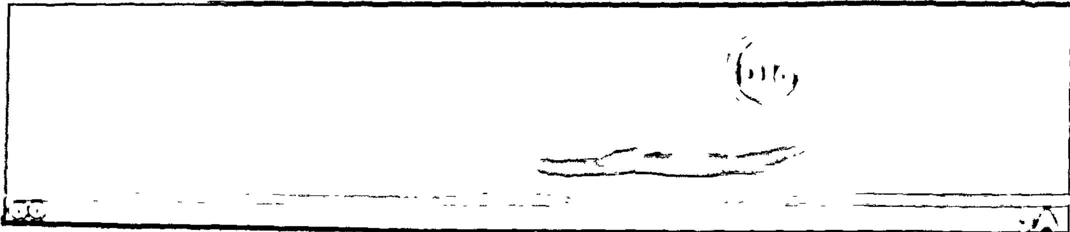
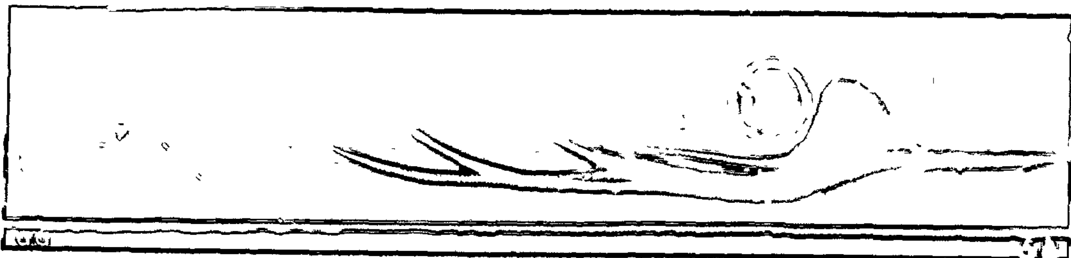
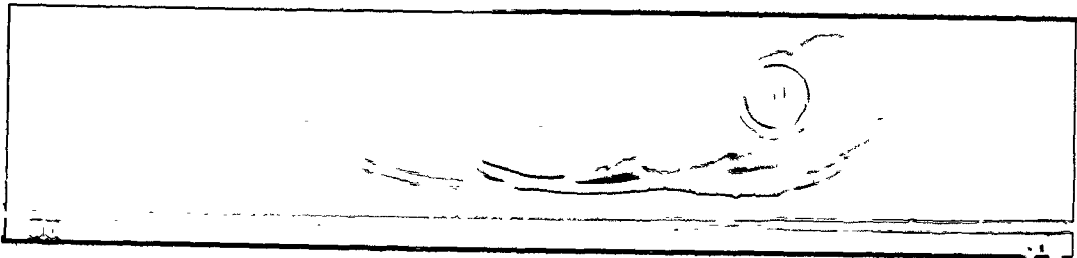
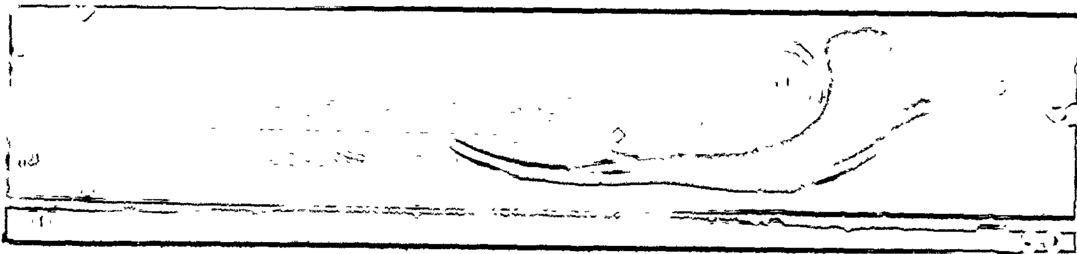
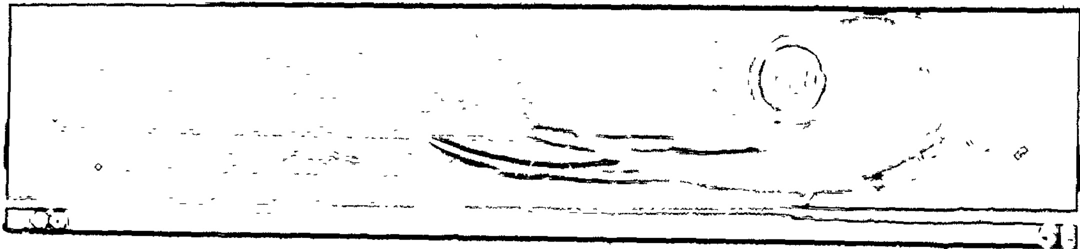
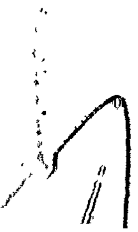
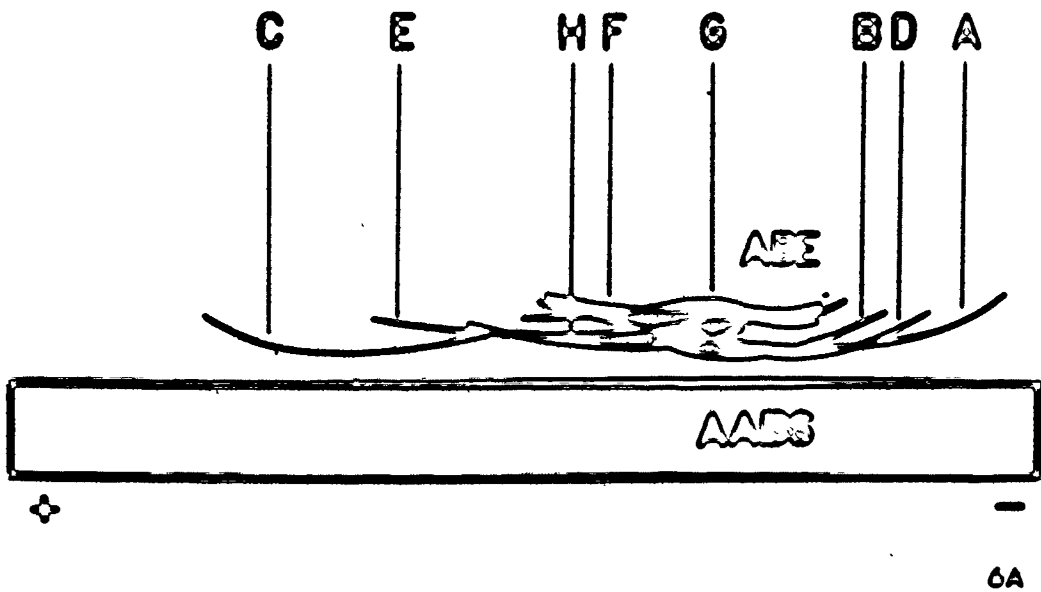
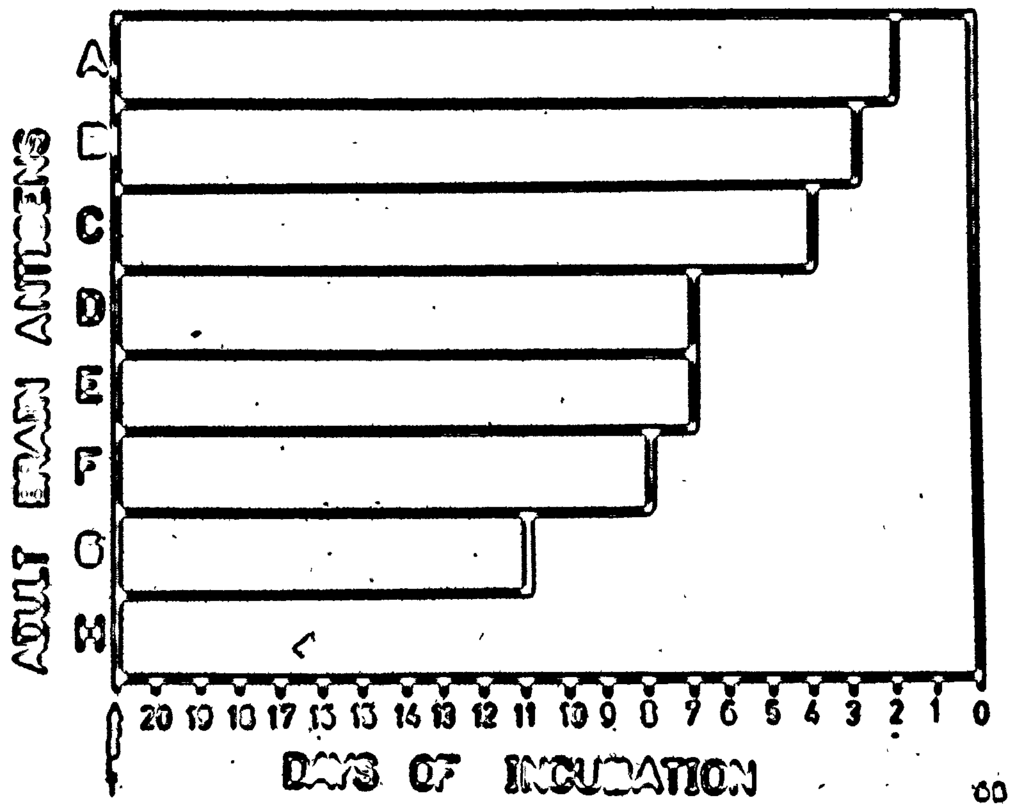







Figure 6A Schematic representation of immunoelectrophoretic plate of adult brain extract (ABE) in the upper well, and the trough containing rabbit anti-chicken brain antiserum absorbed with serum, liver, and kidney extracts (AABS).
Note the antigens have been assigned letter A through H for reference purposes, based on their order of appearance in embryonic extracts.

Figure 6B Summary of ontogenic appearance of adult neural specific antigen found in whole embryo extracts from Day 1 to Day 4, and brain extracts from embryos from Day 5 to Day 20 of incubation.



6A



6B

for reference purposes, based on their order of appearance in embryonic extracts analysed by immunoelectrophoresis. The progressive appearance of these antigens is summarized in Figure 6B.

Since immunoelectrophoresis is a qualitative method, no information could be obtained regarding the quantitative aspects (i.e. changes in concentrations during development) of these neural specific antigens.

3. Characterization of Antigens

The majority of neural specific antigens observed during double immunodiffusion studies were found to be avian-restricted. However, at least one of these antigens also appears in brain extract of adult mouse, hamster and rabbit (Figures 18A). This indicates that at least one neural specific antigen is common to both mammalian and avian brain (i.e. non-class specific). This observation was confirmed by the presence of a single arc during immunoelectrophoretic analysis of extracts of the three mammalian species (Figure 18C). Information on the species specificity of the chicken neural specific antigens was not obtained, since no other avian species were tested.

Trypsin and chymotrypinized ABE exhibited only a single precipitin line during double immunodiffusion. This indicated that the antigenic determinants of the majority, but not all of the neural specific antigens were protein in nature.

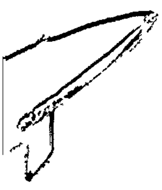
Figure 7A Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit AABS (aa).

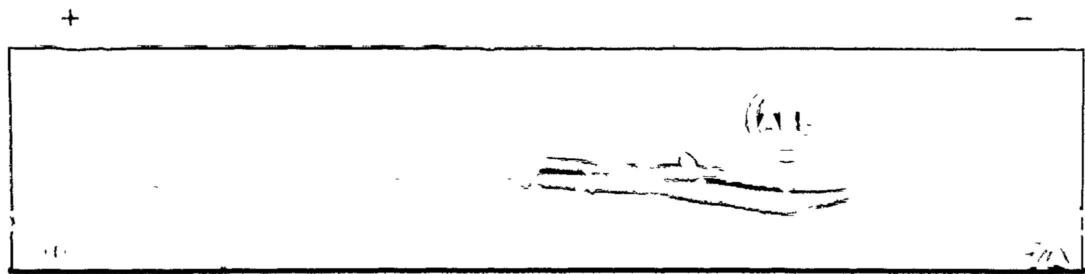
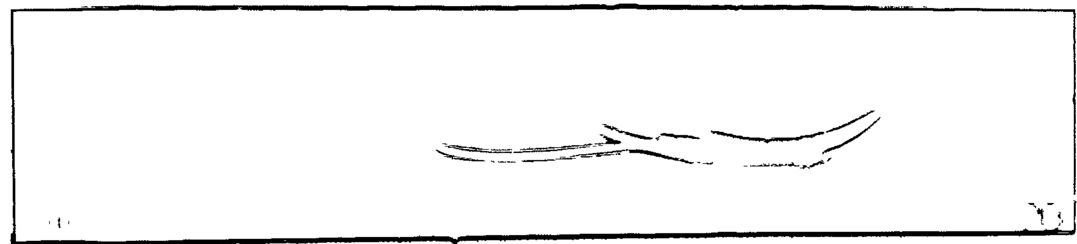
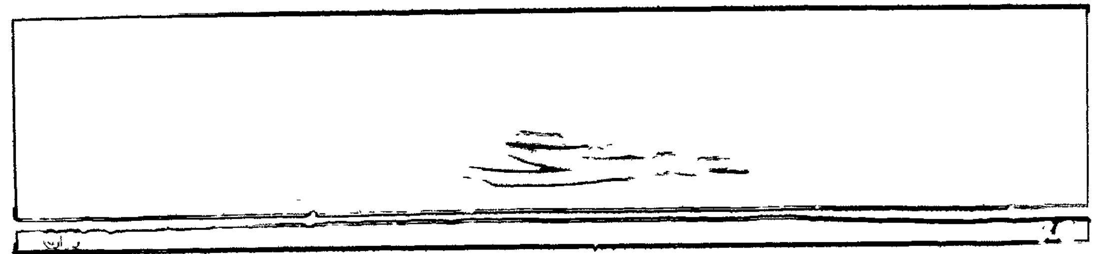
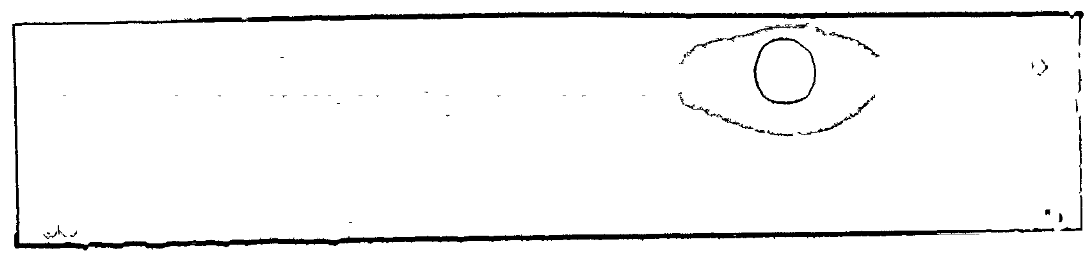
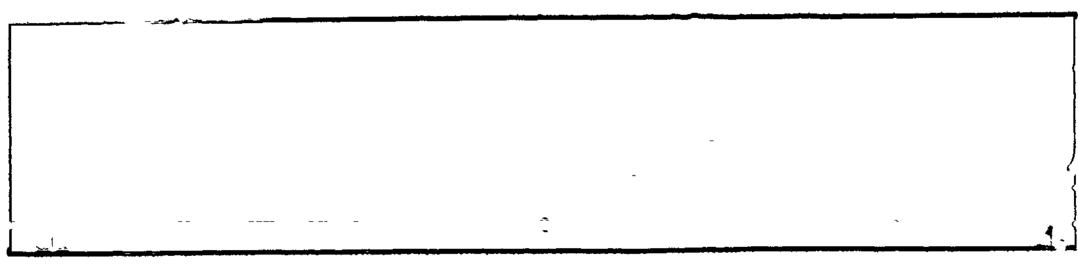
Figure 7B Photograph of immunoelectrophoretic plate with 35% saturated $(NH_4)_2SO_4$ precipitated fraction of ABE in the upper well (P1), and the trough containing rabbit AABS (aa).
Note the majority of antigens display mobilities similar to beta and gamma-globulins, while at least one antigen also possesses alpha-2 globulin mobility.

Figure 7C Photograph of immunoelectrophoretic plate with 36-65% saturated $(NH_4)_2SO_4$ precipitated fraction of ABE in the upper well (P2), and the trough containing rabbit AABS (aa).
Note alpha-2 and alpha-1 globulin mobility expressed by the majority of antigens present in the fraction.

Figure 7D Photograph of immunoelectrophoretic plate with 66-100% saturated $(NH_4)_2SO_4$ precipitated fraction of ABE in the upper well (P3), and the trough containing rabbit AABS (aa).
Note spreading and duplication of precipitin arcs as a result of antibody excess.

Figure 7E Photograph of immunoelectrophoretic plate with 100% saturated $(NH_4)_2SO_4$ precipitated fraction at pH 4.0 in the upper well (P4), and the trough containing rabbit AABS (aa).
Note absence of any detectable precipitin arcs within this fraction.



4. Isolation of One Neural Specific Antigen

I) Ammonium Sulphate Fractionation

ABE was fractionated by the additon of ammonium sulphate as follows: P1 was precipitated at 35% of saturation, P2 was precipitated at 36 to 65% of saturation, P3 was precipitated at 66 to 100% saturation, and P4 was precipitated from the 100% ammonium sulphate saturated supernatant by lowering the pH from 6.7 to 4.0 by the slow addition of 85.0% sulphuric acid. The amount of protein recovered in Fractions P1, P2, P3, and P4 as a percentage of the total soluble protein recovered after ammonium sulphate fractionation was approximately 16.0%, 64.0%, 18.0%, and 2.0% respectively. The immuno-electrophoretic analysis of each fraction developed with AABS is shown in Figure 7A-E.

The majority of antigens demonstrated in Fraction P1 (Figure 7B) showed little anodal migration during electrophoresis at pH 8.6, since most precipitin arcs resulted from radial diffusion around the origin well. The antigens within Fractions P2 (Figure 7C) and P3 (Figure 7D) exhibited greater migration toward the anode, characteristic of acidic proteins.

It was apparent that some overlap of neural specific antigens had occurred during ammonium sulphate fractionation. P1 was found to contain 3 antigens (B, H, and G), P2 was found to contain 6 antigens (A, C, D, E, F, and H), while P3 contained only 2 antigens (F and D).

Fraction P4 (Figure 7E) was found to contain no detectable neural specific antigens by immunoelectrophoresis. Precipitin line duplication in the patterns of Fractions P1 and P3 was the result of an imbalanced system containing an antibody excess.

Fraction P1 was found to exhibit extensive precipitation during prolonged storage, and was also shown to have protease activity when tested using the Bio-Rad protease detection kit. Fraction P2 did not share these characteristics and possessed high protein concentration. The spectrum of electrophoretic mobilities exhibited by the Fraction P2 antigens suggested that they might be separated by ion exchange chromatography.

II) Diethylaminoethyl (DEAE)-Cellulose Chromatography

The neural specific antigens of Fraction P2 were separated on the basis of their net electric charges by column chromatography on DEAE-cellulose, using step wise elution with four buffers of increasing ionic strength. A representative chromatogram obtained from monitoring protein at 280 nm is shown in Figure 13A.

Fraction P2F1 was composed of material which did not bind to the DEAE-cellulose matrix, and was removed from the column with buffer containing 0.10 M KCl. Fraction P2F2 was obtained after elution with 0.2 M KCl, Fraction P2F3 by elution with 0.3 M KCl and Fraction P2F4 with the final elution buffer containing 1.0 M KCl. This system was chosen arbitrarily with the purpose of obtaining a few antigens within each fraction.

The amount of protein recovered in Fractions P2F1, P2F2, P2F3, and P2F4 as a percentage of the total soluble protein recovered after DEAE-cellulose fractionation was approximately 68.0%, 25.0%, 6.0%, and 1.0% respectively. The four fractions obtained were compared by immunoelectrophoresis developed with AABS (Figure 8A-E).

The antigens present in Fraction P2F1 (Figure 8B) showed little if any anodal or cathodal migration indicating that these antigens have little net ionic charge at pH 8.6 and presumably no negative charge at pH 7.6, allowing passage through the column (Anion Exchanger) without retention. Smooth continous lines which were apparent in the Fractions P2F2 (Figure 8C) and P2F4 (Figure 8E) indicate antigens closely related immunochemically (i.e. similar antigenic determinants), but exhibiting differences in their electrophoretic mobility. However, Fraction P2F3 (Figure 8D) contained two anodal migrating antigens which were antigenically dissimilar, but possessed similar electrophoretic mobilities.

Fractions P2F2, P2F3, and P2F4 appeared to contain similar antigenic material when compared immunoelectrophoretically at pH 8.6. Possible explanations are: a) overlap of antigens as a result of step wise elution; and b) the proteins possess similar antigenic sites, but exhibit differences in net ionic charge at pH 7.6, resulting in fractionation on DEAE-cellulose.

One antigen found in Fraction P2 (Figure 8A) as the fastest anodal migrating precipitin line (i.e. Antigen C) was not observed in

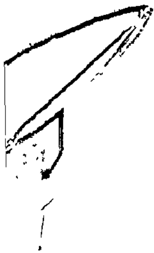
Figure 8A Photograph of immunoelectrophoretic plate with 36-65% saturated $(NH_4)_2SO_4$ precipitated fraction of ABE in the upper well (P2), and the trough containing rabbit AABS (aa).

Figure 8B Photograph of immunoelectrophoretic plate with 0.1 m KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F1), and the trough containing rabbit AABS (aa). Note antigens of this fractions display beta and gamma-globulin mobilities at pH 8.6.

Figure 8C Photograph of immunoelectrophoretic plate with 0.2 M KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F2), and the trough containing rabbit AABS (aa). Note antigens of this fraction develop smooth continuous lines exhibiting alpha-1 or alpha-2 to gamma-globulin mobilities.

Figure 8D Photograph of immunoelectrophoretic plate with 0.3 M KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F3), and the trough containing rabbit AABS (aa).

Figure 8E Photograph of immunoelectrophoretic plate with 1.0 M KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F4), and the trough containing rabbit AABS (aa). Note similarities of precipitin arc with that seen in Figure 8C of Fraction P2F2.



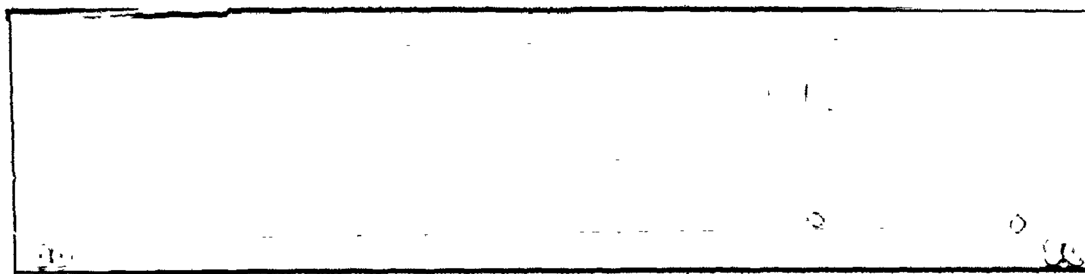
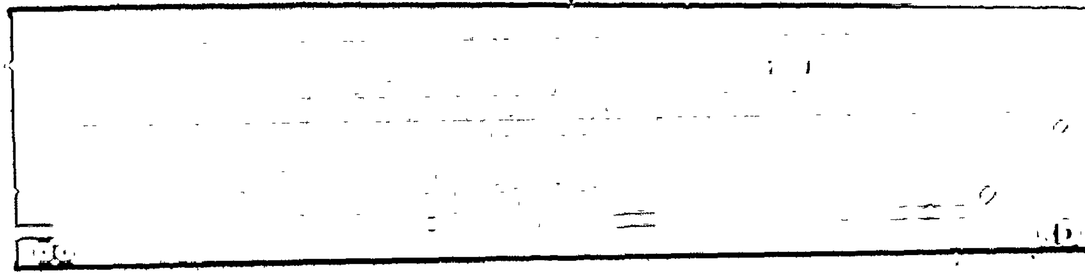
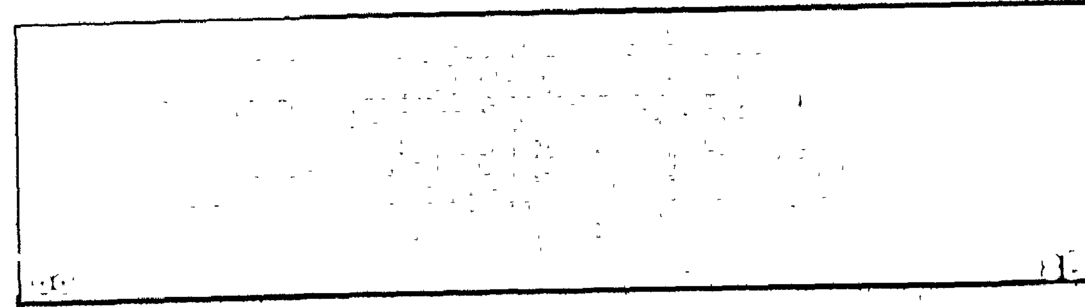




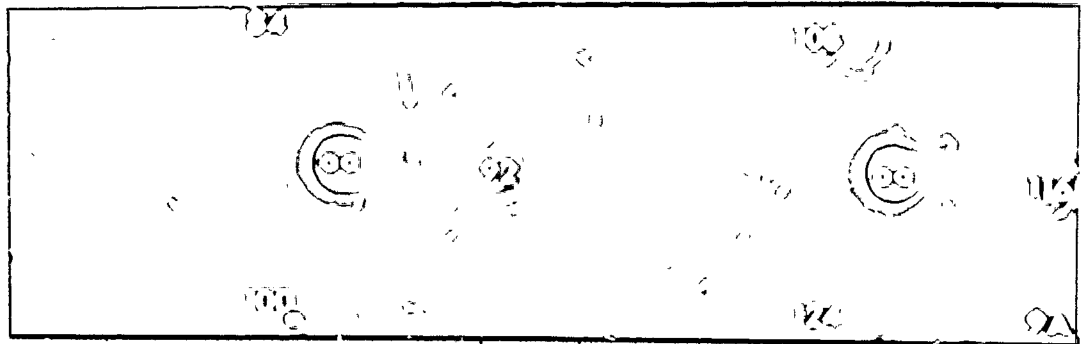
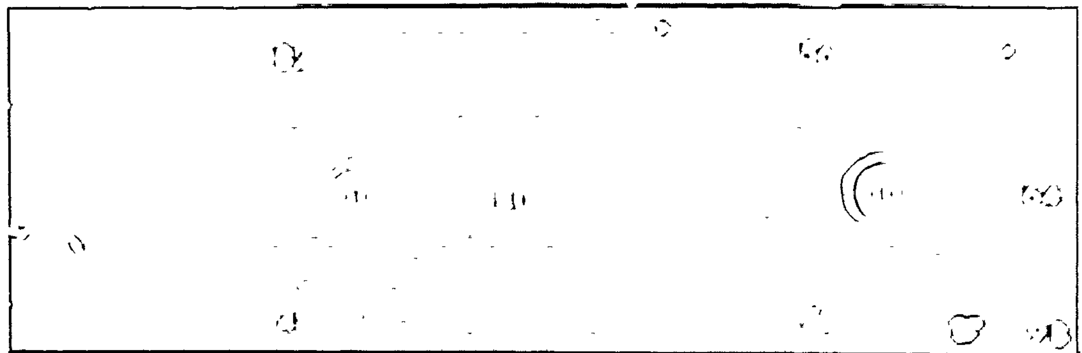
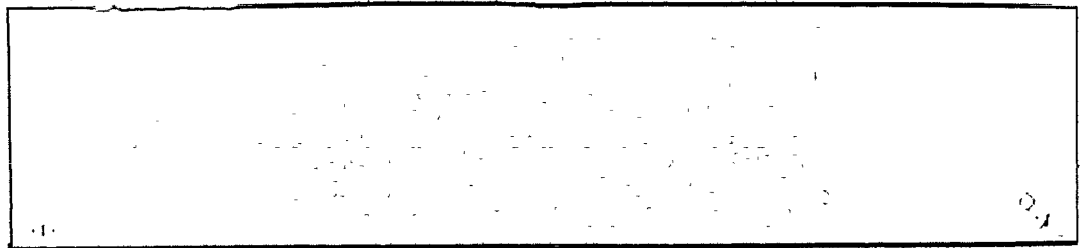
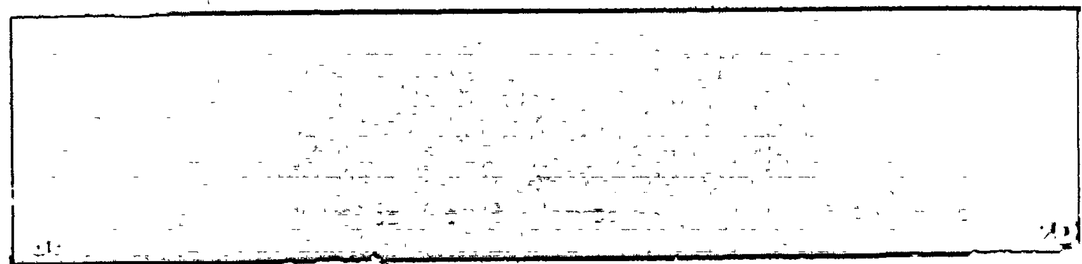
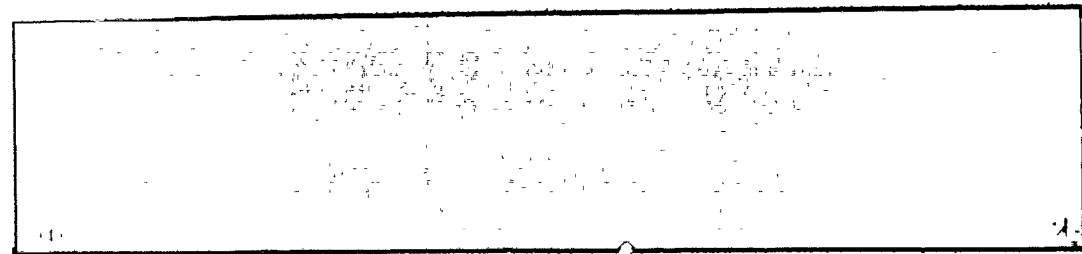
Figure 9A Photograph of double immunodiffusion plate containing rabbit AABS (aa) in the central well, and the peripheral wells containing fractions 84 to 128, obtained during G-150 SF chromatography of Fraction P2F2.
Note the high molecular weight (> 150,000 daltons) neural specific antigens are localized in fractions 92 to 96.

Figure 9B Photograph of double immunodiffusion plate containing rabbit AABS (aa) in the central well, and the peripheral wells containing fractions 132 to 176, obtained during G-150 SF chromatography of Fraction P2F2.
Note fractions 132 to 140 contain a single precipitin line corresponding to a low molecular weight antigen (≈65,000 daltons).

Figure 9C Photograph of immunoelectrophoretic plate with 0.2 M KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F2), and the trough containing rabbit AABS (aa).

Figure 9D Photograph of immunoelectrophoretic plate with high molecular weight (> 150,000 daltons) fraction from G-150 SF Sephadex chromatography of P2F2 in the upper well (P2F21), and the trough containing rabbit AABS (aa).
Note the presence of two antigens, one with alpha-1 and the other with alpha-2 globulin mobilities.

Figure 9E Photograph of immunoelectrophoretic plate with low molecular weight (75,000 - 55,000 daltons) fraction from G-150 SF Sephadex chromatography of P2F2 in the upper well (P2F22), and the trough containing rabbit AABS (aa).
Note single precipitin arc with alpha-1 globulin mobility similar to that shown in Figure 9D of Fraction P2F21.

any of the subsequent DEAE-cellulose fractions, indicating the buffer system employed failed to elute this antigen from the column.

The antigens within Fraction P2F2 were chosen for further isolation because subsequent studies revealed their characteristics to be sufficiently different from other neural specific antigens to permit separation.

III) Sephadex Chromatography (Gel Filtration)

The neural specific antigens within Fracton P2F2 were separated on the basis of their molecular size (i.e. weight) and shape by column chromatography on G-150 SF Sephadex (10-40μ). A representative chromatogram obtained from monitoring the elution fractions for protein at 280 nm is shown in Figure 14A. Double immunodiffision analyses of the elution fractions, developed with AABS, indicated two peaks containing neural specific antigens. The first, (P2F21), from fraction numbers 92 to 96 was shown to contain at least two and possibly three precipitin lines (Figure 9A). The second, (P2F22), from fraction numbers 132 to 140 (Figure 9B) developed only a single precipitin line. The amount of protein recovered in Fractions P2F21 and P2F22 as a percentage of the total soluble protein recovered in the two fractions after G-150 SF Sephadex chromatography was approximately 69.0% and 31.0% respectively.

Figure 9D demonstrates the immunoelectrophoretic pattern obtained from Fraction P2F21 developed with AABS. At least two

distinct antigens were visualized along the axis of migration. However, immunoelectrophoresis of P2F22 revealed only a single immunoprecipitin arc possessing alpha-1 globulin mobility (Figure 9E). Apparent from both the immunodiffusion and immunoelectrophoretic analysis, Fraction P2F22 contained a single neural specific antigen, corresponding to a molecular weight between 75,000 and 55,000 daltons. The name given to this isolated neural specific (organ specific) antigen was chicken neural antigen-1 (CNA-1).

IV) CNA-1 Enrichment

CNA-1, once isolated from other neural specific antigens was further enriched on DEAE-Sephadex A-50 and Sephadex G-100 SF. The resulting chromatograms obtained from monitoring the elution fraction for protein at 280 nm are shown in Figure 10A and 10B respectively. During both chromatographic procedures, fractions were also assayed by double immunodiffusion developed with AABS. Only a single precipitin line in fractions containing CNA-1 was observed.

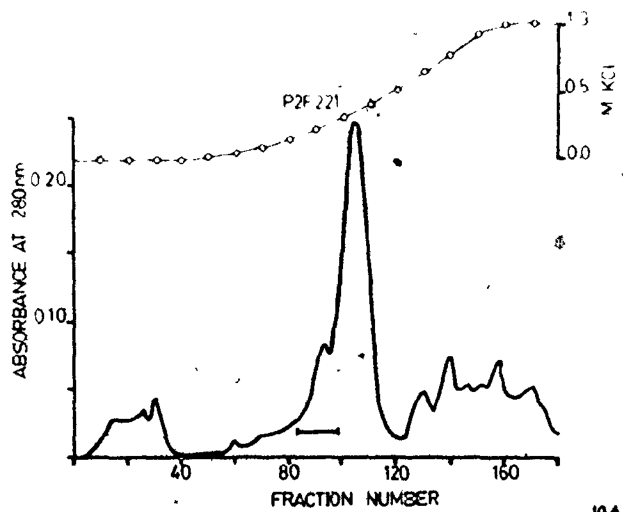
Fraction P2F221 pooled and concentrated after DEAE-Sephadex chromatography accounted for approximately 18.0% of the protein applied, while Fraction P2F2211 recovered from the Sephadex G-100 SF column accounted for approximately 60.0% of the total soluble protein applied to that column.

Pooled fractions from each isolation procedure were also tested by immunoelectrophoretic analysis developed with rabbit anti-chicken

Figure 10A DEAE-Sephadex A-50 chromatogram of Fraction P2F22. Details of experimental conditions were described under Materials and Methods, section 9. Bar represents pooled Fraction P2F221 which contained a single neural specific antigen (CNA-1) assayed by double immunodiffusion developed with rabbit AABS.

Figure 10B Sephadex G-100 SF (10-40μ) chromatogram of Fraction P2F221. Details of experimental conditions were described under Materials and Methods, section 9. Bar represents pooled Fraction P2F2211 which contained a single neural specific antigen (CNA-1) assayed by double immunodiffusion developed with rabbit AABS.

Figure 10C Photograph of Bio-Rad protease detection plate containing 15 μl of Fractions ABE, P2, P2F2, P2F22, P2F221, and P2F2211 obtained during the isolation of the neural specific antigen (CNA-1). Note the clear halo (arrow) surrounding the well containing Fraction P2F221, demonstrating protease activity.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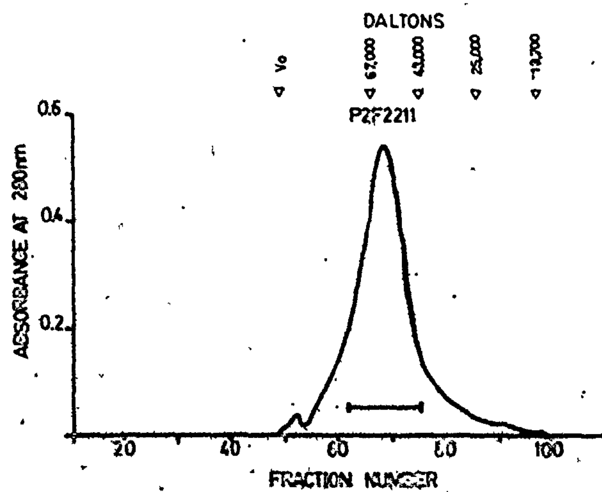
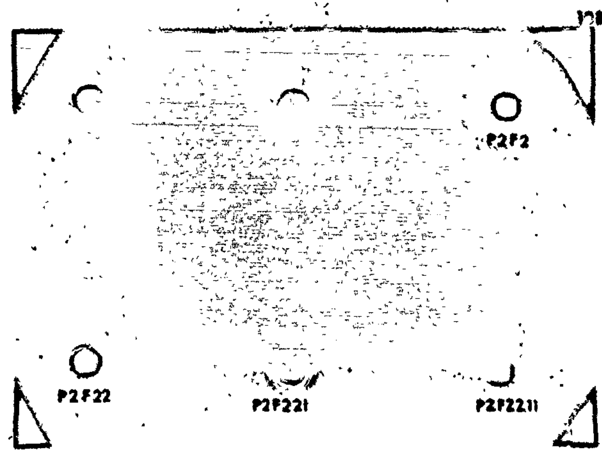
0.10
40
80
120
160
FRACTION NUMBER
1.0
0.5
0.0
M KCl
10A


DALTONS
Vo
67,000
43,000
25,000
13,700
P2F2211
ABSORBANCE AT 280nm
0.6
0.4
0.2
20
40
60
80
100
FRACTION NUMBER


P2F2
P2F22
P2F221
P2F2211

brain antiserum to detect contaminating antigens within each fraction (Figure 11A-F). In this way successive elimination of common tissue antigens could be visualized. The ABE fraction (Figure 11A), the P2 fraction (Figure 11B), and the P2F2 fraction (Figure 11C) as expected contained a large number of both neural and non-neural antigens. However, Fraction P2F22 (Figure 11D) and Fraction P2F221 (Figure 11E) were observed to have little contamination from common tissue antigens. The isolated neural specific antigen CNA-1, appeared to be the only source of antibody-antigen precipitation within the immunoelectrophoresis of Fraction P2F2211 (Figure 11F). No contaminating antigenic material was detected with this antiserum. Fraction P2F2211 was the source of CNA-1 injected into a rabbit for the induction of anti-CNA-1 antiserum (ACNA-1).

V) Protease Activity

Fractions ABE, P2, P2F2, P2F22, P2F221, and P2F2211 obtained during the isolation of CNA-1 were also tested for the presence of protease activity on an agarose-substrate (casein) plate. Figure 10C depicts 15 µl of each sample after incubation at room temperature for 3 hours. It was observed that high protease activity was detected in Fraction P2F221 by the clear halo surrounding its well. Since further purification of CNA-1 and subsequent characterization of the antigen would be hampered by such activity, a number of protease inhibitors were tested with Fraction P2F221. The specific inhibitors,

Figure 11A Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit ABS (a).

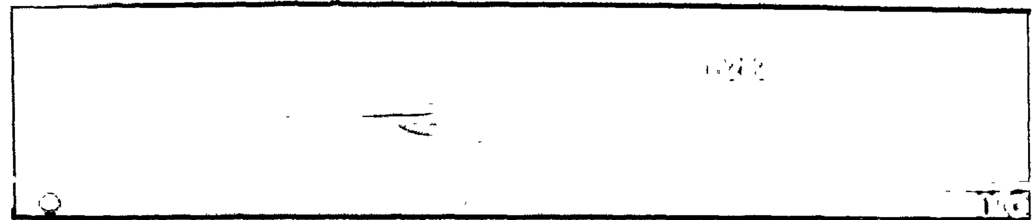
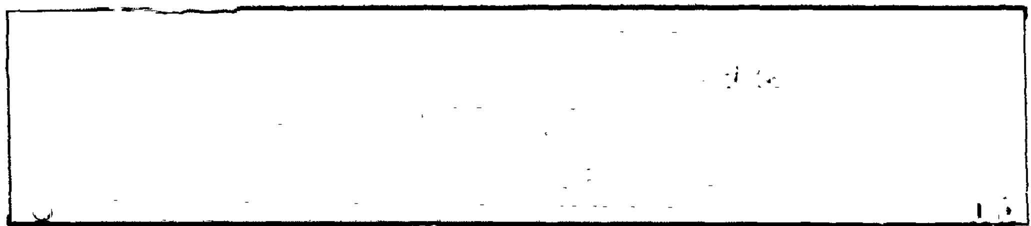
Figure 11B Photograph of immunoelectrophoretic plate with 36-65% saturated ammonium sulphate precipitated fraction of ABE in the upper well (P2), and the trough containing rabbit ABS (a).

Figure 11C Photograph of immunoelectrophoretic plate with 0.2 M KCl eluted fraction from DEAE-cellulose chromatography of P2 in the upper well (P2F2), and the trough containing rabbit ABS (a).

Figure 11D Photograph of immunoelectrophoretic plate with low molecular weight fraction (i.e. 75,000 - 55,000 daltons) from G-150 SF chromatography of P2F2 in the upper well (P2F22), and the trough containing rabbit ABS (a).

Figure 11E Photograph of immunoelectrophoretic plate with 0.2 M KCl eluted fraction from DEAE-cellulose Sephadex chromatography of P2F22 in the upper well (P2F221), and the trough containing rabbit ABS (a).

Figure 11F Photograph of immunoelectrophoretic plate with the peak fractions from G-150 SF chromatograph of P2F221 in the upper well (P2F2211), and the trough containing rabbit ABS (a).

N-α-p-tosyl-L-lysine chloromethyl ketone HCl which irreversibly inactivates papain and trypsin, and L-1-tosylamide-2-phenylethylchloromethyl ketone which irreversibly inactivates chymotrypsin, were found to be ineffective, as were the less specific inhibitors, ε-amino-n-caproic acid, soybean trypsin inhibitor, aprotinin, and phenylmethylsulfonylfluoride at all concentrations tested. Therefore the protease which was present in Fraction P2F221 apparently was a non-trypsin or analogous protease (non-serine type) which could not be inactivated by commercially available inhibitors. For this reason, changes were made in the subsequent purification protocol for CNA-1.

5. Production of Monovalent Antiserum

Anti-CNA-1 serum (ACNA-1) from a rabbit injected with the CNA-1 enriched Fraction P2F2211 was found to exhibit precipitin reactions with antigens of both neural and non-neural origin, when tested by double immunodiffusion in agarose gels (Figure 12A), and to form a number of precipitin arcs during the immunoelectrophoresis of ABE (Figure 12C).

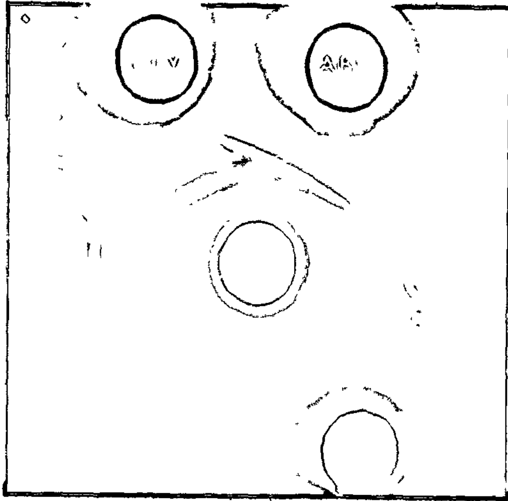
Absorption of ACNA-1 with serum, liver, and kidney extracts revealed both a neural specific and a monovalent antiserum (AACNA-1) when tested by double immunodiffusion (Figure 12B), and by immunoelectrophoresis of ABE (Figure 12D).

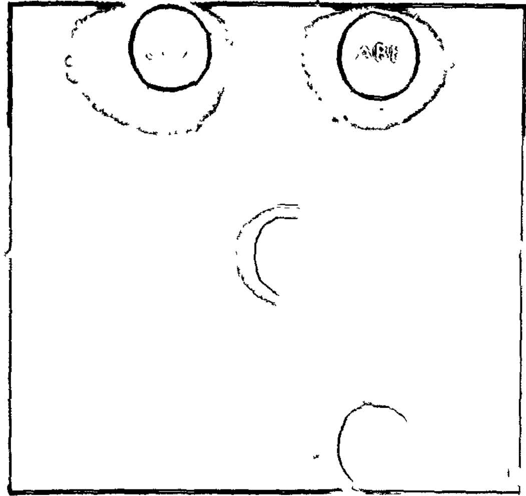
Figure 12A Photograph of double immunodiffusion plate containing rabbit ACNA-1 (b) in the central well and the following tissue extracts in the peripheral wells: liver (LIV), brain (ABE), serum (SER), and kidney (KID).
Note numerous precipitin lines common to both neural and non-neural tissue extracts.

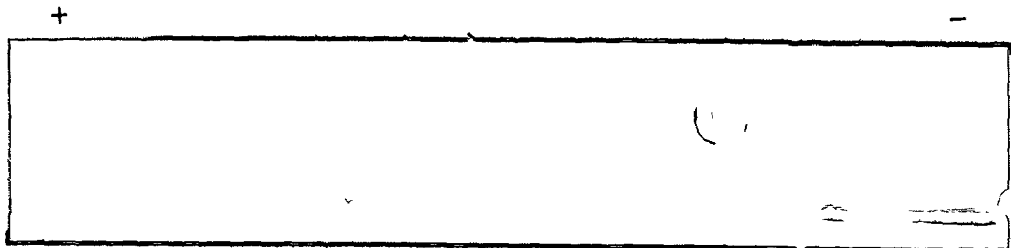
Figure 12B Photograph of double immunodiffusion plate containing rabbit AACNA-1 (ab) in the central well and the following tissue extracts in the peripheral wells: liver (LIV), brain (ABE), serum (SER), and kidney (KID).
Note single precipitin line with brain extracts only, and no reaction with extracts of non-neural origin.

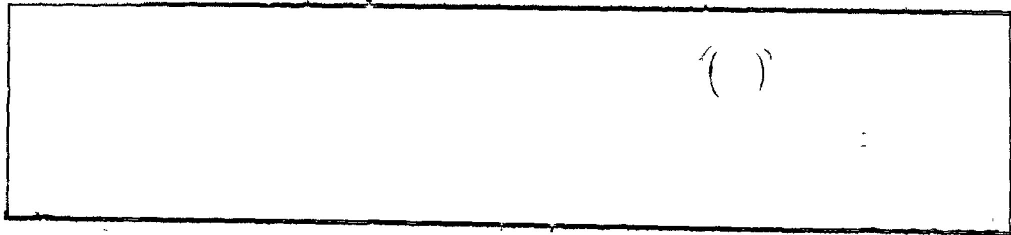
Figure 12C Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit ACNA-1 (b).
Note numerous precipitin arcs displaying alpha-1 globulin mobility.

Figure 12D Photograph of immunoelectrophoretic plate with adult brain extract (ABE) in the upper well, and the trough containing rabbit AACNA-1 (ab).
Note single continuous precipitin line along the axis of migration.









6. Purification of Chicken Neural Antigen-1 (CNA-1)

The purification of CNA-1 is summarized in Table 1. The results were based on 200 adult chicken brains (624.8 gm wet weight) producing 19.6 gm of saline soluble protein (ABE). The concentration of CNA-1 was observed to increase from 1.3 to 100% of the total soluble protein as measured by quantitative rocket immunoelectrophoresis. A 76.9 - fold purification was achieved with a 0.9% final yield.

Changes were made in the purification protocol for CNA-1 which were different from that used during its isolation and enrichment. This was due in part, to the co-purification of a protease within Fraction P2F221, and the apparent contamination of Fraction P2F2211 with antigenic material resulting in the induction of antibodies directed against non-neural tissue antigens. This also indicated that the immunoelectrophoretic analyses of fractions obtained during isolation and enrichment of CNA-1 were not sensitive enough to be used to monitor antigenic contaminants during subsequent purification. For this reason the highly sensitive and quantitative methods of crossed immunoelectrophoresis and fused rocket immunoelectrophoresis were employed.

A protein elution chromatogram of the DEAE-cellulose chromatography of Fraction P2 (36-65% ammonium sulphate fraction) is shown in Figure 13A. The resulting fused rocket immunoelectrophoresis

TABLE I

PURIFICATION OF CHICKEN NEURAL ANTIGEN-1 (CNA-1)[a]

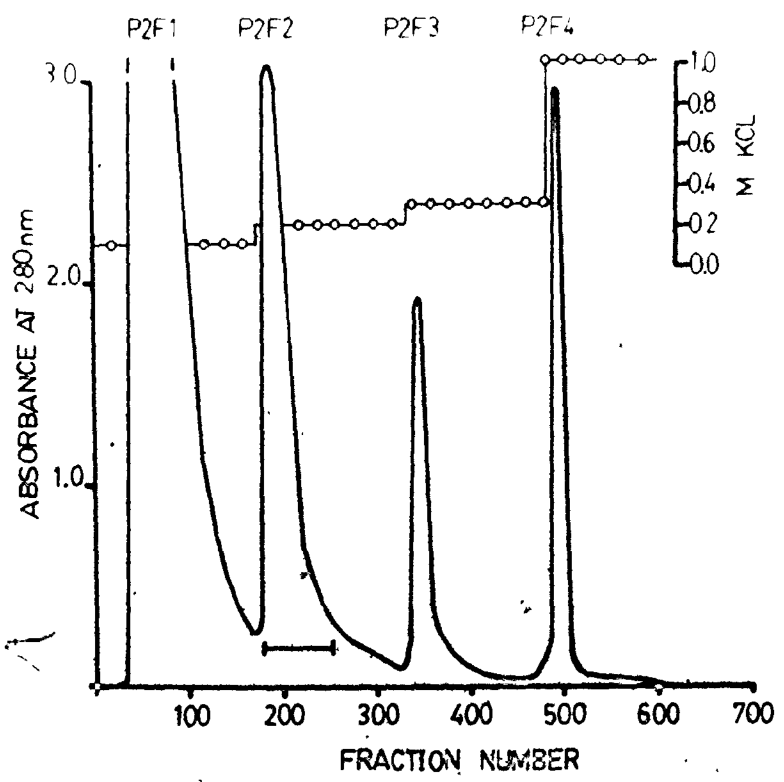
Fraction	Total Soluble Protein mg.	% CNA-1 of Total Soluble Protein[b]	Total CNA-1 mg.	Purification Fold	Yield %
Adult Brain Extract (ABE)	19600.0	1.3	254.8	-	100.0
36-65% Ammonium Sulphate Fraction (P2)	6300.0	3.7	233.1	2.8	91.5
DEAE-Cellulose 0.2 M KCl Fraction (P2F2)	1360.0	4.1	55.8	3.2	21.9
Sephadex G-150 SF Low MW Fraction (P2F22)	230.0	15.6	35.9	12.0	14.1
Preparative Polyacrylamide Electrophoresis (P2F22E1)	41.0	62.8	25.6	48.3	10.0
DEAE-Sephadex A-50 Fraction (CNA-1)	2.4	100.0	2.4	76.9	0.9

(a) Results are based on 200 adult chicken brains (624.8 gm. wet weight). Details of experimental procedures for each purification step are described in Material and Methods.

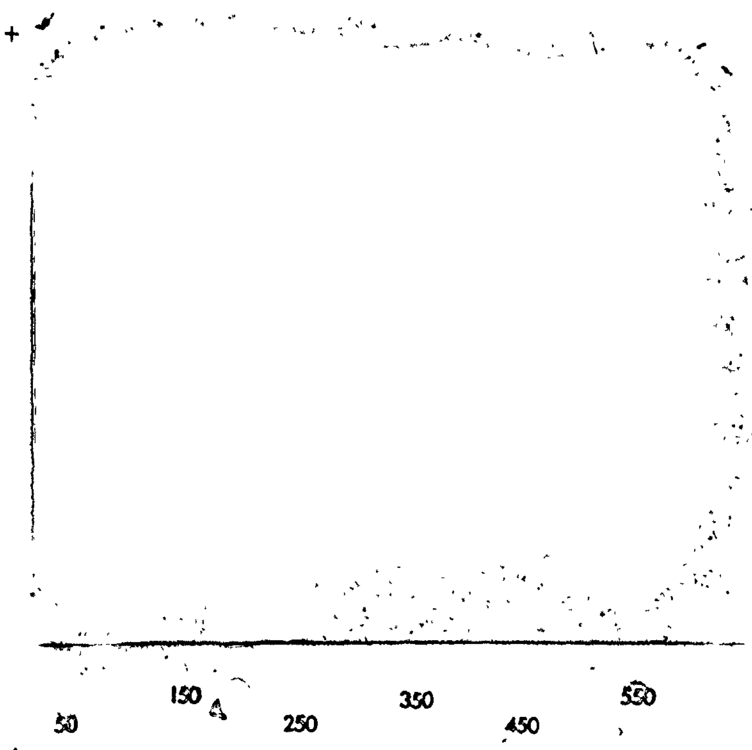
(b) % CNA-1 of total soluble protein as determined by quantitative rocket immunoelectrophoresis, with DEAE-sephadex purified CNA-1 used as the standard.

Figure 13A DEAE-cellulose chromatogram of the 36-65% saturated ammonium sulphate precipitated Fraction P2. Details of experimental conditions were described under Materials and Methods, section 7. Fraction P2F1 was removed from the column with starting buffer containing 0.1 M KCl. Fraction P2F2 was obtained after elution with 0.2 M KCl, Fraction P2F3 by elution with 0.3 M KCl, and Fraction P2F4 with the final elution buffer containing 1.0 M KCl. The bar represents pooled Fraction P2F2 chosen for further separation experiments.

Figure 13B Photograph of fused rocket immunoelectrophoretic analysis of DEAE-cellulose chromatography of 36-65% saturated ammonium sulphate precipitated Fraction P2. Details of experimental conditions were described under Materials and Methods, section 13-II. Eluted fractions were electrophoresed into the antibody-agarose gel containing rabbit ACNA-1 (b).
Note strong, multiple rocket precipitins corresponding to the 0.2 M KCl eluted Fraction P2F2.



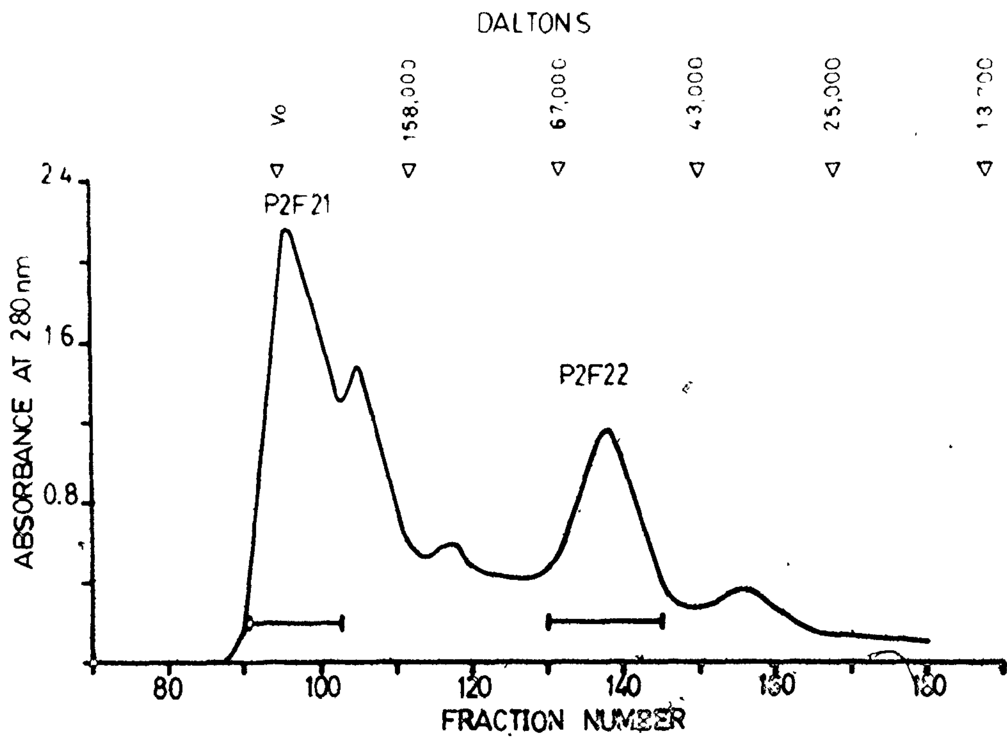
13A



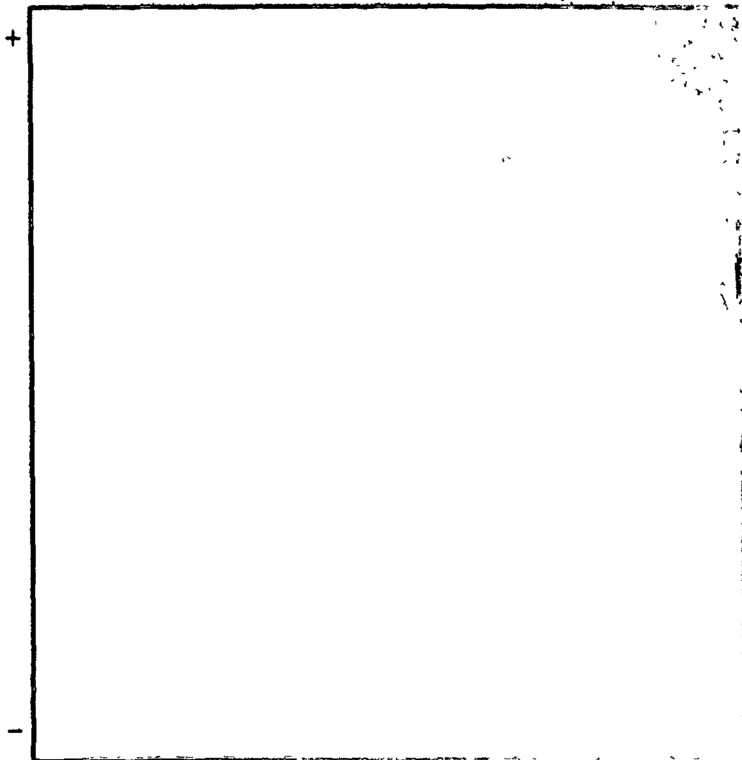
13B

Figure 14A Sephadex G-150 SF (10-40μ) chromatogram of the 0.2 M KCl eluted Fraction (P2F2), obtained from DEAE-cellulose chromatography. Details of experimental conditions were described under Materials and Methods, section 8. Fraction P2F21 corresponds to a peak of high molecular weight proteins excluded from the column at its void volume (i.e. > 150,000 MW), while P2F22 represents a second peak of lower molecular weight proteins. The bars represent pooled eluted fractions shown to contain the CNA-1 determinant.

Figure 14B. Photograph of fused rocket immunoelectrophoresis with intermediate gel, obtained from Sephadex G-150 SF chromatography of Fraction P2F2. Details of experimental conditions were described under Materials and Methods, section 13-III. The intermediate gel contained rabbit AACNA-1 (ab), while the reference gel (upper gel) contained rabbit ABS (a).
Note two precipitin peaks within the monovalent intermediate gel corresponding to CNA-1 containing fractions of high and low molecular weights. Also, there is extensive contamination within both peaks by common tissue antigens as revealed in the reference gel.



14A



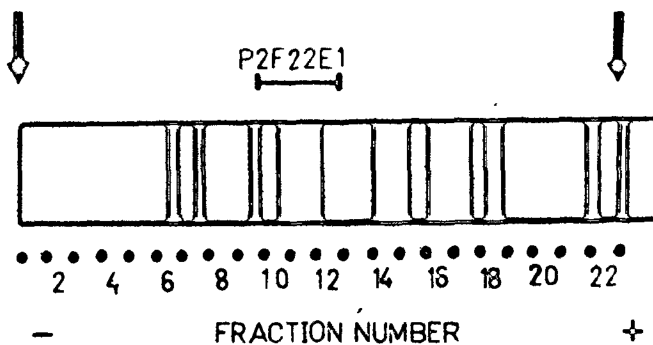
of the eluted fractions is demonstrated in Figure 13B. The reference gel contained polyvalent ACNA-1. The precipitin pattern revealed many antigens within the P2F2 fraction, while only weak cross-reacting antigens within Fraction P2F1 and P2F3 were displayed by this antiserum.

The chromatogram obtained from monitoring the elution fractions for protein at 280 nm after Sephadex G-150 SF chromatography of Fraction P2F2 is depicted in Figure 14A, and the resulting fused rocket immunoelectrophoretic analysis of eluted fractions is shown in Figure 14B. The complexity of antigenic material giving rise to the ultraviolet absorption chromatogram was revealed in the reference gel. In this case, polyvalent antiserum (ABS) was used in the reference gel and monovalent antiserum (AACNA-1) was incorporated within the intermediate gel. Two major precipitant peaks were observed within the intermediate gel, indicating Fraction P2F2 contains both a high molecular weight (> 150,000 MW) and a low weight (≈ 65,000 MW) CNA-1 component. Numerous common antigenic contaminants were also observed within the reference gel corresponding to CNA-1 rich fractions. Further purification by preparative electrophoresis in 7.5% polyacrylamide gel was undertaken on the low molecular weight CNA-1 component only (Fraction P2F22).

A longitudinal section of a stained preparative gel demonstrating numerous protein bands is diagrammatically represented in Figure 15A. Unstained gels, cut in 0.5 cm discs, homogenized and

Figure 15A Diagrammatic representation of preparative polyacrylamide electrophoresis of the low molecular weight Fraction P2F22, obtained from Sephadex G-150 SF chromatography. Details of experimental conditions were described under Materials and Methods, section 14. Bar represents pooled fractions observed to contain CNA-1.

Figure 15B Photograph of fused rocket immunoelectrophoresis with intermediate gel, obtained from preparative polyacrylamide electrophoresis of Fraction P2F22. Details of experimental conditions were described under Materials and Methods, section 13-III. The intermediate gel contained rabbit AACNA-1 (ab), while the reference gel (upper gel) contained rabbit ACNA-1 (b).
Note single precipitin peak within the monovalent intermediate gel corresponding to CNA-1 containing fractions. However, non-neural specific antigens are also present, as demonstrated in the reference gel.



15A

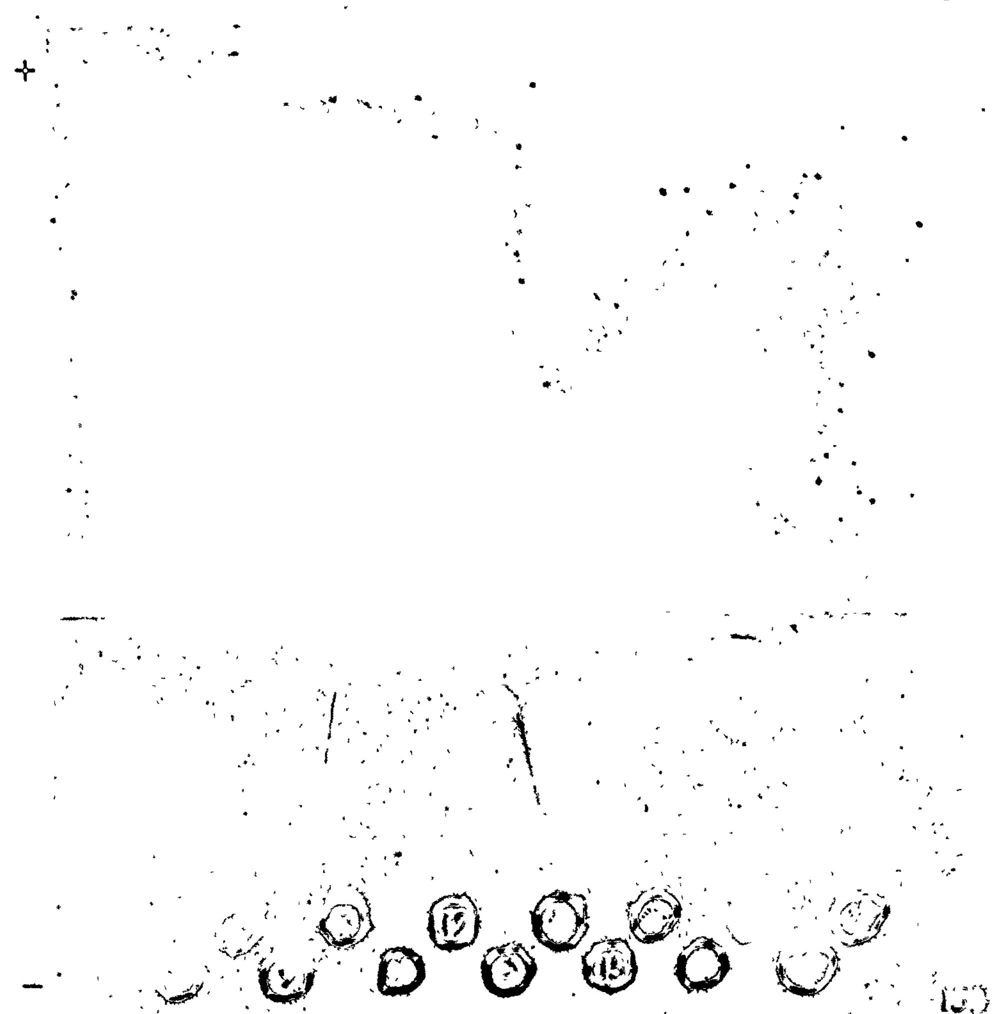
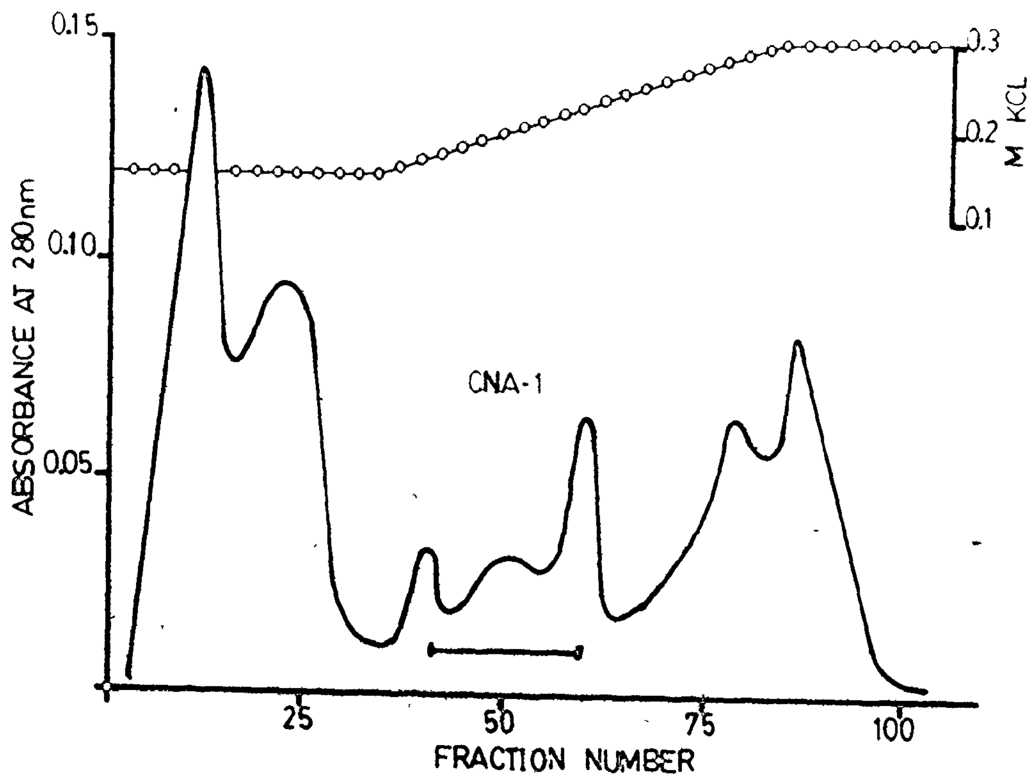
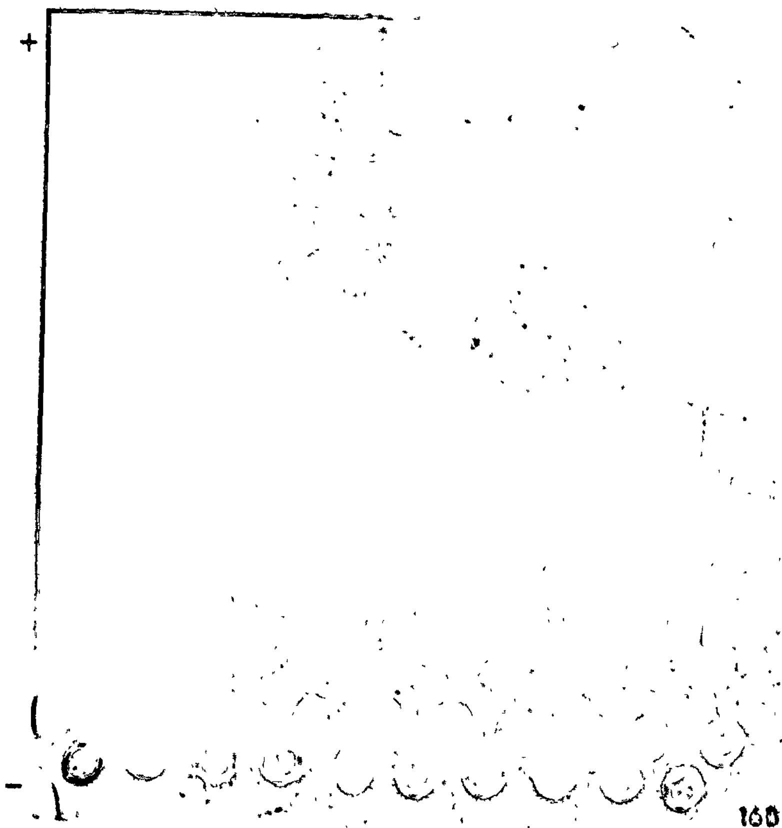


Figure 16A Final DEAE-Sephadex A-50 chromatography of Fraction P2F22E1 obtained from preparative polyacrylamide electrophoresis. Details of experimental conditions were described under Materials and Methods, section 15. Bar represents pooled fractions observed to contain purified CNA-1.

Figure 16B Photograph of fused rocket immunoelectrophoresis with intermediate gel, obtained from final DEAE-Sephadex chromatography of Fraction P2F22E1. Details of experimental conditions were described under Materials and Methods, section 13-III. The intermediate gel contained rabbit AACNA-1 (ab), while the reference gel (upper gel) contained rabbit ACNA-1 (b).
Note single continuous precipitin line in the intermediate gel corresponding to CNA-1 containing fractions. However, fractions 12 to 36 demonstrate CNA-1 which has not bound to the DEAE-Sephadex at pH 7.0 in the presence of 0.15 M KCl, while fractions 40 to 60 were bound firmly at this ionic concentration and not fully eluted until higher KCl concentrations (0.2 M KCl) were used. The reference gel indicates that no contaminating antigens are present in fractions 40 to 56 which were pooled for the purified CNA-1.



16A



16B

eluted in buffer, were analyzed by fused rocket immunoelectrophoresis (Figure 15B). Polyvalent ACNA-1 antiserum was incorporated within the reference gel, while AACNA-1 antiserum was mixed into the intermediate gel. Only one precipitin peak, corresponding to the CNA-1 rich fractions was observed within the intermediate gel. At least two contaminating antigens were also contained within these fractions as demonstrated in the reference gel. A third strongly precipitating antigen was clearly separate from CNA-1 containing fractions and easily excluded from the pooled Fraction P2F22E1.

Final DEAE-Sephadex chromatography of Fraction P2F22E1 resulted in the chromatogram shown in Figure 16A. Purified CNA-1 was demonstrated by fused rocket immunoelectrophoresis (Figure 16B) containing a monovalent intermediate gel (AACNA-1) and a polyvalent reference gel (ABS). CNA-1 rich fractions eluted at approximately 0.2 M KCl did not contain contaminating antigens as demonstrated by the reference gel. However, the premature elution of CNA-1 containing components during washing of the column, may demonstrate heterogeneity of the CNA-1, with components expressing slightly different net charges.

Each step during the purification of CNA-1 was monitored by crossed immunoelectrophoresis. Analysis of each fraction was performed into an antibody-agarose gel containing ABS antiserum (Figure 17A-F). A qualitative decrease of contaminating antigenic material was seen with each purification step. Quantitatively, CNA-1 was also observed to increase in concentration with respect to total soluble protein

Figure 17A Photograph of crossed immunoelectrophoretic analysis of 85.0 μg of adult brain extract (ABE). The antibody-agarose gel contained rabbit ABS (a).
Note multiple precipitin peaks, and arrow indicating position of CNA-1.

Figure 17B Photograph of crossed immunoelectrophoretic analysis of 54.1 μg of 36-65% saturated ammonium sulphate precipitated Fraction P2. The antibody-agarose gel contained rabbit ABS (a).
Note arrow indicating position of CNA-1.

Figure 17C Photograph of crossed immunoelectrophoretic analysis of 43.8 μg of 0.2 M KCl eluted Fraction P2F2 from DEAE-cellulose chromatography. The antibody-agarose gel contained rabbit ABS (a).
Note arrow indicating position and increasing concentration of CNA-1 with respect to total soluble protein.

Figure 17D Photograph of crossed immunoelectrophoretic analysis of 38.5 μg of low molecular weight Fraction P2F22 obtained from Sephadex G-150 SF chromatography. The antibody-agarose gel contained rabbit ABS (a).
Note decrease in number of contaminating precipitin peaks. Arrow demonstrates presence of CNA-1.

Figure 17E Photograph of crossed immunoelectrophoretic analysis of 5.1 μg of preparative polyacrylamide electrophoretically isolated Fraction P2F22E1. The antibody-agarose gel contained rabbit ABS (a).
Note only one contaminating antigen can be demonstrated in the reference gel. The CNA-1 containing precipitin peak is marked by the arrow.

Figure 17F Photograph of crossed immunoelectrophoretic analysis of 3.5 μg of final DEAE-Sephadex purified CNA-1. The antibody-agarose gel contained rabbit ABS (a).
Note a single precipitin peak with no contaminating antigenic peaks visualized in the reference gel.

ABE

17A

P2F22

17D

P2

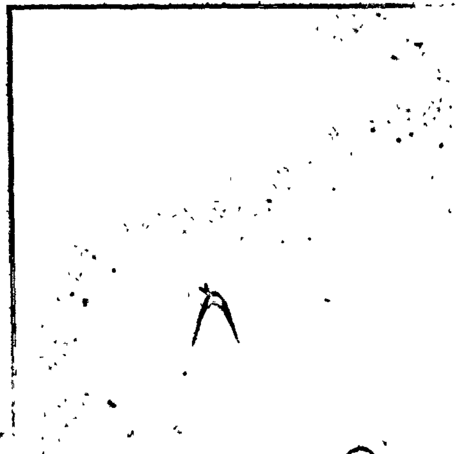
17B

P2F22E1

17E

P2F2

17C



CNA-1

17F

within each fraction as determined by the area beneath the CNA-1 precipitin peaks (arrows). Purity of CNA-1 is demonstrated by a single precipitin peak shown in Figure 17F of DEAE-Sephadex purified CNA-1. This pattern was confirmed with polyvalent ACNA-1 antiserum within the reference gel (not shown). After analytical polyacrylamide disc gel electrophoresis, CNA-1 exhibited a single protein band (Figure 21A).

7. Characterization of Chicken Neural Antigen-1 (CNA-1)

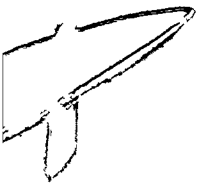
The antigenic determinant of CNA-1 was hydrolyzed by the proteolytic activity of both trypsin and chymotrypsin. Enzyme digested ABE demonstrated no precipitin line when tested by double immunodiffusion developed with AACNA-1 antiserum. This indicated the antigenic site and possibly the entire molecule was proteinaceous in nature.

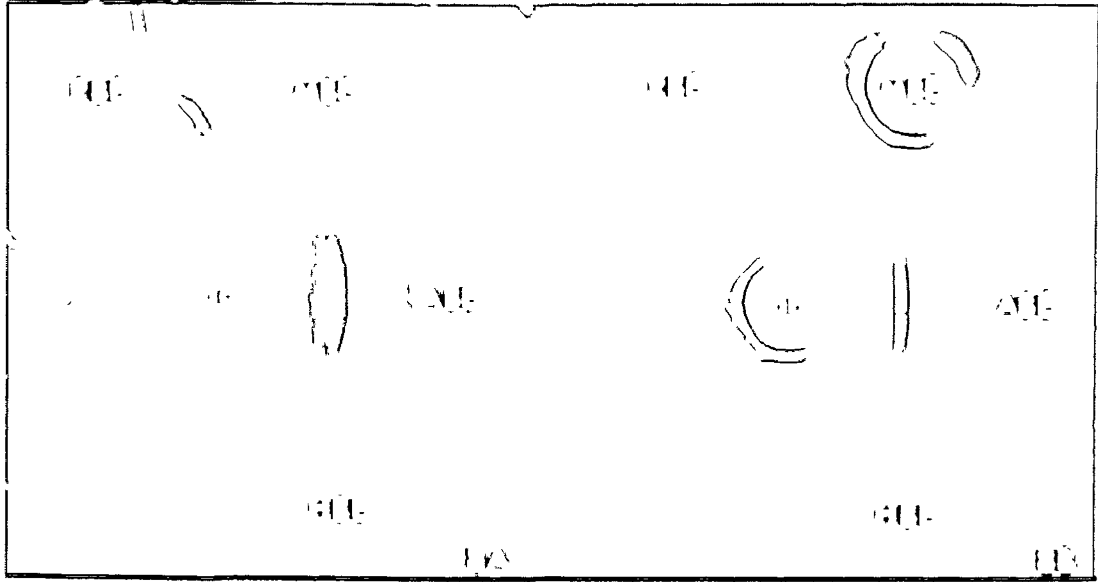
CNA-1 was absent in adult brain extracts of three mammalian species (rabbit, mouse, and hamster) when tested by double immunodiffusion (Figure 18B) and immunoelectrophoresis (Figure 18C) developed with AACNA-1 antiserum. Therefore CNA-1 was shown to be at least partially a class specific antigen not found in mammalian nervous tissue. However, no information could be obtained on the species specificity of CNA-1 since no other avian species were examined.

Figure 18A Photograph of double immunodiffusion plate containing rabbit AABS (aa) in the central well and the following adult brain extracts in the peripheral wells: rabbit (RBE), mouse (MBE), chicken (ABE), and hamster (HBE). Note cross reaction of antiserum with brain extracts of mammalian species, although not as numerous as with chicken brain.

Figure 18B Photograph of double immunodiffusion plate containing rabbit AACNA-1 (ab) in the central well and the following adult brain extracts in the peripheral wells: rabbit (RBE), mouse (MBE), chicken (ABE), and hamster (HBE). Note single precipitin line with adult chicken brain extract only, and no cross reaction with brain extracts of adult mammalian species.

Figure 18C Photograph of immunoelectrophoretic plate with adult brain extracts of: mouse (MBE), rabbit (RBE), chicken (ABE), and hamster (HBE), in the wells. The troughs were filled with either rabbit AABS (aa) or rabbit AACNA-1 (ab). Note that mouse, rabbit, and hamster brain extracts developed with absorbed anti-chicken brain antiserum displayed at least one precipitin arc, while no precipitin formation is observed with these extracts developed with absorbed anti-CNA-1 antiserum.





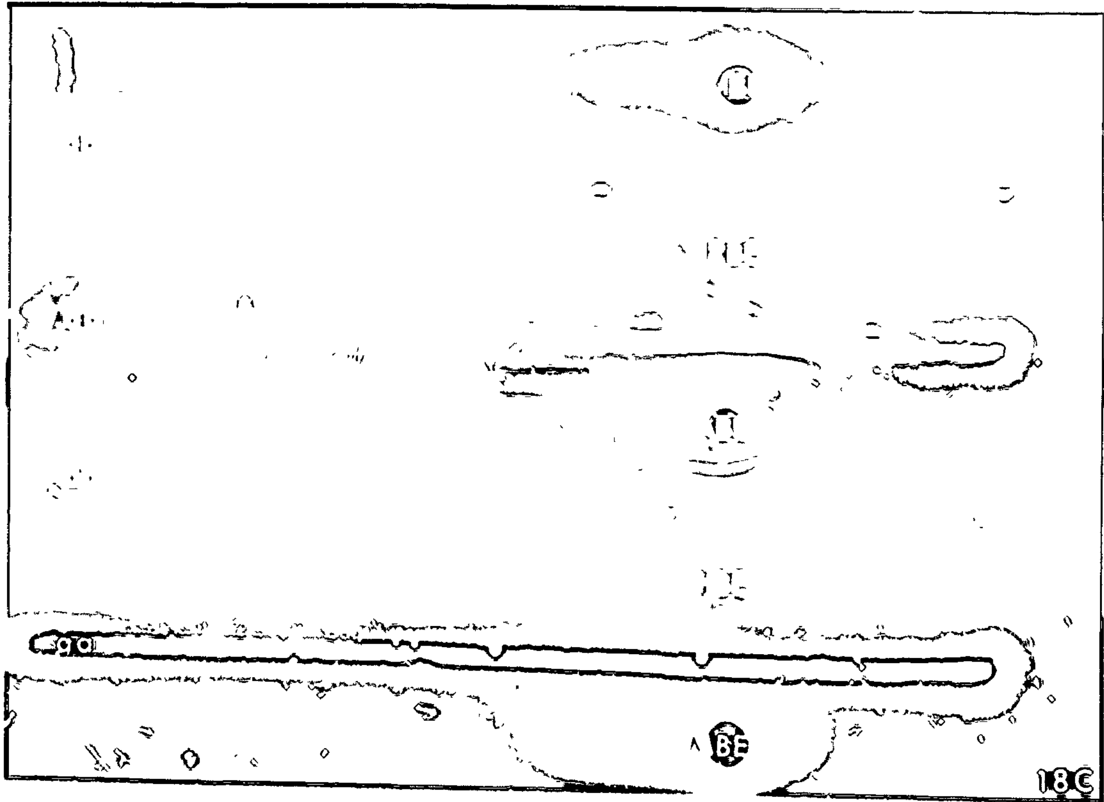
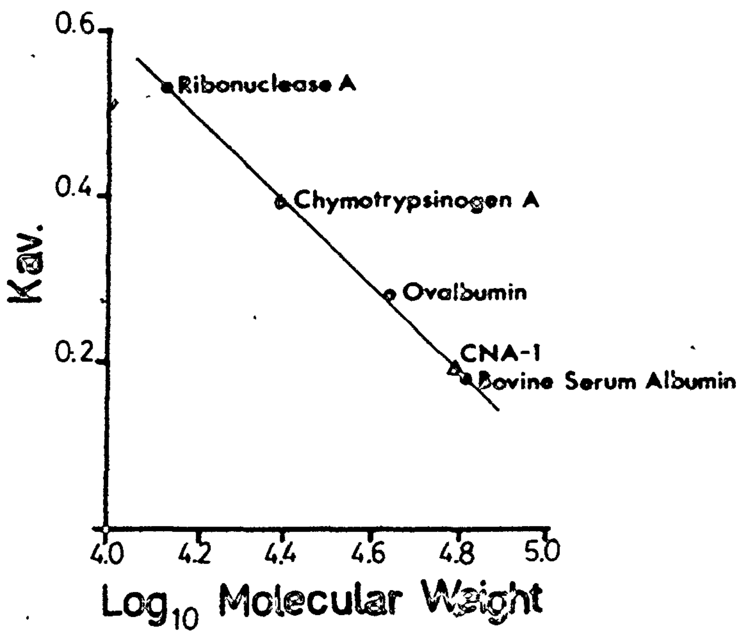
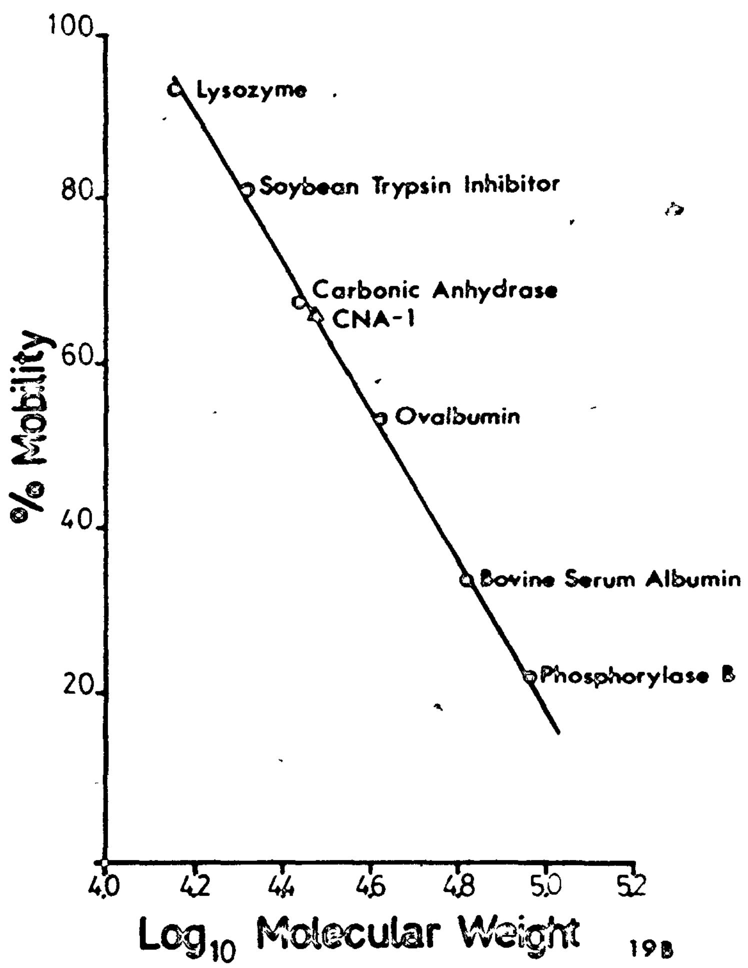


Figure 19A Estimation of molecular weight of CNA-1 by gel filtration in Sephadex G-100 SF (10-40μ). Details of experimental conditions were described under Materials and Methods, section 17-Ia. Standard proteins used and their molecular weights are: ribonuclease A, 13,700; chymotrypsinogen A, 25,000; ovalbumin, 43,000; and bovine serum albumin, 67,000.

Figure 19B Estimation of molecular weight of CNA-1 by sodium dodecylsulphate polyacrylamide gel electrophoresis. Details of experimental conditions were described under Materials and Methods, section 17-II. Standard proteins used and their molecular weights are: lysozyme, 14,300; soybean trypsin inhibitor, 21,000; carbonic anhydrase, 29,000; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase B, 94,000.



19A



19B

8. Molecular Weight Determination

I) Molecular Exclusion Chromatography

The molecular weight, determined by molecular exclusion chromatography of Sephadex G-100 SF (10-40μ), for CNA-1 purified from Fraction P2F22 (low molecular weight), was estimated to be 65,000 daltons (Figure 19A). The molecular weight of CNA-1 isolated within Fraction P2F21 (high molecular weight) could only be estimated on Sephacryl S300 SF (40-105μ). The CNA-1 containing component was eluted at the exclusion limit for this column (i.e. void volume), indicating the molecular weight of this component to be in excess of 1.5 x 10^6 daltons (Figure 20A).

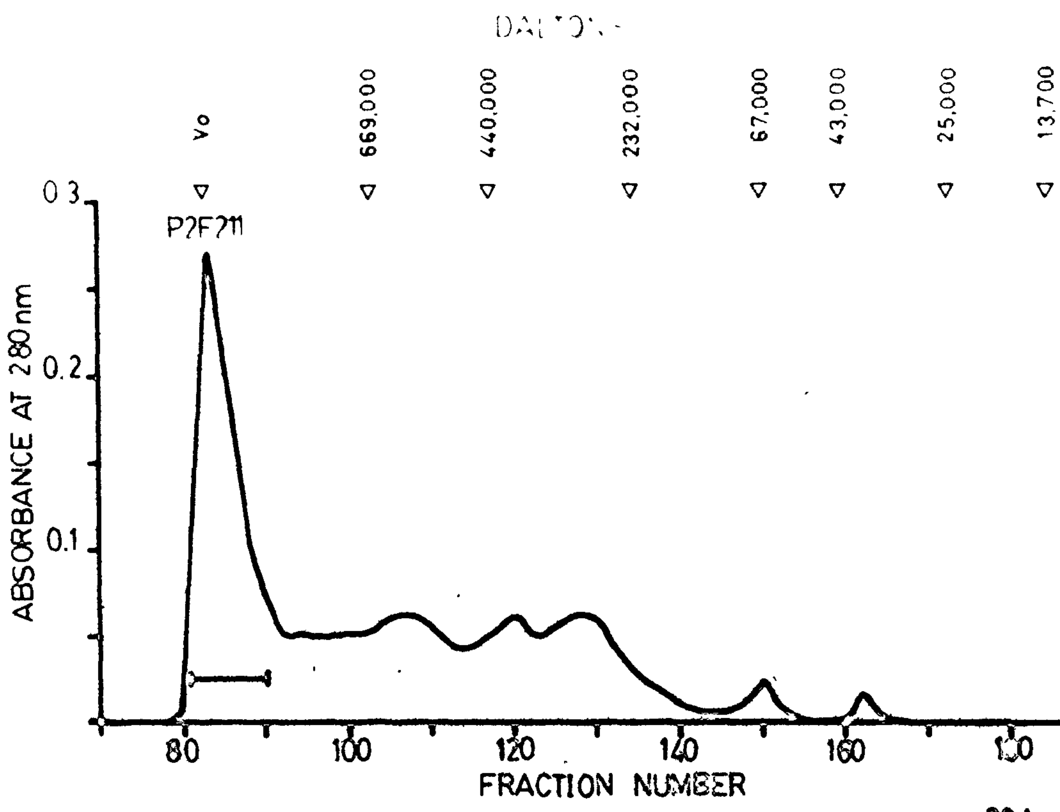
II) Sodium Dodecylsulphate (SDS) Polycrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis has been used for determining the molecular weight of polypeptide chains in oligomeric proteins. As demonstrated in Figure 21B a purified sample of CNA-1 was subjected to SDS polyacrylamide gel electrophoresis according to the method of Yamada and Weston (1974). One protein band was observed, and a molecular weight of 30,000 daltons was calculated (Figure 19B). This indicated the antigen consisted of two subunits with the same molecular weight. A second band, felt to be an artifact in the system, at a molecular weight of 27,000 daltons was observed in both CNA-1 samples and protein standards.

Figure 20A Sephacryl S-300 SF (40-105μ) chromatogram of the high molecular weight Fraction P2F21, obtained from Sephadex G-150 SF chromatography. Details of experimental conditions were described under Materials and Methods, section 17-Ib. Fraction P2F211 corresponds to the peak of high molecular weight proteins excluded from the column at its void volume (i.e. > 1,500,000 MW). The bar represents pooled eluted fractions shown to contain the CNA-1 determinant. Standard proteins used and their molecular weights are: thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; bovine serum albumin, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; and ribonuclease A, 13,700.

Figure 20B Photograph of double immunodiffusion plate containing rabbit anti-CNA-1 antiserum absorbed with serum, liver, and kidney extracts (ab) in the central wells, and the peripheral wells containing fractions 80 to 102, obtained during Sephacryl S-300 SF chromatography of Fraction P2F21.

Note that a high molecular weight (> 1,500,000 MW) component containing the CNA-1 determinants was localized in eluted fractions 82 to 88. No further evidence of cross reacting fractions was found for the remainder of the column.



20A

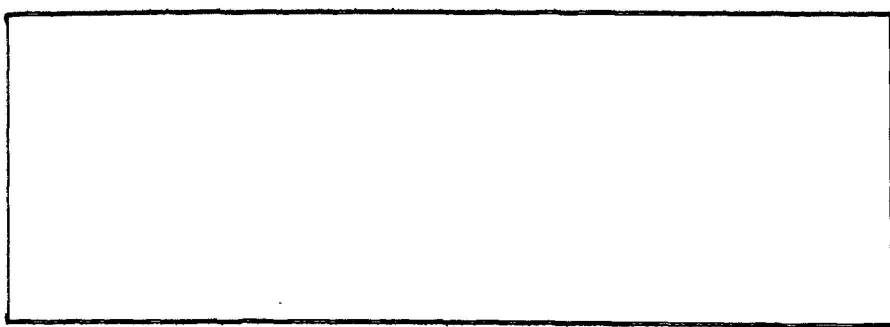


Figure 21A

Photograph of final DEAE-Sephadex purified CNA-1 after analytical polyacrylamide gel electrophoresis. Details of experimental conditions were described under Materials and Methods, section 14.
Note single homogeneously staining protein band.

Figure 21B

Photograph of final DEAE-Sephadex purified CNA-1 and appropriate protein standards after electrophoresis in 10% sodium dodecylsulphate polyacrylamide gel. Details of experimental conditions were described under Materials and Methods, section 17-II. Standard proteins used and their molecular weights are: lysozyme, 14,300; soybean trypsin inhibitor, 21,000; carbonic anhydrase, 29,000; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase B, 94,000.
Note CNA-1 protein band showing similar electrophoretic mobility (MW 30,000) as carbonic anhydrase. A second thin band (MW 27,000) which was thought to be artifactual, is observed in both standard and purified protein samples (arrow).

Phosphorylase B

Bovine Serum Albumin

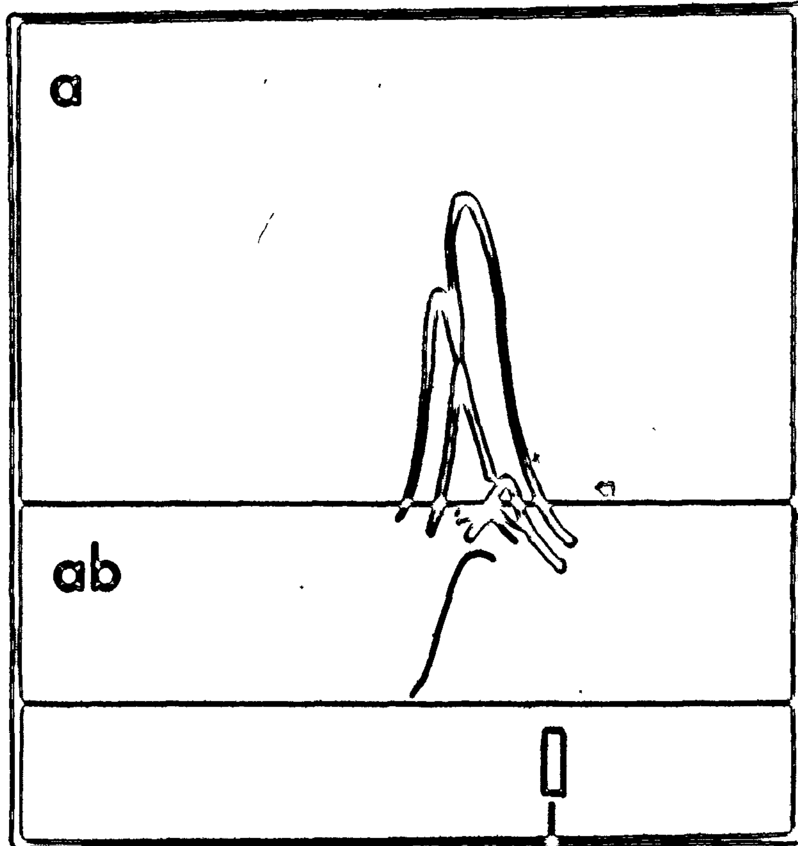
CNA-1

Dye

21A

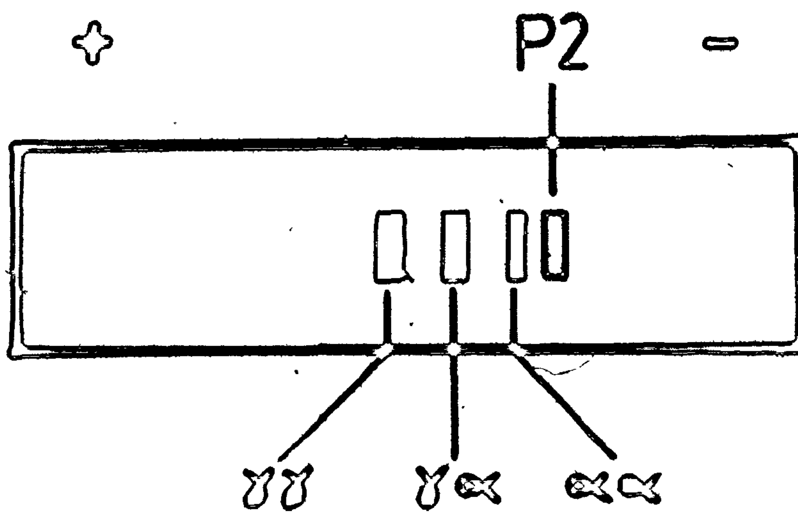
Figure 22A Diagrammatic representation of a crossed immunoelectrophoresis with intermediate gel of Fraction P2. Details of experimental conditions were described under Materials and Methods, section 13-III. The intermediate gel contained rabbit AACNA-1 (ab), while the reference gel (upper gel) contained rabbit ABS (a).
Note single precipitin peak within the monovalent intermediate gel corresponding to CNA-1. No enolase activity could be demonstrated for this peak.

Figure 22B Diagrammatic representation of a first dimension gel run simultaneously with the first dimension of the crossed immunoelectrophoresis. The gel was stained for enolase activity under the experimental conditions outlined in Materials and Methods, section 18.
Note three distinct brain enolase isozymes could be demonstrated ($\gamma\gamma$, $\gamma\alpha$, and $\alpha\alpha$).



+
−

22A



22B

Precipitation in agarose could not be demonstrated for CNA-1 treated with 0.1% SDS, indicating the anti-CNA-1 antibody was directed against the native protein structure and not against the dissociated subunits. For this reason, no immunoassay system was available to detect SDS treated CNA-1 containing components isolated but not purified within the high molecular weight Fraction P2F21.

9. Enolase Activity Staining

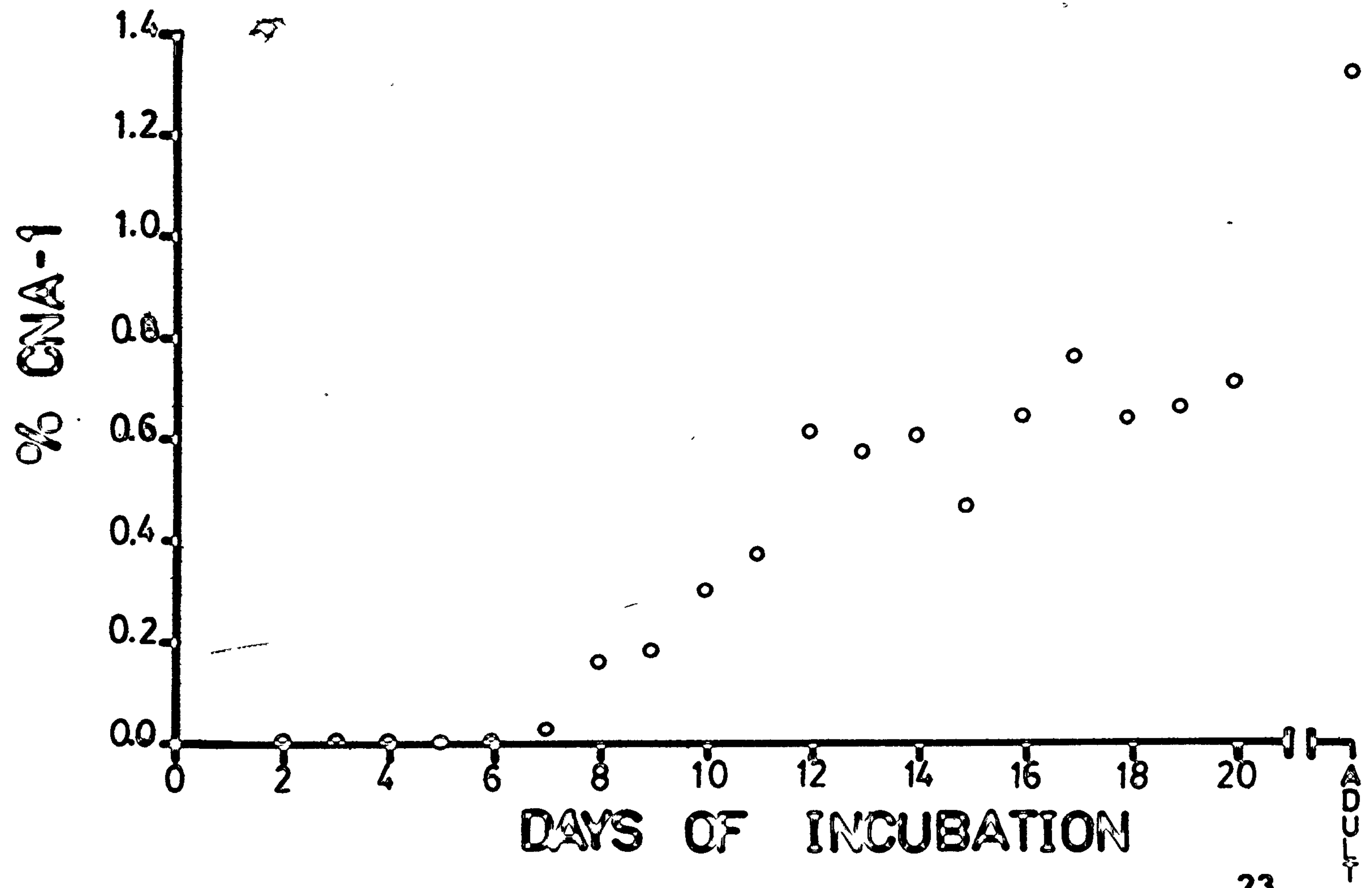
The three brain isozymes ($\gamma\gamma$, $\gamma\alpha$, and $\alpha\alpha$) of enolase (2-Phospho-D-glycerate hydro-lyase, E.C. 4.2.1.11) were clearly demonstrated by the method of Dave et al. (1966) after agarose electrophoresis of Fraction P2.

The CNA-1 precipitin peak observed within the intermediate gel during crossed immunoelectrophoresis (Figure 22A) did not demonstrate enolase activity after staining. Also the peak was not found to correspond to the mobility expressed by the neural specific enolase $\gamma\gamma$ electrophoresis under similar conditions (Figure 22B). This indicated CNA-1 was dissimilar to the 14-3-2 protein which has been demonstrated to exhibit enolase activity.

10. CNA-1 Levels During Chicken Embryonic Development

The results of rocket immunoelectrophoretic analysis of embryonic whole brain extracts from Days 5 to 20 of incubation, and whole embryo extracts from Days 1 to 4 of incubation, assayed with monovalent AACNA-1 are shown in Figure 23.

Figure 23 Pattern of CNA-1 accumulation during the embryonic development of the chicken brain, expressed as % CNA-1 of the total soluble protein obtained in saline extracts of embryonic and adult brains. Details of experimental conditions were described under Materials and Methods, section 13-IV. Note that CNA-1 is not detected earlier then the seventh day of incubation. There is a steady accumulation of CNA-1 within the developing embryonic brain which continues during post-hatching development until adult levels are reached.


% CNA-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
ADULT
DAYS OF INCUBATION

The earliest day at which CNA-1 was detected was on the seventh day of incubation. At this time the concentration of CNA-1 was small, but measurable (0.03%). This appearance correlates well with that observed for Antigen E, shown during the ontogenic appearance of neural specific antigens (Results, section 2) to appear by the seventh day and exhibit alpha-1 globulin mobility.

CNA-1 was found to increase in concentration from the seventh day to prehatching (Day 20). However, the prehatching level of CNA-1 was observed to be 0.7% of the total soluble protein. This is approximately half of the adult level which was shown to be 1.3% of total soluble protein obtained from whole brain extracts. It therefore appears that CNA-1 continues to accumulate within the chicken brain during the posthatching period until adult levels are achieved.

11. Immunohistochemical Localization of CNA-1

Immunohistochemical localization of CNA-1 within the adult chicken brain was undertaken using both indirect immunofluorescence and indirect immunoperoxidase techniques. After incubation with rabbit AACNA-1 antiserum directed against the CNA-1 antigen, fluorescence was most predominant on neuronal plasma membranes. However, the antigen could not be localized on all neurons, and was absent in control tissue sections of adult chicken liver and adult mouse cerebellum and brainstem.

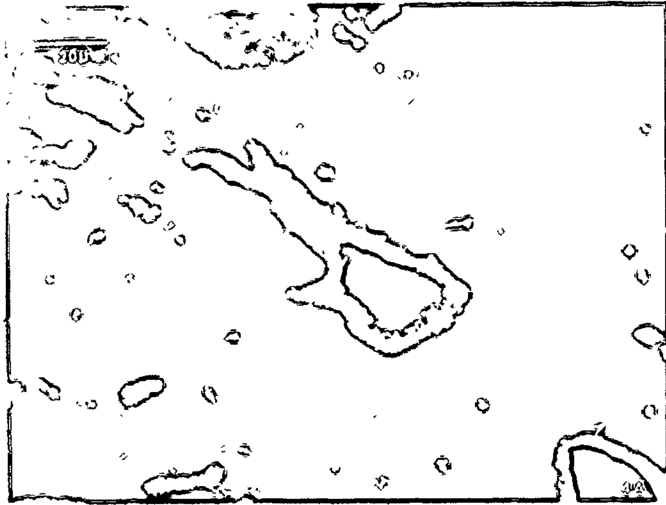
In the cerebellum of adult chickens, fluorescence was clearly demonstrated on plasma membranes and processes of large neurons located in the nucleus medialis cerebelli (Figure 24A-B). The cytoplasm and nucleus of these neuronal cells lacked this fluorescence. Similar observations confirming this distribution of CNA-1 were obtained during immunoperoxidase staining. Both the neuronal plasma membrane and processes were selectively stained by the deposition of the peroxidase reaction product (Figure 24C, 26A and 26C). However, as was the case for all control slides, no selective staining of neuronal cell membranes and processes was seen (Figure 26B). The background staining which was observed using both immunohistochemical techniques resembled transversely sectioned neuronal processes rather than macroglial labelling. However, this possibility could not be entirely ruled out.

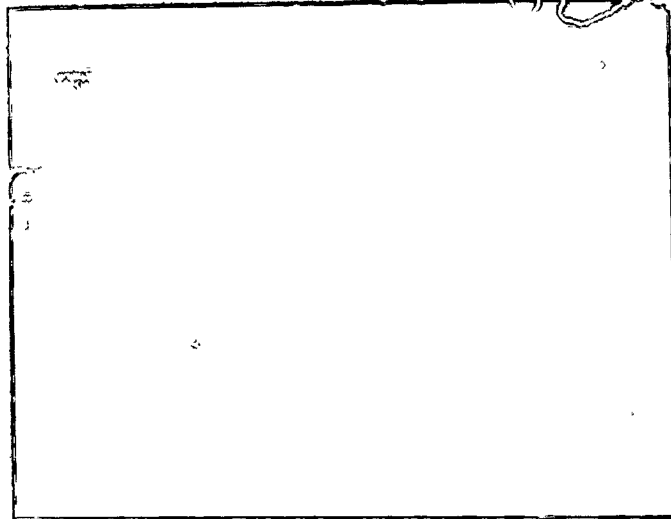
The adult cerebellar cortex has three definitive layers which were easily identifiable after nerve cell staining (Figure 25C). They are named in order from the outer cortical surface: the molecular layer, the Purkinje cell layer, and the granular layer. The distribution of fluorescent label throughout the three layers was distinctive (Figure 25A-B). The molecular layer exhibited no selective labelling. However, the large Purkinje cells within the second layer demonstrated a faint fluorescent border, indicating the presence of CNA-1 on the plasma membrane of these neurons, but in significantly lower concentration than was observed for the larger neurons of the deep nuclei. Within the granular layer immunofluorescent labelling was

Figure 24A High power photomicrograph of a large neuron within a cryostat section of adult chicken cerebellar roof nuclei, treated with rabbit AACNA-1 antiserum and FITC conjugated swine anti-rabbit antiserum for immunofluorescence. Note strongly fluorescent labelling of neuronal plasma membrane and processes, but absence of label within cytoplasma and nucleus. Bar represents 20μ.

Figure 24B Low power photomicrograph of large neurons within a cryostat section of adult chicken cerebellar roof nuclei, treated with rabbit AACNA-1 antiserum and FITC conjugated swine anti-rabbit antiserum for immunofluorescence. Note prominent labelling of neuronal plasma membrane and processes. Background fluorescence may be partially attributed to transversely sectioned neuronal processes. Bar represents 50μ.

Figure 24C Low power photomicrograph of large neurons within a cryostat section of adult chicken cerebellar roof nuclei, treated with rabbit AACNA-1 antiserum and horseradish peroxidase conjugated swine anti-rabbit antiserum for immunoperoxidase labelling. Note similar distribution of peroxidase reaction product as was demonstrated for immunofluorescence (Figure 24B). Bar represents 50μ.





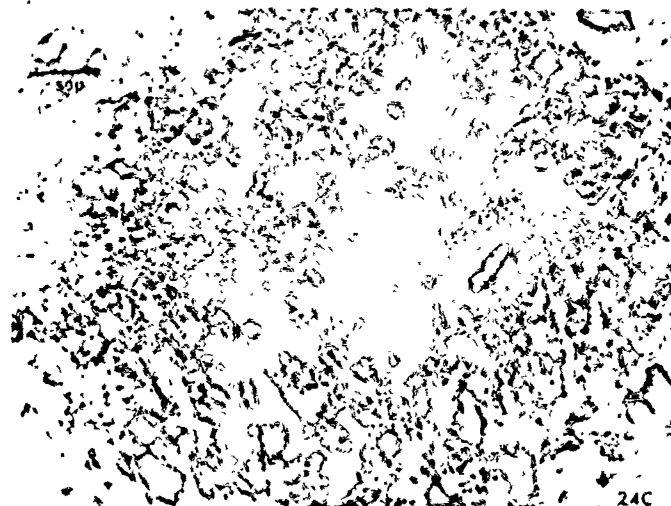
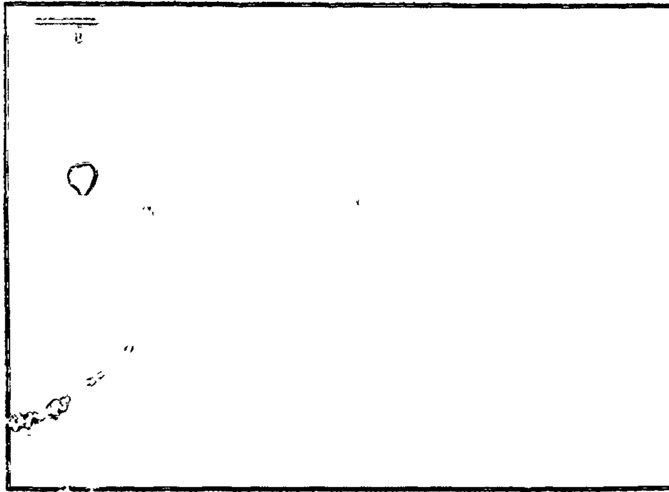
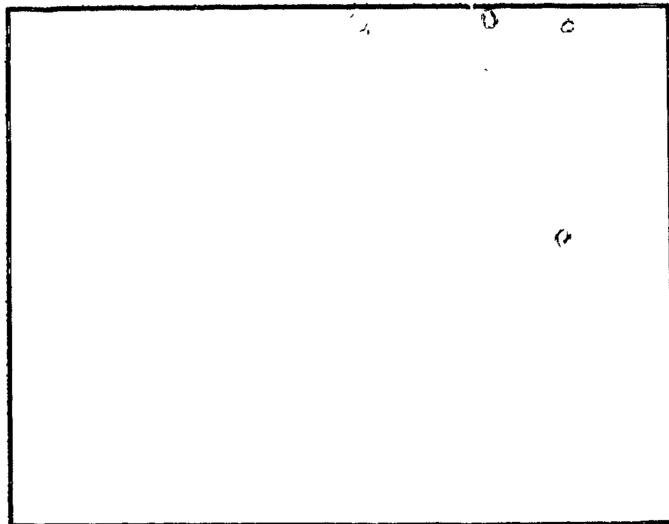

24C

Figure 25A Low power photomicrograph of a cryostat section of adult chicken cerebellum, treated with rabbit AACNA-1 antiserum and FITC conjugated swine anti-rabbit antiserum for immunofluorescence. Note fluorescent labelling within Purkinje cell (PURK) and granular (GRAN) layers, but absence of label within molecular (MOL) layer of cerebellar cortex. Bar represents 50μ.

Figure 25B High power photomicrograph of a cryostat section of adult chicken cerebellum, treated with rabbit AACNA-1 antiserum and FITC conjugated swine anti-rabbit antiserum for immunofluorescence. Note sparse fluorescent labelling of Purkinje cell (PURK) plasma membrane, and labelling of scattered cells within granular layer whose morphology and distribution conform to that of granule cells. Bar represents 20μ.

Figure 25C High power photomicrograph of a cryostat section of adult chicken cerebellum, similar to that shown in Figure 25B, but histologically stained for nissil substance by the acidic cresyl violet method.
Note deep staining of nerve cells within the three layers of the cerebellar cortex: the molecular layer (MOL), Purkinje (PURK) cell layer, and granular layer (GRAN). Bar represents 10μ.





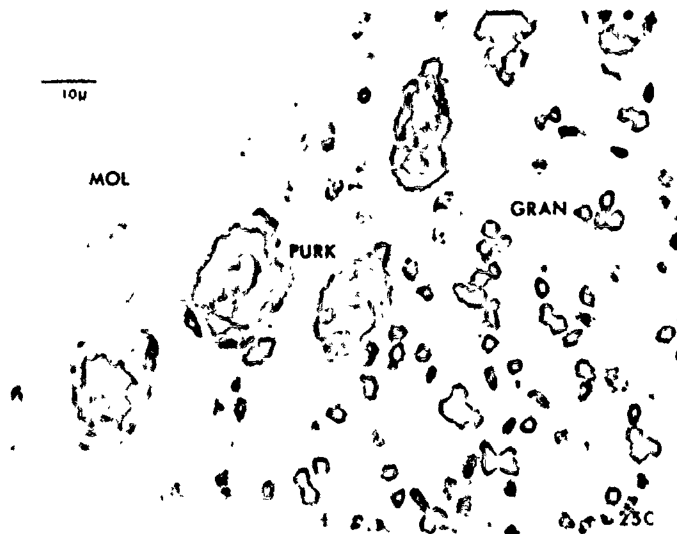
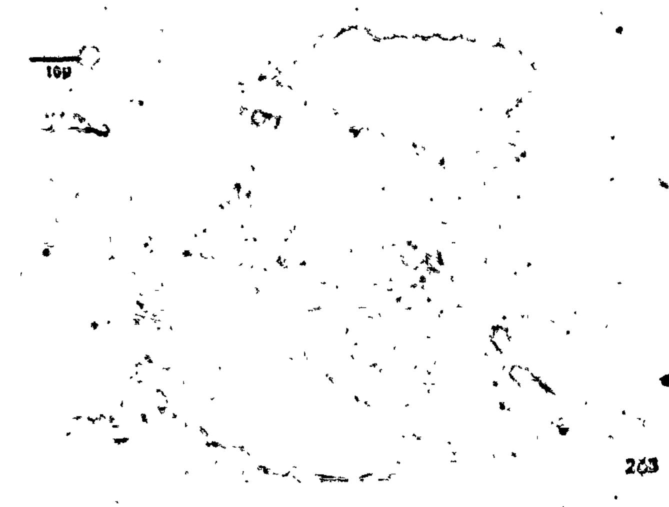

10µ
MOL
PURK
GRAN
23C

Figure 26A High power photomicrograph of a large neuron within a cryostat section of adult chicken cerebellar roof nuclei, treated with rabbit AACNA-1 antiserum and horseradish peroxidase conjugated swine anti-rabbit antiserum for immunoperoxidase labelling.
Note prominent deposition of peroxidase reaction product on neuronal plasma membrane (arrow). Bar represents 10μ.

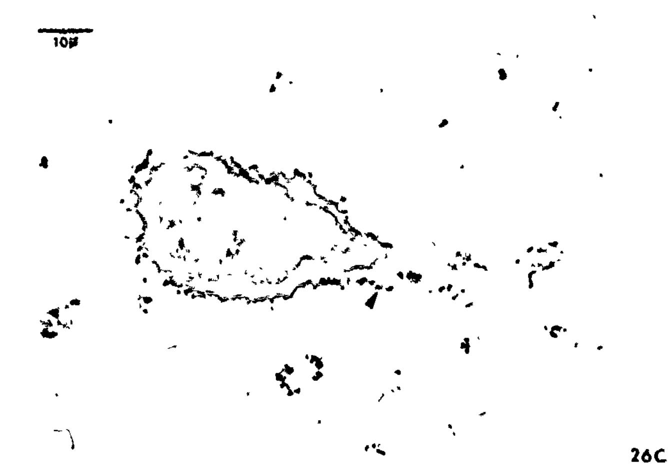
Figure 26B High power photomicrograph of a large neuron within a cryostat section of adult chicken cerebellar roof nuclei, treated with normal rabbit serum (i.e. preimmune) and horseradish peroxidase conjugated swine anti-rabbit antiserum for immunoperoxidase labelling.
Note absence of deposition of peroxidase reaction product on neuronal plasma membrane (arrow) in this control slide. Bar represents 10μ.

Figure 26C High power photomicrograph of a large neuron within a cryostat section of adult chicken cerebellar roof nuclei, treated with rabbit AACNA-1 antiserum and horseradish peroxidase conjugated swine anti-rabbit antiserum for immunoperoxidase labelling.
Note prominent deposition of peroxidase reaction product on neuronal plasma membrane and processes (arrow) similar to that demonstrated by immunofluorescence (Figure 24A). Bar represents 10μ.

10μ
26A



10μ
26B



10μ
26C

present in scattered cells whose morphology and distribution conform to that of microneurons (granule cells).

In other regions of the chicken brain, the localization of CNA-1 was also distinctive. No specific labelling could be demonstrated on the neurons of the cerebral hemisphere, but characteristic labelling of neuronal membranes and processes was observed within brainstem nuclei.

DISCUSSION

In the field of immunoembryology, antibodies directed against adult antigens have been used to study the synthesis and localization of antigenic substances during the course of development. The rationale behind such studies is that the appearance during development of antigens which react with antiserum against specific adult antigens is considered to reflect the synthesis of similar substances in the embryo. When the adult antigen is characteristic of a particular organ or tissue, the appearance in the embryo of cross-reacting substances may be taken as an indication of differentiation in that tissue (Clayton, 1960).

The presence in the early embryo of antigens serologically identical to those of adult avian brain has been well established (Burke et al., 1944; Ebert, 1950; Schalekamp, 1963; McCallion and Langman, 1964). However, those antigens which by immunochemical criteria were found to be restricted to nervous tissue (i.e. neural specific) have not previously been extensively studied.

In the present study, the survey of antigens within extracts of whole chicken brains has yielded results similar in many respects to earlier studies performed with mammalian brain (MacPherson and Liakpoulou, 1966; Kosinki and Grabar, 1967 and Hatcher and

MacPherson, 1969), and avian brain (Schalekamp, 1963 and McCallion and Langman, 1964). However, substantially more non-neural and neural specific antigens were detected. At least eight adult chicken neural specific antigens were visualized along the axis of migration during immunoelectrophoresis of adult brain extract (ABE) which had been developed with anti-brain antiserum absorbed with serum, liver and kidney extracts (AABS). The differences between these observations as opposed to previous studies of chicken brain can be accounted for by differences in the quality of antiserum used for the detection of the antigens. To obtain a polyvalent antiserum as potent as possible, a high antigenic burden (30.0 mg/ml total protein) and long periods of immunization (7-8 months) were employed. The duration of the immunization period is known to affect the compostion of the antiserum. As the period increases, more antigenic sites will elicit an antibody response (Bock, 1972). The use of optimal conditions for immunoelectrophoresis (high concentrations of antibody and large amounts of antigen) during this study may also account for the higher number of antigens detected, since antibodies of low titer or antigens of low concentration could be visualized.

Immunoelectrophoresis based on the method of Grabar and Williams (1953), employs two independent criteria to distinguish proteins from one another, first segregating the proteins according to their electrophoretic mobilities and then identifying them by the highly specific immune reaction. The geometry of the diffusion format

provides an added refinement. Then antigen-antibody pairs precipitate independently, not as straight lines, but as elliptical arcs. SimIlar to the intersecting lines on a double immunodiffusion plate, the crossed arcs indicate that this reaction is between immunochemically different proteins. A line drawn through an arc at the point furthest from the path of electrophoretic migration, and perpendicular to its path, will indicate the centre of concentration of each antigen on the plate regardless of the antigen's relative concentration (Williams, 1960). Despite the differences in mobilities, certain proteins are so closely related chemically that some antisera will not distinguish between them, explaining why the antibody-antigen precipitation forms a smooth continuous line rather than several intersecting arcs. It must also be kept in mind that although immunoelectrophoresis is a sensitive technique for the detection and distinction of antigens in protein mixtures, it has two principal limitations; it detects only soluble antigens, and their detection depends on the presence in the immune serum of corresponding antibodies. Thus the number of constituents detected must be considered as a minimum.

Based on these parameters and limitations, the electrophoretic patterns obtained for the adult neural specific antigens can be used both as a template for the study of individual antigens in extracts of embryonic brain, and to trace their ontogenic appearance during early neural development. Of the eight neural specific antigens which the AABS antiserum was able to demonstrate in extracts of adult brain (Figure 6A), one was shown to possess prealbumin mobility (C), one with alpha-1 globulin mobility (E), three with alpha-2 globulin mobility

(H, F, G), one with beta globulin mobility (B), one with gamma globulin (D) and the final antigen possessed both beta and gamma globulin mobilities (A). Continuous precipitin lines with gamma globulin mobility were also observed from Antigens E, F and G.

Extracts of embryonic whole brain from Day 5 to 20 and whole embryos from Day 1 to 4 of incubation when compared by immunoelectrophoresis with AABS, clearly demonstrated a sequential appearance of these adult neural specific antigens. Antigens A through G were detected between Day 2 and 11 of incubation. Antigen H however, was not observed within any of the embryonic extracts, and was considered to be specific to posthatching neural tissue. Therefore the majority of adult neural specific antigens were detectable during the period of early establishment of the nervous system (neuralization) through the period in which the definitive cytoarchitecture of the brain is established.

Also worthy of mention was the change in precipitin patterns of specific antigens during electrophoretic analysis, although the relative position of the antigen remained constant. After the adult antigenic complement was established by the 11th day, the patterns became more complex with the appearance of smooth continuous precipitin lines rather than arcs. This indicated the presence of antigenically similar molecules with differences in electrophorectic mobilities. For example Antigen E first appeared by the 7th day of incubation, and up until the 18th day of incubation expressed a concentric arc with

alpha-1 globulin mobility. However, prior to hatching the precipitin pattern showed a continuous line with antigen components showing little anodal migration. Possible explanations for this observation are: an increasing complexity of the antigenic molecule with subsequent development, binding of the antigen by new synthesized proteins altering its electrophoretic mobility but not affecting its antigenicity, or simple experimental artifact due to excessive antigen concentration resulting in electrophoretic smearing of the protein bands. The exact mechanisms for the phenomenon requires further investigation. However, structural changes in brain glycoproteins by the addition of terminal sialic acid residues has been observed by Margolis and Gomez (1974) during neural development. This addition of sialic acid residues may represent one of the major modifications of cell surface and other glycoproteins during brain development as well as suggest that these negatively charged groups may be of importance in regulating intercellular adhesions, synaptogenesis and other cell-cell interactions (Margolis and Gomez, 1974).

The overall temporal appearance within the embryo of the neural specific antigens described in this study (Day 2-11) were similar to those previously described independently by Schalekamp (1963) (i.e. Day 2-14) and by McCallion and Langman (1964) (i.e. Day 5-12). However, in both of these studies, only three neural specific adult antigens were detected and differences in the temporal appearances of individual antigens were also apparent. Croisille (1969) suggested that they were in fact describing six different antigens. The lack of uniformity of

antisera produced within different rabbits to a given tissue extract, under slightly different courses of immunization has been the subject of some concern. During the present study an antibody pool was established from the serum of six rabbits, injected with the same antigenic solution and employing the same injection schedule. In this way inter-rabbit differences in antibody specificities would be minimized. Therefore the possibility exists, that some antigens detected during the present study were the same as those previously described in the earlier studies. However, the additional demonstration of at least one neural specific antigen that was found to be antigenically similar to an antigen within mammalian brain extract (i.e. non-class specific), as well as at least one neural specific antigen not demonstrable within the embryonic chicken brain clearly demonstrates the differences and quality of the antiserum employed.

ABE which had undergone hydrolysis by the proteolytic action of trypsin and chymotrypsin for 6 hours at 37°C continued to display a single precipitin line during double immunodiffusion when developed with AABS. This demonstrated that although a majority of the neural specific antigenic determinants within the ABE were proteins, at least one antigenic site was not proteinaceous or susceptible to these enzymes. This observation was similar to that described by MacPherson and Liakopoulou (1966) for brain antigens in rat brain extract.

The isolation of a single neural specific antigen from the chicken adult brain extract (ABE) was accomplished by several procedures, including, ammonium sulphate fractionation, DEAE-cellulose

chromatography using a stepwise elution of increasing ionic strength and G-150 SF sephadex chromatography. The antigen which demonstrated only a single precipitin line during double immunodiffusion and a single precipitin arc expressing alpha-1 globulin mobility during immunoelectrophoresis when developed with AABS was designated chicken neural specific antigen-1 (CNA-1). CNA-1 was further enriched and partially isolated from other non-neural brain antigens by DEAE-sephadex A-50 chromatography using a parabolic salt gradient and the G-100 SF sephadex chromatography. When isolated, CNA-1 corresponded well with Antigen E of the adult neural specific antigens.

Anti-CNA-1 serum (ACNA-1) from a single rabbit injected with an enriched CNA-1 fraction (P2F2211) exhibited precipitin reactions with antigens of both neural and non-neural origin. However, after absorption with chicken serum, liver and kidney extracts the antiserum was demonstrated to be monovalent for CNA-1 (AACNA-1) when tested by double immunodiffusion and immunoelectrophoresis. However, a smooth continuous precipitin line was observed during immunoelectrophoresis of ABE (Figure 12D). This indicated that the AACNA-1 recognized antigenically similar molecules expressing different mobilities. It must be kept in mind that the anti-CNA-1 antiserum was produced against a CNA-1 enriched fraction which exhibited only a single arc with alpha-1 globulin mobility when tested with AABS. Therefore the production and subsequent characterizaton of anti-CNA-1 serum revealed three important observations: that the enriched CNA-1 fraction (P2F2211) was contaminated with common tissue antigens capable of

inducing antibody production; that immunoelectrophoresis developed with polyvalent ABS failed to detect the contaminating antigens; and that the CNA-1 antigen was molecularly more complex than had been originally anticipated during its isolation.

An added complication arose during the isolation procedure with the detection of a protease co-purifying with CNA-1 and becoming activated following DEAE-Sephadex chromatography. Electrophoretic studies on Fraction P2F221 revealed that digestion of the CNA-1 molecule had taken place, and degradation products containing the antigenic site could be detected. For this reason, inhibition of the protease was attempted using a number of commercially available protease inhibitors. All inhibitors tested at all concentrations proved to be ineffective. Serine proteases such as trypsin and chymotrypsin would be inhibited by either soybean trypsin inhibitor, aprotinin or PMSF. Cysteine proteases such as papain similarly would be inhibited by the action of TPCK and TLCK. The presence within the protein fraction of EDTA would eliminate the metalloenzymes such as the carboxypeptidases and aminopeptidases. However, a second group of acid proteases, the cathepsins, are not inhibited by either the trypsin type inhibitors or by molecules which interfere with its sulfhydryl groups (i.e. TPLK, PMSF). The optimal pH of these enzymes however, is between pH 2.5 and 4. The protease detected during this study were very active in buffer at pH 7.0. Therefore the identity of some representative groups of proteases could be eliminated.

The separation of acid and neutral proteases from rat brain has been investigated by Marks and Lajtha (1965). The acid proteases were demonstrated to be of the cathepsin type, particularly cathepsin D. However, marked inhibition of both acid and neutral protease was observed with the specific chymotrypsin inhibitor TPCK. The protease found during the present study was not inhibited by TPCK, therefore the precise nature of this brain protease has not been elucidated and requires further investigation. Fortunately the protease activity was eliminated by preparative polyacrylamide electrophoresis of Fraction P2F22 at pH 8.6 in the absence of EDTA and 2-mercaptoethanol. This step was therefore implemented in the subsequent purification of CNA-1.

CNA-1 was purified by modifying the procedure initially utilized for the isolation of the neural specific antigen. After ammonium sulphate fractionation, DEAE-cellulose chromatography and G-150 SF sephadex chromatography, CNA-1 was purified from the low molecular weight fraction by preparative polyacrylamide electrophoresis and DEAE-Sephadex chromatography using a linear salt gradient. The presence of contaminating antigens of very close isoelectric pH, charge and molecular weight, which could be identified by quantitative immunoelectrophoresis, made the purification of CNA-1 a tedious task.

Both fused rocket and crossed immunoelectrophoresis were used to monitor the purification procedures because of their superior qualitative and quantitative powers over simple immunoelectrophoresis. However, as well as the limitation of immunoelectrophoresis described earlier, the quantitative immunoelectrophoretic techniques utilized

within this study also suffer from one additional limitation. These methods only allow for the investigation of proteins with an isoelectric pH below 8.6 (i.e. the isoelectric pH of immunoglobulin), (Bock *et al.*, 1971). Therefore basic proteins could not be detected unless the charge of the proteins was changed by carbomalation prior to electrophoresis (Weeke, 1968), which was not undertaken during this study.

Within these limitations, quantitative immunoelectrophoretic techniques are outstanding tools for immunochemical identification of individual antigens within samples of unknown antigen compostion when compared with a reference system. The identification procedures allow quantitation of individual antigens by means of polyvalent antisera even in samples containing a multitude of different antigens. Similarly the specificity and titres of precipitating antibodies can be determined by comparison with a reference antiserum, even in complicated systems (Axelsen and Bock, 1972).

The purity of CNA-1 preparation was subjected to several tests for homogeneity. Only one precipitin peak was detectable after crossed immunoelectrophoresis of CNA-1 into a polyvalent antiserum (ABS or ACNA-1) containing gel, indicating the absence of contaminating antigens. Analytical disc polyacrylamide gel electrophoresis of this preparation also demonstrated only one homogeneous band after protein staining. Since disc polyacrylamide gel electrophoresis permits high resolution, analysis of extremely small samples of complex protein mixtures can be performed. Therefore the combination of this test

with the highly sensitive quantitative immunochemical tests, indicated that the CNA-1 preparation was unlikely to contain other contaminating proteins.

Preliminary characterization of CNA-1 confirmed the proteinaceous nature of the antigen. The proteolytic action of both trypsin and chymotrypsin were observed to destroy its antigenicity. Similarly, the neural and avian specificities of the antigen were also demonstrated immunochemically. No cross activity with mammalian brain extracts could be detected during double immunodiffusion and immunoelectrophoresis when developed with AACNA-1 antiserum. The restriction on the interspecies distribution of this antigen may be at the class level. Thus, the conservation of this antigen during evolution appears to differ from that of the soluble, low molecular weight, acidic proteins, S-100, GFA and NSP, which are specific to the nervous system and present in a wide variety of vertebrate species (Levine and Moore, 1965; Moore and Perez, 1968; Dahl and Bignami, 1973).

The use of monovalent antiserum (AACNA-1) within the intermediate gel during the fused rocket immunoelectrophoretic analysis of G-150 SF sephadex chromatography of Fraction P2F2, revealed both a high and low molecular weight component that contained the CNA-1 antigenic determinant. The molecular weight of both the purified low molecular weight CNA-1 component and the isolated high molecular weight CNA-1 component were estimated by molecular exclusion chromatography on Sephadex G-100 SF and Sephacryl S-300 SF

respectively. However, molecular exclusion chromatography does not measure the true molecular weight of an unknown protein but rather its Stokes' Radius, which is the radius of a perfect unhydrated sphere having the same rate of passage through the column as that of the CNA-1 containing components (Andrews, 1965).

Subunit studies by sodium dodecylsulphate gel electrophoresis indicated that the purified 65,000 MW CNA-1 was composed of two subunits of identical molecular weight (i.e. 30,000 daltons). SDS is known to dissociate a large number of water soluble proteins to their constituent polypeptides in the presence of a reducing agent (Weber and Osborn, 1969). SDS readily binds to protein at a constant ratio, producing complexes with constant charge per unit mass. The SDS protein complexes are rod-shaped, the length of which varies uniquely with the polypeptide molecular weight (Reynolds and Tanford, 1970). The non-specific nature of the binding of SDS to a wide variety of proteins representing many different structural types, implies that complex formation requires conformational changes of similar characteristics with each complex having a uniform type of structure (Weber and Osborn, 1969 and Reynolds and Tanford, 1970).

The small differences in the calculated molecular weight for the CNA-1 component based on molecular exclusion chromatography (MW 65,000) and SDS polyacrylamide gel electrophoresis (MW 60,000) may be indicative of differences in the molecular structure of CNA-1 and globular proteins, since exclusion limits during molecular exclusion chromatography are relatively low for linear, randomly coiled

molecules, and relatively high for molecules possessing more compact structures such as globular proteins (Andrews, 1965).

The exact relationship between the high and low molecular weight components that contain the CNA-1 antigenic determinant is unknown. Subunit aggregation during the extraction and chromatographic procedures may be one possibility. It is well known that some enzymes associated with the metabolism of neurotransmitters possess molecular weight heterogeneity (i.e. glutamic acid decarboxylase; 16,000 - 129,000 daltons, Matusda et al., 1973; Wu et al., 1973). This possibility is supported by the similar electrophoretic mobilities expressed by both the high and low molecular weight CNA-1 containing components during immunoelectrophoresis. However, confirmation of this relationship will require disaggregation and subunit analysis of the high molecular weight component in the presence of ionic detergents and denaturing conditions. The loss of CNA-1 antigenicity observed under these conditions would necessitate the further purification of the large molecular weight species. Other possible relationships between both CNA-1 containing molecules such as mechanical shearing, or cleavage of the large molecule by the action of proteolytic enzymes, or extraction procedures can not be entirely discounted. This phenomenon would however, require non-random cleavage of the large molecule with the subsequent formation of the 65,000 dalton molecular weight species.

Immunohistochemical techniques have played an important role in understanding the functional significance of brain proteins (for Review see Livett, 1978). In the present study, both immunofluorescence and

immunoperoxidase procedures were utilized in the localization of CNA-1 within the central nervous system and within the cerebellum specifically. The purpose of using these histochemical techniques was threefold: 1) to confirm the neural and species specificity of CNA-1; 2) to determine if CNA-1 was associated with either a specific neural cell type or brain region, and 3) to determine whether CNA-1 was associated with the nerve cell surface, the cell cytoplasma or both. The results of this study confirmed both the neural and species specificity of CNA-1 by the lack of demonstrable fluorescence within tissue sections of adult chicken liver and adult mouse cerebellum. Also CNA-1 labelled fluorescence was absent from tissue sections which had been treated with either preimmune serum or AACNA-1 absorbed with purified CNA-1, thus confirming the monovalent nature of the AACNA-1 antiserum.

CNA-1 was demonstrated to be localized specifically to the cell surface of some neuronal cell types. However, even within the plasma membrane of a given neuron, the distribution was not uniform. CNA-1 was present on the perikaryal membrane and in some neurons was found in extended regions of the primary dendritic membranes as illustrated in the neurons of the medial cerebellar nuclei. The axonal membranes of all neurons studied appeared to lack the antigen, at least in sufficient amounts to be detected as demonstrated by the lack of fluorescence observed within the white matter of the cerebellum. Again the labelling of the dendritic membranes was not uniform, since the diverse dendritic tree of the Purkinje cells could not be demonstrated

within the molecular layer of the cerebellar cortex. This discontinuous distribution of an apparently membrane bound antigen is of particular interest, since the plasma membrane of the neuron in both the axon and cell body are known to be continuous and the membrane is thought to be in a fluid state at physiological temperatures (Hubbel and McConnell, 1968). To account for this restricted mobility of neuronal membrane antigens, Rostas and Jeffrey (1975), proposed that a regulatory area of non-fluid membrane existed within the membrane matrix. This area functions as a general mechanism for the partitioning of membrane components into discrete regions of highly differentiated cell membrane. Similarly the soluble nature of CNA-1 would suggest that the antigen is a "peripheral" membrane protein. The association of a peripheral protein with a membrane has been suggested to be due to specific binding to an "integral" protein (i.e. amphipathic molecule within the membrane matrix) at a site where the latter protein protrudes from the membrane into the aqueous plane (Singer, 1977). The self-aggregation of peripheral proteins while attached to integral protein molecules, could provide a mechanism to restrict the lateral mobility of integral molecules in the plane of the membrane (Painter _et al_., 1975).

The fact that CNA-1 is not distributed within the cytoplasm of the neuronal cell body or its processes is also of interest. This suggests that the antigen, if synthesized within the cell body, is either present there in an incomplete form which is not recognized by the antibody, or its concentration in the cell body is very low due to a short metabolic half-life.

It can be concluded from this preliminary study of the immunohistochemical localization of CNA-1, that the antigen is localized in specific regions of the neuronal cell surface and that not all neurons express the antigen.

Immunohistochemical techniques have previously been applied in search of surface membrane antigens specific to given cell types within the CNS (Poduslo et al., 1977; Schachner et al., 1977b). These studies indicate that cells from different tissues can express distinctive tissue specific or cell type specific antigens on their surfaces. This antigenic distinctiveness at the cell surface may be the means whereby cells identify one another. Cell to cell association as in tissue or synapse formation could conceivably be predicted upon such recognition (Joseph and Oldstance, 1974). This area of research is of particular interest in developmental neurobiology because of the belief that a knowledge of the antigenic composition of neuronal membranes will provide insight into the molecular basis of neuronal specificity. However, before such an association can be made with regards to the ontogenic appearance of CNA-1 and specific neuronal cell types, the exact subcellular localization of CNA-1 must be elucidated. The punctuated deposition of immunoperoxidase reaction product on the perikaryal and primary dendritic membranes of neurons within the deep cerebellar nuclei is suggestive of synaptic distribution. The association of CNA-1 with either pre or post synaptic membranes would greatly influence the interpretation placed on data concerning the ontogeny of CNA-1 within the developing embryonic brain. The

appearance of postsynaptic membrane proteins may be related to postmitotic neuronal differentiation, whereas presynaptic (terminal axon) associated proteins may reflect axonal innervation or interneuronal connections. These two events may differ significantly with respect to the specific ontogenic appearance of proteins during the establishment of the neural cytoarchitecture. Although the present study has not addressed itself to this problem, several approaches could be utilized to determine the precise relationship of CNA-1 to the neuronal membrane. For example, immunoelectron microscopy utilizing the electron dense immunoperoxidase reaction product could be used to determine if CNA-1 is related to the synaptic complex. Preliminary studies have revealed that the antigenicity of CNA-1 was maintained during cytological fixation of chicken neural tissue when employing the periodate-lysine-paraformaldehyde fixative and method of McLean and Nakane (1974). This fixative would be required since simple formaldehyde fixation would not produce sufficient ultrastructural preservation or detail required for electron microscopy.

A second approach would involve density gradient fractionation of intact neuronal subcelluar organelles and membrane fractions. Subsequent immunochemical analysis of each fraction for the presence of CNA-1 would also contribute to the subcellular localization of CNA-1.

A number of studies have undertaken similar approaches in the demonstration of proteins of synaptosomes, synaptic vesicle membranes, and synaptic plasma membranes (Kornguth _et al_., 1969; Lim and Hsu, 1971). During the homogenization of brain tissue under isotonic

conditions the synapses or nerve terminals are pinched off from the axon and the cell bodies or dendrites, and resealed to form synaptosomes (Whittaker et al., 1964). The greater part of the external limiting membrane (synaptic plasma membrane) of the synaptosome is presynaptic in origin, but depending on the composition of the homogenization medium, a postsynaptic attachment may be present, connected by the synaptic cleft material to the membrane of the nerve terminal (Van Leeuwen et al., 1976). Both the synaptic plasma membrane and the synaptic vesicle membrane fractions are obtained from hypo-osmotically shocked synaptosomes.

Antiserum directed against a synaptic plasma membrane fraction, purified from one day old chicken forebrain tissue has been prepared by Livett et al., (1974), and extensively studied by Rostas and Jeffrey (1977 a, b, c). The antigens demonstrated by their polyvalent antiserum were described as neuronal and avian specific, as well as being either protein or glycoprotein in character. These antigens, although present in both the central and peripheral nervous system were localized by immunofluorescence to regions which are known to be rich in preterminal axons and synaptic terminals (i.e. cerebellar white matter and molecular layer). The antigens were also localized to extended regions of the axonal membrane, but absent from the dendritic membrane or neuronal cell bodies, either on the perikaryal membrane or in the cytoplasm. This characteristic distribution of these chicken synaptic plasma membrane antigens is clearly in contrast to that described for CNA-1 in the present study. The observation that

no change in the immunofluorescent pattern of these antigens after absorption of the anti-synaptic plasma membrane antiserum with a soluble fraction (obtained from osmotically shocked synaptosomes, also confirms the unlikeliness of CNA-1 being included in the integral antigens described by Livett et al., 1974, and Rostas and Jeffrey (1977 a, b, c). However, this does not totally negate a possible association of CNA-1 with the neuronal synaptic complex. The characteristic cellular and regional localization of CNA-1 is suggestive that CNA-1 may be associated with a specific type of synapse with a defined function (i.e. inhibitory synapse). This possibility would necessitate further indepth localization studies in relation to specific neurotransmitters for confirmation.

The localization of L-glutamic acid decarboxylase (E.C. 4.1.1.15, GAD), a soluble enzyme which catalyzes the synthesis of the central inhibitory neurotransmitter γ-aminobutyric acid (GABA), has been studied within the mouse cerebellum both at the cellular and subcellular level utilizing the immunoperoxidase technique (Saito et al., 1974a; McLaughlin et al., 1974). Previous biochemical studies demonstrated GAD to be in high concentration within synaptosomes, and suggested a synthetic capacity of GABA formation within the nerve terminal (Roberts and Hammerschlag, 1972). In the mouse cerebellar cortex, a highly localized peroxidase reaction product was observed to be associated with the Purkinje cells and within the granule cell layer. However only diffuse stain appeared within the molecular layer or within the neuronal cell bodies. In addition, neurons within the

deep cerebellar nucleus (nucleus interpositus) displayed dense staining in a punctate distribution around both the neuronal somata and their proximal dendrites. Numerous punctate deposits were also observed around the Purkinje cells which corresponded to the expected distribution of inhibitory basket-cell endings. The deposits within the granule cell layer, were suggestive of axonal endings of Golgi type II neurons upon the dendrites of granule cells (Saito et al., 1974a). These observations found immunohistochemically were subsequently confirmed ultrastructurally. In additon, deposits of reaction product were demonstrated presynaptically on the synaptic vesicle and mitochrondrial membranes. However, the internal portions of these organelles were not labelled. GAD was also not identifiable within the cytoplasm of any Purkinje cell or associated with any organelle within the cell soma. These observations suggested that specific synaptic connections within the cerebellum were inhibitory and that the presynaptic components of these synapses used GABA as their neurotransmitter (McLaughlin et al., 1974).

It is readily apparent that the cellular distribution of GAD is remarkably similar to that observed for CNA-1 within the chicken cerebellum. However, a number of differences between the two proteins can be described. First, the molecular weight of GAD has been estimated to be approximately 85,000 by high speed sedimentation equilibrium analysis. GAD was also shown to dissociate into two identical subunits of 44,000 daltons in the presence of 6 M guanidine HCL and 0.1 M 1-mercaptoethanol (Wu et al., 1973). In the presence of

SDS however, a number of subunits were observed and it has been suggested that GAD is a hexamer made up of 15,000 dalton subunits (Matsuda *et al.*, 1973). CNA-1 in contrast has not been shown to possess similar physical characteristics although differences in methodology employed may be significant between the cited studies and the present study.

Antiserum to mouse brain GAD has also been demonstrated to cross react with the decarboxylases from brains of several mammalian species and partially cross react with avian and amphibian brain GAD during double immunodiffusion (Saito *et al.*, 1974b). Again, in contrast, CNA-1 has not been found to induce cross reacting antibodies to mouse brain extracts, and also failed to demonstrate any immunohistochemically labelled CNA-1 within mouse cerebellar tissue. Therefore it may be speculated that although CNA-1 is possibly localized to similar neuronal membrane sites (i.e. inhibitory synapses) within the adult chick cerebellum, CNA-1 is immunochemically distinct from L-glutamic acid decarboxylase. However, further biochemical and immunochemical studies are required to support this hypothesis.

CNA-1 also differs significantly in both its immunohistochemical localization and physical characteristics from other immunochemically identified brain specific proteins such as S-100, GFA and alpha-2 glycoprotein. Furthermore, the absence of detectable enolase activity associated with CNA-1, as well as other physical characteristics eliminates the possibility that CNA-1 is similar to the brain enolases (E.C.4.2.1.11) containing either the

$\gamma\gamma$ or $\alpha\gamma$ subunits.

The rocket immunoelectrophoretic analyses of brain extracts of chicken embryos revealed CNA-1 in detectable amounts by the seventh day of incubation. Also the antigen was observed to increase in concentration prior to hatching with respect to the total concentration of extractable soluble protein within the brain. However, the prehatching level of CNA-1 (Day 20) was noted to be only 54.0% of that attained in the adult chicken brain. Based on this observation it is assumed that CNA-1 continues to accumulate within the brain during the posthatching period until the adult levels are achieved. Confirmation of this assumption must await further analysis of brain extracts obtained from chickens shortly after hatching to maturity.

As previously discussed, until a precise subcellular localization of CNA-1 as well as a more encompassing study of its regional distribution is performed, the association of CNA-1 with the differentiation, migration, or cellular interaction (i.e. synaptogenesis) of specific neuronal cell types, must be regarded as speculative only. However, a brief discussion of cellular events occurring within the developing chicken brain and specifically within the cerebellum during the period of CNA-1 appearance and accumulation is in order.

Within the cerebellum, as in other regions of the CNS, the establishment of the final definitive patterns of cellular relationships (cytoarchitecture) is the result of several processes that are patterns in space and time, namely histogenesis, cell

migration, cell differentiation and cell death (Jacobson, 1978). The largest neurons of the cerebellum (i.e. Purkinje, Golgi type II and neurons of deep cerebellar nuclei) are generated between Day 3 and Day 6 of incubation after migration from the ventricular proliferative zone. DNA synthesis within this zone declines by the eighth day and ceases by the twelfth day of incubation (Hanaway, 1967). The Purkinje cell population commences and ceases before that of any other cell type. However, they remain quiescent for several days after their generation and migration and grow very slowly until proliferaton of the external granule cells occurs (Jacobson, 1978). The granule, stellate and basket neurons, as well as some of the cerebellar glial cells are derived from cells of the external granular layer. This layer develops mainly during the sixth day of incubation and gradually increases in thickness until a maxium is reached by the fifteenth day of incubation. Although some cells are released to migrate inward by Day 8, a massive inward migration of cells occurs from Day 15 on (Hanaway, 1967). The external granular layer gradually disappears at the time of hatching or soon afterward. When the granule cells reach their position within the internal granular layer, their migration ends and teleodendria sprout from the cell bodies. The teleodendria of the granule cells and of the Golgi type II cells together with the terminals of the mossy fibers form the cerebellar glomeruli (Mugnaini and Forstronen, 1967).

Synaptogenesis starts before neurogenesis is completed, and thus young neurons migrate to their definitive levels, by-passing

neurons upon which synapses have already formed or are in the process of formation. However, synaptogenesis may in some cases by delayed. The presynaptic endings and the cells on which they will synapse may be juxtaposed for days before they form synaptic connections (Jacobson, 1978).

There is no general rule about the developmental order of the presynaptic and postsynaptic structure, but in each case the order is apparently invariant. Synthesis of transmitters in the presynaptic neuron and presumably its release at the presynaptic terminal occurs independently of development of the postsynaptic specialization within the chicken CNS (Zukin *et al.*, 1975). However, development of the postsynaptic membrane specialization is sometimes seen prior to arrival of the presynaptic terminal (Jacobson, 1978).

It is apparent that CNA-1 is immunochemically demonstrable and found to accumulate during a period (Day 7 to hatching) which closely parallels complex cellular and morphological events occuring within the embryonic cerebellum as well as the entire developing central nervous system. Until our knowledge of the properties of specialized membranes and factors (possibly proteins specific to a cell type or function) which govern the establishment of cellular recognition (cell-cell interactions), is considerably increased a satisfactory understanding of ontogenic events will not be possible.

The association of CNA-1 with either the plasma membrane or synaptic complex of neurons of defined function which has been outlined in the above discussion, is based on a number of observations and

several assumptions which remain to be experimentally investigated before the significance of the neural specific antigen CNA-1 in neural development and adult function is fully realized.

SUMMARY

1. Polyvalent anti-brain antiserum (ABS) was induced in rabbits against saline soluble chicken brain extract (ABE). Absorption of ABS antisera with adult liver and kidney extracts yielded a polyvalent but neurospecific antiserum (AABS). Eight adult "neural specific" antigens were demonstrated during immunoelectrophoresis developed with AABS.
2. Seven of the neural specific antigens appeared sequentially during embryonic neural development of the chicken (Day 2 to Day 11). An eighth antigen appeared to be limited to posthatching neural development.
3. By immunochemical criteria, the majority of neural specific antigens, but not all, were demonstrated to be restricted to avian brain. Similarly the majority of neural specific antigens, but not all, were susceptible to proteolysis by trypsin or chymotrypsin, suggesting a protein structure.
4. Separation of the neural specific antigens was attempted by combining ammonium sulphate fractionation, DEAE-cellulose chromatography and G-150 SF Sephadex chromatography. One neural specific antigen (CNA-1), which exhibited alpha-1-globulin mobility during immunoelectrophoresis was isolated and partially

purified with additonal DEAE-Sephadex and G-100 SF sephadex chromatographic steps.

5. Polyvalent anti-CNA-1 antiserum (ACNA-1) was induced in rabbits against partially purified CNA-1. Absorption of ACNA-1 antiserum with adult liver and kidney extracts yielded a monovalent and neurospecific antiserum (AACNA-1).

6. CNA-1 was purified from ABE by 35-65% ammonium sulphate fractionation, DEAE-cellulose chromatography, G-150 SF sephadex chromatography, preparative polyacrylamide gel electrophoresis and DEAE sephadex A-50 chromatography. Each step of the purification procedure was monitored using quantitative immunoelectrophoretic techniques, incorporating ABS, ACNA-1, and AACNA-1 antiserum. CNA-1 accounted for about 1.3% of the total soluble proteins within ABE. A 76.9%-fold purification was achieved with a 0.9% yield.

7. The purified CNA-1 was demonstrated to be homogeneous by the following criteria; analytical polyacrylamide gel electrophoresis, SDS polyacrylamide gel electrophoresis and crossed immunoelectrophoresis employing either the polyvalent antisera ACNA-1 or ABS.

8. By immunochemical criteria, CNA-1 was demonstrated to be both avian restricted and neural specific. The CNA-1 antigenic determinant was found to be protein in nature, since treatment with either chymotrypsin or trypsin resulted in a loss of antigenicity.

9. CNA-1 antigen expressed molecular weight heterogeneity on molecular exclusion chromatography (i.e. > 1,500,000 and 65,000 daltons), possibly due to aggregation of the smaller component. SDS treatment of the 65,000 dalton CNA-1 component revealed two identical subunits of 30,000 daltons each.
10. Using immunohistochemical techniques (i.e. immunofluorescence or immunoperoxidase) CNA-1 was localized specifically on the cell surfaces of particular classes of neurons within the adult chicken cerebellum (i.e. Purkinje neurons, granule cells, and large neurons of deep cerebellar nuclei). Distribution of CNA-1 resembled synaptic distribution, particularly associated with inhibitory functions (i.e. presynaptic nerve endings of Purkinje cells, basket cells and Golgi type II cells).
11. CNA-1 was detected as early as the seventh day of incubation in embryonic brain extracts and found to accumulate within the developing embyronic brain from Day 7 to hatching. CNA-1 was detectable during the period in which neurogenesis and synaptogenesis is known to occur within the chicken CNS. Prehatching (Day 20) levels were only 54.0% of adult CNA-1 levels, therefore accumulation continues until adult levels are achieved.

APPENDIX 1

Abbreviations of Units of Measurements

cm	Centimeter
g	Force of Gravity
gm	Gram
mA	Milliampere
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
nm	Nanometer
N	Normal
μ	Micron
μg	Microgram
μl	Microliter
U	Unit

APPENDIX II

Abbreviations of Chemicals

ADP	Adenosine diphosphate
BIS	N,N'-methylene-bis-acrylamide monomer
DEAE-	Diethylaminoethyl-
EDTA	Ethylenediamine tetracetic acid
FITC	Fluorescein isothiocyanate
Glycerate-2-P	Glycerate-2-phosphate
IgG	Immunoglobulin G
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD+	Nicotinamide adenine dinucleotide (oxidized form)
PMSF	Phenylmethylsulfonylfluoride
SDS	Sodium dodecylsulphate
TEMED	N,N,N'N'-tetramethylethylenediamine
TLCK	N-α-p-tosyl-L-Lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
Tris	Tris-hydroxymethylamino-ethane

APPENDIX III

Miscellaneous Abbreviations

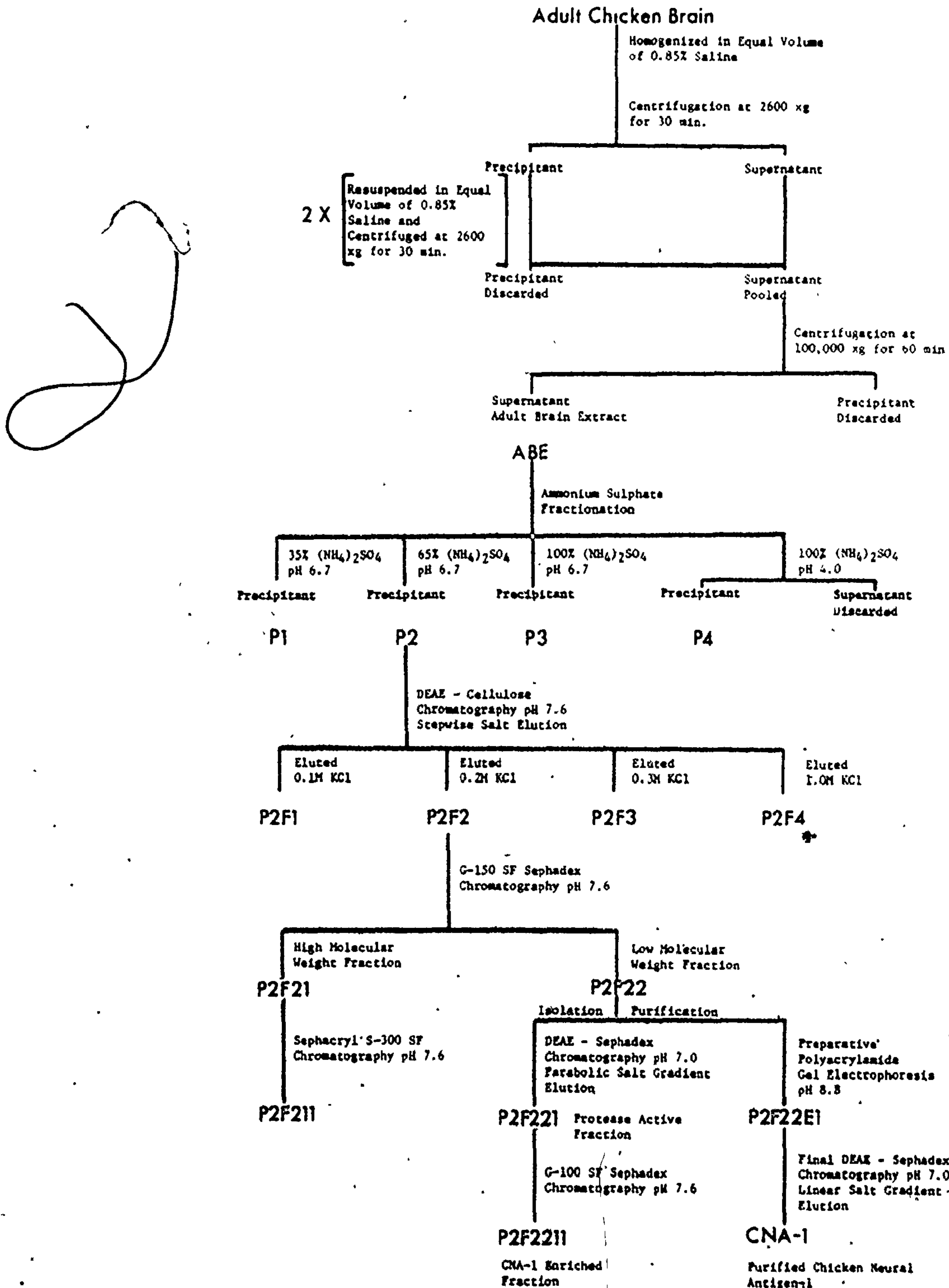
α	Alpha
ABE	Adult brain extract
ABS	Anti-brain antiserum
AABS	Absorbed anti-brain antiserum
ACNA-1	Anti-CNA-1 antiserum
AACNA-1	Absorbed anti-CNA-1- antiserum
CBA-1	Chick brain acidic protein Fraction 1
CNA-1	Chicken neural antigen-1
CNS	Central Nervous System
EEO	Electroendosmosis
e.g.	for example (L. *exampli gratia*)
GFA	Glial fibrillary acidic protein
GABA	γ-aminobutyric acid
GAD	L-glutamic acid decarboxylase
γ	gamma
HBE	Hamster brain extract
i.e.	that is (L. *id est*)
ID	Internal diameter

APPENDIX III (continued)

KID	Adult kidney extract
LIV	Adult liver extract
MBE	Mouse brain extract
M.W.	Molecular Weight
NSE	Neuron specific enolase
NSP	Nerve (neuron) specific protein
OD	Outer diameter
PAP	Phenylalanine rich acidic protein
PAS	Periodic acid - Schiff
PBS	Phosphate buffered saline
RBE	Rabbit brain extract
SER	Adult chicken serum
SF	Super fine
SRANT	Species restricted antigen of rat nervous tissue
UV	Ultra-violet

APPENDIX IV

Flow Chart for the Preparation of Adult Brain Extract, the Isolation of Neural Specific Antigens and the Purification of Chicken Neural Antigen-1.[a]



(a) Details of experimental procedures for each step are described in Materials and Methods.

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